Reinforcing Effect of Optogenetic Stimulation of Dorsal Subiculum

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Reinforcing Effect of Optogenetic Stimulation of Dorsal Subiculum

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This thesis entitled:

Reinforcing Effects of Optogenetic Stimulation of Dorsal Subiculum
written by Brian Alan Cadle
has been approved for the Department of Psychology and Neuroscience

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Ryan Bachtell, Chair

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Jerry Stitzel

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Jerry Rudy

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Tor Wager

2014

The final copy of this thesis has been examined by the signatories, and we
Find that both the content and the form meet acceptable presentation standards
Of scholarly work in the above mentioned discipline.
Abstract: The subiculum is a subregion of the hippocampus that sends projections from the hippocampus to several cortical and limbic structures involved in mediating motivated behaviors such as the medial prefrontal cortex (mPFC), nucleus accumbens (Nacc), amygdala, and hypothalamus. Electrophysiological and pharmacological studies have demonstrated that the subiculum itself can modulate certain motivated behaviors. Prior work has often focused on the ventral portion of the subiculum (vSUB), as electrically stimulating the vSUB can induce reinstatement of cocaine seeking behavior. However, no studies to date have shown whether stimulation of the dorsal subiculum (dSUB) can affect operant responding or motivated behavior. Here we demonstrate that mice placed in custom built operant nose poke chambers will nose poke for optogenetic stimulation of the dSUB. This reinforcing effect was significantly greater than baseline nose poking rates or poking for a light cue and was dependent on the frequency of stimulation, with increased frequency correlating with increased self-stimulation rates.

Stimulating subicular projections to the Nacc also significantly increased self-stimulation rates, indicating a role for dSUB to Nacc projections in mediating the reinforcing effects of self-stimulation. Acute and chronic administration of cocaine had no effect on laser self-stimulation rates, however, administration of dopamine D_1 receptor antagonist SCH-23390 or muscarinic acetylcholine receptor (mAChR) antagonist scopolamine significantly reduced laser self-
stimulation, suggesting the involvement of dopaminergic and cholinergic systems in mediating laser self-stimulation’s reinforcing effects. Together, this data supports a novel role for dSUB projections to the Nacc in mediating control of operant and motivated behaviors.
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Chapter I: Introduction and Background

The hippocampus is a well-studied structure found in the brains of mammals and vertebrates primarily responsible for mediating the formation of long term memories and spatial navigation. It is a curved bilateral structure often described as horn or banana shaped (Figure I-1), located adjacent to and under the cerebral cortex. The hippocampus is primarily comprised of a series of laminar cell layers, referred to as stratum, which can be divided up into linear subregions such as the dentate gyrus, cornu ammonis 3 (CA3), cornu ammonis 2 (CA2), cornu ammonis 1 (CA1), and subiculum. The dentate gyrus receives input from the entorhinal cortex through the perforant pathway, which in turn sends projections to the CA3 through mossy fiber projections. The CA3 then sends projections to the CA2, CA1, and the subiculum through Schaffer collaterals. Finally, the subiculum acts as a major output of the hippocampus, sending glutamatergic projections to adjacent and distant cortical and subcortical targets such as the entorhinal cortex, medial prefrontal cortex (mPFC), nucleus accumbens (Nacc), amygdala, mammillary bodies, and hypothalamus. The hippocampus is considered part of the limbic system, a series of discrete but interconnected brain regions thought to regulate emotions, motivated behavior, the formation of memories, and olfaction (Mega et al, 1997). Prior work in both animals and humans has demonstrated the critical role the hippocampus plays in the formation of long term memories and spatial navigation (Buzsaki et al, 2013), however, more
recent work has demonstrated the additional ability of subregions such as the subiculum to modulate motivated behaviors such as locomotion and drug-seeking (Black et al, 2006, Caine et al, 2001, Martin-Fardon et al, 2008).

The subiculum is a subregion of the hippocampus, located between the CA1 region of the hippocampus and entorhinal cortex. It receives input from CA1 and the entorhinal cortex and sends projections to many cortical and subcortical areas of the brain, such as the medial prefrontal cortex, hypothalamus, nucleus accumbens, mammillary nuclei, septal nuclei, and amygdala (Naber & Witter 1998). Functionally, the subiculum influences a variety of neural processes and behaviors, such as learning and memory, spatial and temporal processing, and motivation (Caine et al, 2001, Cooper et al, 2006, O’Mara 20005, Potvin et al, 2006). However, while studies have demonstrated that the subiculum is a functionally and anatomically distinct region of the hippocampus (O’Mara et al, 2001), few studies have focused on the subiculum compared to the hippocampus proper. Thus, many of the cellular, physiological, and circuit-level functions of the subiculum remain poorly understood.

There is some controversy regarding proper anatomical subdivisions of the subiculum. The subiculum has historically been divided into anywhere from two to four different subregions based on differences in cytoarchitecture and neural projections. Together, they are often described as the “subicular complex” (O’Mara et al, 2001). Contemporary studies typically describe the subicular complex in three areas: The subiculum proper, presubiculum, and parasubiculum. The subiculum proper shares many connections and architectural features with the hippocampus, such as the number of laminar cell layers and connections to CA1 and the entorhinal cortex. It is typically considered part of the hippocampal formation, while the pre- and parasubiculum are more anatomically related to the entorhinal cortex (O’Mara et al, 2001). For
the purposes of this study, “subiculum” will refer to the subiculum proper and, it will be the focus of discussion.

The subiculum itself can be further anatomically divided along two axes: Dorsal-ventral and proximal-distal (referencing the subiculum itself) (Figure I-2).

Due to the complex three-dimensional shape of the hippocampal formation, coronal sections of the brain divide the subiculum into dorsal and ventral regions through most of the anterior portion of the rodent brain (Paxinos and Watson, 2006). Numerous studies have suggested functional differences between the dorsal and ventral hippocampal formation (Dougherty et al, 2012, Fanselow and Dong, 2010, Wang et al, 2013, Zhang et al, 2014). Tract tracing studies identifying projection targets of the subiculum have also found differences in projections between the dorsal and ventral regions of the subiculum (Naber & Witter, 1998).

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<th>Dorsal-distal subiculum</th>
<th>Dorsal-proximal subiculum</th>
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<tbody>
<tr>
<td>retrosplenial cortex</td>
<td>perirhinal cortex</td>
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<td>presubiculum (dorsal)</td>
<td>prelateral cortex</td>
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<td>medial mammillary nucleus (CM)</td>
<td>nucleus accumens (RL)</td>
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<td>medial entorhinal cortex (CL)</td>
<td>lateral septum (dorsal)</td>
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<td>medial mammillary nucleus (RL)</td>
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<th>Ventral-distal subiculum</th>
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<td>lateral septum (ventral)</td>
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<td>medial mammillary nucleus (CM)</td>
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<tr>
<td>medial entorhinal cortex (RM)</td>
<td>amygdala</td>
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<td>medial mammillary nucleus (RL)</td>
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<td>lateral entorhinal cortex (RM)</td>
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Figure I-2. Projections of the subiculum.
Figure listing the targets of projections of the dorsal and ventral proximal (light gray) and distal (dark gray) subiculum. CM – caudomedial, CL – caudolateral, RL – rostromedial, RM – rostromedial. From Naber & Witter, 1998.
Nacc. The dSUB also projects to the prelimbic area of the mPFC. The ventral subiculum (vSUB), on the other hand, sends projections to the infralimbic area of the mPFC as well as the hypothalamus and amygdala. In addition to the dorsal-ventral subdivisions of the subiculum, there are proximal-distal subdivisions of the dSUB and vSUB, with distal areas of the dSUB and vSUB projecting to nearby areas such as the presubiculum and entorhinal cortex and proximal areas projecting to distant cortical and subcortical areas such as the mPFC, Nacc, and amygdala.

It is likely that functional differences arise from the differences in projections along the proximal-distal axis, though there is little data examining the proximal-distal subiculum. Electrophysiological studies have found variations in firing properties in subicular neurons along the proximal-distal axis (Kim and Spruston, 2011, Sharp and Green, 1994), but few behavioral studies have attempted to make the distinction. This is likely due to the difficulty in individually targeting the relatively compact and coterminous regions with cannulae or electrodes. Indeed, even studies examining behavioral differences between the dSUB and vSUB have occasionally found quite different or even contradictory results (Black et al, 2004, Martin-Fardon et al, 2008). This difficulty in determining the functional differences between subdivisions of the subiculum underscores the need for further investigation of discrete subicular pathways and how they contribute to behavior. Optogenetics, or the use of genetically delivered light-sensitive ion channels to modulate neural activity, is an ideal technique for determining which subregions and targets of the subiculum mediate specific behaviors and is the primary technique used in the current study.

At a cellular level, the subiculum is mostly comprised of a homogenous population of pyramidal glutamate neurons (O’Mara et al, 2001). A key characteristic of these subicular neurons is their propensity to generate a “burst” of action potentials (i.e., brief, high frequency
firing) both *in vivo* and *in vitro* in response to current injection or synaptic activation (Staff et al, 2000, Taube 1993). Functionally, bursting is hypothesized to provide a way for subicular neurons to selectively signal salient stimuli and gate information from the hippocampus. Thus, burst firing has been shown to significantly increase probability of vesicle release and can drive changes in up (depolarized) and down (hyperpolarized) states in target regions such as the Nacc (Battaglia et al, 2004, Lisman, 1997). Burst firing is considered a form of non-synaptic plasticity that that can induce long term plasticity in target regions (Pike et al, 1999).

Neurons in the subiculum can switch between single-spiking and burst firing behavior (Moore et al, 2009), providing a mechanism though which the subiculum can bidirectionally control the gain on signals sent to target regions. Bursting is driven by an afterdepolarization (ADP) caused by an influx of Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels open following Na\(^{+}\)-dependent action potentials (Jung et al, 2001). Switching from bursting to single-spiking can occur through inactivation of voltage-gated Na\(^{+}\) channels, while metabotropic receptors can bidirectionally affect electrical induction of burst firing (Cooper et al, 2005, Moore et al, 2009).

For example, bath application of metabotropic glutamate receptor type 1 (mGluR1) and muscarinic acetylcholine receptor (mAChR) antagonists prior to the induction of bursting decreases the occurrence of burst firing in slices. Bath application of mGluR5 antagonist 2-Methyl-6-(phenylethynyl)pyridine (MPEP) increases burst firing (Moore et al, 2009).

Another important characteristic of subiculum neurons is that they project to a single target region (O’Mara et al, 2001). This is in contrast to pyramidal CA1 neurons, which often project to multiple neuronal networks. Coupled with the ability to switch between bursting and single-spiking behavior, this provides a possible mechanism for the subiculum to selectively...
activate individual target regions in order to drive a wide variety of specific behaviors, such as drug-seeking or locomotion.

The subiculum mediates wide variety of behaviors, such as locomotion, spatial and temporal cognition, reinforcement, cue- and drug-induced reinstatement of drug seeking (Cooper et al, 2006, Herman et al, 1998, O’Mara, 2005, Potvin et al, 2007). Due to the complex shape of the hippocampus and practical limitations in the implantation of cannulae and electrodes, most behavioral studies have treated the dSUB and vSUB as functionally separate regions (Figure I-3). Animals with lesions in the dSUB or vSUB display a variety of impairments, including learning and memory deficits (Riegert et al, 2006), increased psychostimulant locomotor response (Caine et al, 2001, Riegert et al, 2006), increased stress response (Mueller et al, 2006), and decreased spatial navigation abilities (Potvin et al, 2009).

Pharmacologically inactivating the vSUB with tetrodotoxin or lidocaine impairs cue- and cocaine-induced reinstatement of drug-seeking behavior in some studies (Sun and Rebec, 2003), but not others (Black et al. 2004, Martin-Fardon et al, 2008), while electrical stimulation of the vSUB following extinction training primes reinstatement of cocaine-seeking behavior (Vorel et al, 2001). Manipulation of the dSUB, on the other hand, has

Figure I-3. Dorsal and ventral subiculum behavioral contributions.

Studies have indicated that the dorsal (red) and ventral (blue) subiculum are functionally distinct behavioral structures. Lesions or pharmacological inactivation in either region leads to deficits in processes listed.
no effect on cue- or drug-mediated reinstatement of drug seeking behavior but affects the maintenance of drug taking (Black et al, 2004). Instead, it appears that the dSUB may be involved in the acquisition of conditioned reinforcement. Thus, pharmacological inactivation of the dSUB impairs the acquisition of drug-seeking behavior (Martin-Fardon et al, 2008). Taken together, this data demonstrates a clear ability of the subiculum to modulate a wide variety of behaviors, however, the particular pathways and discrete regions involved each behavior remain controversial.

One possible pathway for subicular modulation of appetitive behavior is proposed by Grace et al., (2010), whereby activation of the vSUB results in activation of the Nacc and ventral tegmental area (VTA), ultimately leading to increases in dopamine (DA) levels in the Nacc. Prior work has shown that activation of the subiculum can lead to increases in DA levels in the Nacc (Blaha et al, 1997). Interestingly, the vSUB does not project directly to the ventral tegmental area (VTA), the primary source of dopamine innervation in the mesocorticolicimbic circuit. Instead, there is an indirect loop involving the vSUB, Nacc, ventral pallidum, and VTA, whereby the vSUB activates the Nacc. This inhibits the ventral pallidum, resulting in decreased inhibition of the VTA and an increase in DA levels of VTA targets (Sesack and Grace, 2010). This circuit has been proposed to affect schizophrenia, and blocking activity in the vSUB lowers VTA DA neuron activity and blocks amphetamine induced hyperlocomotion (Grace, 2010). Together, this data shows the functional relevance of vSUB to Nacc projections in modulating DA release in the Nacc and behavior. To date, no studies have examined the functional relevance of dSUB projections to regions such as the Nacc and how they might also affect behavior.

Optogenetics is a technique that genetically inserts light-sensitive ion channels in neuronal membranes to selectively activate or deactivate specific neural populations, and it is an
attractive technique for the functional dissociation of neural networks. Here we used viral mediated transfection of the opsin gene Channelrhodopsin (ChR2) to stimulate the dSUB and its targets. ChR2 responds to blue light exposure by conducting Na\(^+\) ions into the neuron, thus depolarizing it. Once the protein is expressed in a neuron, exposure to light, typically through an optic cannula, allows researchers to optically stimulate not only that region but also terminals in target regions, thus ensuring that any ensuing behavioral effects are due to a specific set of projections. Here, we inserted ChR2 into the dSUB to determine the behavioral effects of stimulation of the dSUB and its projections to the Nacc using an operant behavioral task.

Intracranial self-stimulation (ICSS) is a behavioral technique first described in the 1950s used to measure positive reinforcement (Olds and Milner, 1954). In ICSS, subjects perform a specific behavior in order to receive stimulation of a brain region. While ICSS originally used implanted electrodes, recent work has shown that optogenetic stimulation can support ICSS as well (Steinberg et al, 2014). Optogenetic ICSS is an attractive model for investigating the effects of stimulation of discrete brain regions and their targets due to the ability to selectively activate subpopulations of neurons and neural projections to target regions of transfected neurons using light. Several studies have already demonstrated that optogenetic stimulation of neural projections to the Nacc readily facilitates ICSS, clearly demonstrating a link between stimulation of a particular region and its reinforcing effects (Steinberg et al, 2014, Stuber et al, 2011). The present study sought to use optogenetic ICSS to characterize the reinforcing effects of optogenetic stimulation of the dSUB and how dSUB projections contribute to reinforcement.
Chapter II: Operant Chamber Design and Testing

Introduction

ICSS was first described in a series of experiments by Olds and Milner (1954), who found that rats preferred to remain in an area where they received electrical stimulation of the septal area. Further experiments demonstrated that rats would perform specific actions, such as lever pressing, for electrical stimulation of the septal region. Additionally, rats would increase their behavior in response to increasing stimulation. As such, ICSS is a measure for positive reinforcement, or increases in a behavioral response following a stimulus, such as presentation of a reward or electrical or optogenetic stimulation of a particular region. Experiments electrically stimulating other brain regions found that animals will readily stimulate the lateral hypothalamus, medial forebrain bundle, Nacc, and VTA. Recent studies have begun to reexamine ICSS using optogenetic stimulation, which allows not only for self-stimulation of a particular region, but also cell types and individual projections.

To facilitate the measurement of an operant behavior in response to optogenetic stimulation, we designed and built custom acrylic operant chambers equipped with two nose poke holes, infrared sensors, and light cues. Nose pokes were chosen as the behavioral output as opposed to lever presses due to the comparative ease of the motor response and increased likelihood of responding. Operant chambers were tested and modified as needed to verify three operant criteria: baseline responding levels, reinforcement of operant responding to rewarding stimuli such as sucrose, and discrimination between the active (stimulation linked) and inactive (control) nose poke holes.

Materials and Methods
Optogenetic Operant Chamber Materials and Construction

Sheets of acrylic plastic used in the construction of the operant chambers were cut to specification and acrylic glue was obtained through Colorado Plastic Products (Louisville, CO). Acrylic hinges, stainless steel bars, magnetic latches, 1/4” galvanized wire mesh flooring, tray handles, screws, and nuts were obtained from a local hardware store. Cables, plugs, wires, infrared sensors, resistors, and LEDs were obtained from Sparkfun (Boulder, CO). Lasers (445 nm, 1 W) were obtained from Lasever, Inc. (Ningbo, China) and laser mounts were obtained from Old School Industries (Dacono, CO).

Operant chambers were assembled using cut pieces of acrylic, acrylic glue, and wire mesh to create a rectangular box with 6 individual operant chambers with doors and a removable tray for bedding. Infrared sensors and LEDs were then screwed onto nose poke holes drilled in the door and rear wall of each operant chamber. Control of LEDs, lasers, sucrose delivery, and nose poke counting was performed using a pair of USB-6008 data acquisition boards (National Instruments, Austin, TX) connected to a computer running Labview software (National Instruments, Austin, TX).

Animals

Wild-type male C57Bl/6 and C3H/HeJ mice were bred at the Institute for Behavioral Genetics, University of Colorado at Boulder, Boulder, CO, and group housed. Animals were given food and water ad libitum. All experiments were conducted during the dark period of a reverse (12:12) light/dark cycle. All procedures were completed in accordance with the Guide for the Care and Use of Animals and approved the Institutional Animal Care and Use Committee at the University of Colorado Boulder.
**Baseline nose pokes and bias testing**

To assess baseline poking levels and test for bias, adult (30-60 day old) male C57Bl/6 mice were placed in operant chambers and allowed to freely nose poke in daily 3 hour sessions for 7 days. Pokes in either nose poke hole were counted but had no outward effects. Pokes for each hole were then averaged across mice for each day.

**Discrimination and contingent cue response test**

Operant responses to a contingent LED light cue were assessed by placing mice in operant chambers daily for 3 hour sessions for 7 days where pokes in the active hole resulted in the presentation of a 2-second white LED light stimulus inside the nose poke hole on a fixed ratio 1 (FR1) scale. Further pokes during presentation of the LED light stimulus were counted but did not extend the duration of the light cue. Pokes in the inactive hole resulted in no outward effects. Pokes in each hole were then averaged across mice for each day.

**Sucrose self-administration**

To assess sucrose seeking behavior in the operant chambers, adult (30-60 day old) C3H/HeJ mice were placed in the operant chambers daily for 3 hour sessions for 7 days where pokes in the active hole resulted in the 2-second delivery of 150 µl of a 15% sucrose solution through a spout located directly above the nose poke hole. Further pokes during sucrose delivery
were counted but resulted in no further effects. Pokes in each hole were then averaged across mice for each day.

**Statistical Analysis**

For each behavioral experiment, total nose pokes in both holes were averaged across mice for each day, and then compared using a student’s t-test for equal sample sizes and variance. The threshold for statistical significance was set at $p< 0.05$. All values are the mean ± SEM unless otherwise stated.

**Results**

**Operant Chamber Construction**

Finished sets of operant chambers had 6 individual operant chambers with doors, magnetic latches, front and rear nose poke holes, mesh floors, and a removable lid and bedding tray (Figure II-1). Final dimensions were: 36.5” wide, 6.25” deep, and 12.5” high. Each individual chamber had dimensions of: 5.75” long, 5.75” wide, and 9.75” high. The bedding tray had final dimensions of: 36” long, 6” wide, and 1.5” high. Lasers hung above the operant chamber and were allowed to turn freely through an electrical commutator. (Figure II-2)

**Baseline nose pokes and bias testing**
To test the baseline levels of nose poking and ensure there was no inherent bias in the operant chambers, mice were placed in operant chambers and allowed to nose poke freely for 3 hour sessions for 7 days. Pokes in either hole resulted in no effects. Across all mice and sessions, mice freely poking averaged 204.54±77.09 pokes in the front hole and 174.94±29.81 pokes in the rear hole per 3 hour session (Figure II-3). There was no significant difference between pokes in either hole (t₁₂=0.27, p=0.40, n=12), demonstrating that mice had no preference for either hole.

**Discrimination and contingent cue response test**

To test the ability of mice to discriminate between the holes and responses to a neutral contingent cue, mice were again placed in the operant chambers and given access to a LED light cue for daily 3 hour sessions over 7 days. Pokes in the active (front) hole resulted in presentation of a white LED light cue for 2 seconds on an FR1 schedule while pokes in the inactive (rear) hole were counted but resulted in no effects. Across all mice and sessions, mice poked the active hole an average of 505.32±99.12 times versus an average of 128.67±25.18 times in the inactive
hole (Figure II-4). Mice poked the active hole significantly more than the inactive hole across all sessions ($t_{12}= 5.96$, $p<0.0001$, $n=12$).

**Sucrose self-administration**

To test operant responses to a natural food reward, mice were again placed in the operant chambers and given access to a 15% sucrose solution for 3 hour sessions over 7 days. Pokes in the active hole resulted in the presentation of a 15% sucrose solution from a spout directly above the active hole over 2 seconds on an FR1 schedule. Mice readily administered sucrose, poking the active hole an average of $760.09 \pm 158.40$ times across all sessions while poking the inactive hole an average of $119.36 \pm 39.34$ times (Figure II-5). Mice poked the active hole significantly more than the inactive hole ($t_{12}=5.35$, $p<0.001$, $N=10$), and showed a slight preference for sucrose over the light cue ($t_{12}=1.91$, $p<0.05$, $N=10$).

**Discussion**

The custom operant chambers successfully demonstrated the ability to facilitate operant responding in mice. Mice had no initial bias to either nose poke hole, and access to a cue or food reward was sufficient to drive increases in responding and appropriate discrimination between
the active and inactive holes. Mice poked more for a light cue than no cue, and slightly more for a sucrose reward than a light cue. This is in line with previous operant data demonstrating that mice will investigate a neutral cue (such as light) but find food more rewarding. Together, this data demonstrates the ability of the custom chambers to successfully facilitate operant behavior \textit{in vivo}. Indeed, published research has used the operant chambers to mediate cocaine self-administration (Pomrenze et al, 2013). Next, we used the operant chambers to test the effect of optogenetic stimulation of the dSUB and its afferents to the Nacc.

![Figure II-5. Sucrose reward responding.](image)

Nose poke rates in the active versus inactive holes in 3-hour sessions over seven days. Poking the active hole resulted in the presentation of 150 µl of a 15% sucrose solution above the nose poke hole. Mice poked the active hole significantly more than the inactive hole across all sessions \(p<0.0001, \, n=10\)
Chapter III: Reinforcing Effects of Optogenetic Stimulation of Dorsal Subiculum

Introduction

Sources of dopaminergic signaling such as the VTA and targets of the VTA such as the Nacc are particularly effective at driving ICSS behavior. Administration of DA antagonists can significantly decrease ICSS (Corbett et al, 1980), and optogenetic stimulation of DA neurons in the VTA and their projections to the Nacc is sufficient to drive ICSS (Steinberg et al, 2014, Stuber et al, 2011). Together, this suggests an important role for DA in mediating the reinforcing effects of ICSS. However, the exact role of DA in mediating the reinforcing effects of ICSS is unclear, as DA has been proposed to both mediate direct reward (Baudonnat et al, 2013, Wise, 2008) and to serve as a signal of novelty and error prediction (Steinberg et al, 2013). While the dSUB does not project directly to the VTA, it does send projections to regions that the VTA innervates, such as the Nacc and mPFC. Additionally, activation of the vSUB can lead to increases in DA in target regions, suggesting that the subiculum has the potential to support ICSS behavior.

Prior work has investigated the effects of electrical stimulation of the vSUB on cocaine seeking behavior (Vorel et al, 2001). However, to date no studies have examined the effects of stimulation of dSUB on the positive reinforcement of behavior, nor have the neural pathways involved in dSUB modulation of behavior been elucidated. Here we use custom operant chambers, viral vector driven expression of ChR2 in the dSUB, and blue laser light to stimulate the dSUB and subicular projections to the Nacc using an ICSS paradigm. Additionally, we tested
whether optogenetic ICSS of the dSUB can be extinguished and reinstated using contingent and non-contingent laser stimulation paradigms.

**Materials and Methods**

**Animals**

Wild-type male C57Bl/6 mice were bred at the Institute for Behavioral Genetics, University of Colorado at Boulder, Boulder, CO, and group housed until undergoing surgical procedures at postnatal day 30-60. Animals were given food and water ad libitum. Behavioral experiments were conducted during the dark period of a reverse (12:12) light/dark cycle. All procedures were completed in accordance with the Guide for the Care and Use of Animals and approved the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

**Stereotaxic Injections of Viral Vectors**

Mice were anesthetized with an injection of a ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine, ip) then placed in a stereotactic head apparatus (Kopf). Ophthalmic ointment was applied to prevent the eyes from drying. A midline scalp incision was made, the skull cleaned with hydrogen peroxide, and small holes drilled at the injection sites using a Dremel tool. Using a 10 µl Hamilton syringe and a 34 gauge metal needle backfilled with 1 µl of silicone oil, mice were bilaterally injected with 1 µl of either a mouse adeno-associated virus (AAV) containing the ChR2 gene fused to an enhanced yellow fluorescent protein gene (EYFP) driven by a Calcium/Calmodulin-dependent protein kinase promoter (CaMKII) (AAV-CaMKIIa-hChR2(H134R)-EYFP, UNC Vector Core) or a control virus containing the gene for the fluorescent protein but not the opsin protein (AAV-CaMKIIa-EYFP, UNC Vector Core).
injection volume and flow rate (1 µl per injection at 0.1 µl/min) were controlled by an injection pump (World Precision Instruments). Animals received bilateral injections into the dSUB (From bregma and brain surface: AP -3.4, ML ±2.1, DV -1.3), and needles were left in place for 10 minutes following each injection to allow diffusion of virus. Animals were given 3-5 weeks prior to behavioral or electrophysiological testing to allow for full transfection, expression, and trafficking of the ChR2 protein to neuron membranes and terminals.

**Placement of Optical Cannulae**

Animals undergoing behavioral testing received custom-made optic cannulae (Numerical aperture – 0.39) implanted into the dSUB or Nacc. One week following virus injection, animals were again anesthetized using a ketamine/xylazine cocktail and placed in a stereotaxic apparatus. A midline scalp incision was made and small holes drilled in the skull above the stimulation sites using a Dremel tool. Optic cannulae were then lowered into the brain with a stereotactic device and secured with dental cement (Stoelting) and steel screws implanted in skull. Stereotactic coordinates for optic cannula were as follows: dSUB (AP: -3.4, ML: ±2.1), nucleus accumbens (AP:+1.5, ML: ±1.1). Length of optical fibers from skull surface: dSUB 1.5 mm, Nacc 4.0 mm)

**Extracellular Field Recordings**

Three to five weeks following virus injection, mice were anesthetized with isoflurane, decapitated, and brains rapidly extracted. Brains were sliced in 350 µm thick sections in ice cold oxygenated artificial cerebrospinal fluid (125 mM NaCl, 25 mM glucose, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 2 mM CaCl2, and 1 mM MgCl2, pH 7.4, bubbled with 5% CO2 and 95% O2) on a Vibratome (Leica). Slices were incubated in oxygenated warm ACSF (35°C) for 30-40 minutes then brought to room temperature prior to recording. Extracellular field
recordings were performed on a MEA1060-Up-BC 60-channel microelectrode array (Multichannel systems). Recordings were taken from the dSUB and Nacc in response to laser light stimulation (2 seconds stimulation, 5 Hz, 10 Hz, and 20 Hz, 10 mW). To verify that Nacc responses were synaptic, Nacc slices were incubated in ACSF containing 20 nM CNQX (an AMPA receptor antagonist) and low calcium ACSF (100 nM). The loss of light-evoked potentials confirmed calcium-mediated synaptic responses.

**Frequency-response laser self-stimulation**

Two to four weeks following cannula implantation and recovery, mice were placed in the operant chambers daily for three days in 3 hour sessions. During each session poking at the active hole resulted in delivery of a blue laser light (445 nm, 2 seconds light delivery, 10 mW) delivered through the optic cannulae to the dSUB. The frequency of laser stimulation (0, 1, 5, 10, or 20 Hz) was randomly reassigned after each three day block until all frequencies were used. Thus, each mouse was randomly switched to another previously untested frequency until all frequencies were tested over the course of 15 days. Pokes during each 3-day block were averaged for each mouse and then averaged across frequencies.

**Dorsal subiculum and nucleus accumbens terminal laser self-stimulation**

Two to four weeks following cannula implantation and recovery, mice were placed in the custom built operant chambers with nose-poke holes and white LED signal lights. Unless otherwise stated, sessions lasted 3 hours. During the first 6 sessions poking the active hole resulted in delivery of a white LED signal light above the hole (2 seconds, FR1) to determine baseline responding for each mouse. Over the next 6 sessions, poking the active hole resulted in the delivery of blue laser light (445 nm, 2 seconds, 20 Hz, 10 mW, FR1) through the optic
cannulae to the stimulation site (dSUB or Nacc). Further pokes during presentation of the light or laser were counted but did not extend the duration of the stimulus. Pokes in the inactive hole were counted but also resulted in no effects. Pokes in each hole were averaged across mice for each training day to determine daily poking rates, and then averaged across each 6 days of training to determine average pokes under each condition.

*Extinction training and laser reinstatement*

Following laser self-stimulation, mice were placed in the operant chambers and given daily extinction training sessions for 5 days. During each 3-hour extinction session the laser was turned off and pokes in both the active and inactive hole were counted but resulted in no outward effects. After 5 daily extinction sessions, mice were tested for the reinstatement of responding using two different protocols. In the first protocol, mice were exposed to non-contingent blue laser light (2 seconds light, 20 Hz, 1/minute) for the first 15 minutes of the 3 hour session to reinstate nose poking. During the entire session, pokes in either hole were counted but resulted in no effects. In the second protocol, mice were re-exposed to the original self-stimulation.
conditions for the first 15 minutes of the 3 hour session, where nose poking in the active hole was reinforced with 2 seconds of laser light delivery (20 Hz). After 15 minutes, the laser was turned off and further pokes in either hole were counted but resulted in no effects.

**Immunohistochemistry**

To verify proper locations of viral vector injections and optic cannulae, animals undergoing behavioral testing were anesthetized with isoflurane and transcardially perfused with cold paraformaldehyde (4% in saline). Following perfusion, brains were removed and stored in 30% sucrose in saline for 24 hours. Brains were then frozen, sliced at -20°C into 40 µm sections on a cryostat (Leica), and mounted on glass microscope slides. Slides were incubated overnight in blocking solution (5% BSA in 0.1 M PBS with 0.1% Triton-X) at 4°C, followed by incubation in primary antibody overnight (anti-GFP, 1:1000, Molecular Probes) at 4°C. Last, slides were incubated in secondary antibody (Alexa 488, 1:250, Molecular Probes) at room temperature for 2 hours, coverslipped with Vectashield Hardset (Vector Labs), and imaged using a fluorescent microscope (Leica). Animals with improper injection sites, cannula placements, or viral expression were excluded from this study.
Statistical Analysis

Nose pokes in the active hole were averaged across mice and training days for each experimental condition and compared using a paired student’s t-test. The threshold for statistical significance was set at $p < 0.05$. All values are the mean ± SEM unless otherwise stated.

Results

Viral injections and field recordings

We first verified the successful transfection of the ChR2 opsin gene into the dSUB and characterized the functional implications of ChR2 in the dSUB and Nacc. Mice injected with AAV-CamKIIa-hChR2(H134R)-EYFP in the dSUB displayed heavy immunofluorescence in the dSUB and CA1 region of the hippocampus by 21 days post injection (Figure III-1). There was also significant and detectable EYFP expression in projection regions of the dSUB such as the mPFC, Nacc, and amygdala by 35 days.
post injection. We tested functional implications of hChR2 expression in the dSUB by performing extracellular recordings in acute slices taken from the dSUB and Nacc (Figure III-2). Stimulation with 5, 10, and 20 Hz blue laser light evoked field potentials in both regions, with maximum amplitudes similar across all frequencies in both regions (Figure III-3). dSUB slices had an average maximum amplitude of 443.99±130.82 µV across all frequencies, while Nacc slices had an average maximum amplitude of 421.31±113.38 µV across all frequencies. Increasing frequencies of stimulation led to increases in attenuation (final amplitude divided by maximum amplitude) of the response, with dSUB slices having attenuation percentages of 38.69±6.82, 55.22±7.72, and 79.11±7.28 for 5 Hz, 10 Hz, and 20 Hz stimulation, respectively. Nacc slices had attenuation percentages of 25.79±3.31, 42.73±3.73, and 83.69±4.88 for 5 Hz, 10 Hz, and 20 Hz stimulation, respectively. Responses in both regions had high rates of fidelity, with 5 and 10 Hz stimulation having 100% fidelity, and 20 Hz stimulation having 99.2% fidelity.

**Frequency-response Curve**

To establish optimal laser stimulation frequency to support self-stimulation behavior, mice were allowed to self-stimulate the dSUB at 0, 1, 5, 10, or 20 Hz. Increasing frequencies of
stimulation resulted in increased responding, with the frequency-response curve fitting a sigmoidal function (Figure III-4).

**Laser self-stimulation**

To establish baseline poking rates, responding at the active hole resulted in delivery of an LED light cue during the first 6 sessions. This resulted in significant discriminative responding in the active hole over the inactive hole, averaging 114.17±16.92 pokes in the active hole and 75.08±11.40 pokes in the inactive hole in a 3 hour session ($t_5=6.78$, $p=0.001$, $N=15$). Over the subsequent 6 sessions, responding in the active hole resulted in the delivery of 20 Hz laser stimulation to the dSUB (Figure III-5). Mice again responded at the active hole significantly more than the inactive hole and responding was significantly more vigorous than for the light cue. Mice averaged 454.30±143.69 pokes in the active hole for laser stimulation per 3 hour session. Poking rates within each session typically remained stable for each mouse, with mice poking for laser stimulation on an average rate of 2.5 pokes per minute (Figure III-6). Figure III-7 summarizes responding rates at the active hole of mice during each 6 day training block ($t_5=11.7$, $p<0.0001$, $n=15$). Mice injected with a control virus (AAV-CamKIIa-EYFP) showed no significant
alterations in poking rates when switched to laser stimulation (Figure III-8) \( (t_5=0.28, p=0.79, n=8)\).

We next wanted to test whether the reinforcing effects of dSUB stimulation was supported by self-stimulation of dSUB projections to the Nacc. Mice were injected with AAV-CamKIIa-hChR2(H134R)-EYFP in the dSUB and optic cannulae were surgically placed into the Nacc to allow laser stimulation of the dSUB terminals located there. Similar to the dSUB self-stimulation, mice showed significant responding at the hole paired with laser delivery to dSUB terminals (Figure III-9). This was significantly higher than both LED light stimulation \( (t_5=6.89, p<0.001, n=8)\) and inactive responding \( (t_5=15.27, p<0.0001, n=8)\). Figure III-10 summarizes responding at the active hole during light cue and laser self-stimulation conditions.

**Extinction and reinstatement of laser self-stimulation**

We next investigated the rate of extinction of laser self-stimulation behavior and determined the robustness of reinstatement of laser self-stimulation using two paradigms. For extinction training, mice were placed in the operant chambers for 3 hour sessions where nose poking in either hole resulted in no consequences. Responding in the active hole decreased rapidly over successive extinction sessions, and it was no longer significantly different from the

![Figure III-6. Within session responding rates.](image)

Graph of cumulative nose pokes over a 3-hour dorsal subiculum laser self-stimulation session. Mice poked an average of 2.5 pokes per minute. Red bars: individual responding rates. Black bar: average responding rate.
inactive hole by the second day of training (Figure III-11) ($t_{11}=1.97$, $p=0.07$, $n=12$). Following 5 days of extinction training, mice received one of two reinstatement protocols. One group of mice received 15 minutes of non-contingent laser stimulation (2 seconds, 20 Hz, 1/minute) at the beginning of the session, while the second group received 15 minutes of exposure to the initial laser self-stimulation conditions where each poke was reinforced with 2 seconds of blue laser light (20 Hz). Non-contingent laser light delivery was insufficient to reinstate nose poking in the active hole ($t_5=0.32$ $p=0.76$, $n=6$, data not shown). A brief 15 minute re-exposure to contingent laser delivery resulted in a small but significant increase in poking in the active hole over the 3 hour session ($t_{11}=3.65$, $p<0.01$, $n=12$). However, this significant increase is primarily due to increases in poking rates during the 15 minute laser self-stimulation period (Figure III-12). Figure III-13 compares responding rates on the

![Figure III-7. Light cue versus dorsal subiculum self-stimulation.](image)

Bar graph depicting the average responding rate for all mice over each six day training block. Mice significantly increased responding rates at the active hole for laser stimulation compared to the light cue ($p<0.001$, $n=15$).

![Figure III-8. Control virus laser effects.](image)

Responding at the active and inactive hole during light cue presentation (first six days) and laser presentation (final six days, blue line) in mice injected in the dorsal subiculum with a control virus lacking the channelrhodopsin gene.
last day of laser self-stimulation to the final day of extinction and reinstatement responding.

Discussion

Here we demonstrated optogenetic stimulation of the dSUB and subicular afferents to the Nacc to drives ICSS behavior in mice. This confirms a causal relationship between activation of excitatory dSUB neurons projecting to the Nacc and the positive reinforcement of operant behavior, and this data joins a large body of literature demonstrating the ability of stimulation of projections to the Nacc to drive ICSS behavior. The reinforcing effect was dependent on activation of neurons in the dSUB and terminals in the Nacc, as infusions of laser light had no effect on animals injected with a control virus. Reinforcement was also frequency dependent, with increases in stimulation frequency leading to increases in behavioral responses.

This suggests that optogenetic stimulation of the dSUB drives behavior through increased output
of the dSUB and actions on its targets rather than through a specific firing frequency. In rodents, animals engaging in exploratory behavior exhibit electrical oscillations in the hippocampus at a frequency of 6-10 Hz, known as the “theta rhythm.”

While the function of theta rhythms in the hippocampus is not clearly understood, optogenetic stimulation of the dSUB at theta rhythm frequencies did not significantly increase responding compared to higher frequencies such as 20 Hz.

Responses at the active hole were quickly abolished during extinction training, as mice poked both the active and inactive holes at similar levels after one day of training. Mice also quickly reinstated responding, but only during exposure to ICSS conditions. Once the laser was turned off, mice rapidly responded at rates similar to extinguished animals. The temporal specificity of the behavioral response may reflect the speed of action of optogenetic stimulation, or it may demonstrate that the neural signal mediating dSUB self-stimulation is also temporally specific, such as an error or novelty signal.
Figure III-12. Contingent versus non-contingent laser stimulation.

Examples of within session poking rates for a mouse receiving contingent (blue) and non-contingent (black) stimulation of dorsal subiculum during the first 15 minutes of the session.

Figure III-13. Extinction and re-exposure summary.

Bar graphs summarizing active hole responding during the last day of self-stimulation (ICSS), last day of extinction training (Ext), non-contingent laser re-exposure (Laser), and contingent laser re-exposure (ICSS Exp).
Chapter IV: Effect of Cholinergic and Dopaminergic Systems on Laser Self-Stimulation

Introduction

Cholinergic signaling plays an important role in hippocampal function, mediating both learning and memory and spatial navigation (Deiana et al, 2011, Hasslemo, 2006, Power et al, 2003, Shor et al, 2009). Both nicotinic and muscarinic receptors are able to modulate intrinsic excitability and synaptic plasticity in hippocampal neurons (Yakel, 2012). Systemic injection of scopolamine, a muscarinic acetylcholine receptor antagonist, impairs a variety of hippocampal-dependent tasks, such as the Morris water maze (von Linstow Roloff et al, 2007), radial maze (Masuoka et al, 2006), and spatial discrimination tasks (Carli et al, 1997). At a cellular level, muscarinic activity can affect both long term plasticity and plasticity of burst firing in the subiculum (Shor et al, 2009, Moore et al, 2009), as muscarinic antagonism lowers LTP at CA1-subiculum synapses and blocks induction of burst firing. Together, this suggests that muscarinic acetylcholine receptors play an important role in subicular function. Here we tested the effects of nicotinic and muscarinic cholinergic systems by systemically administering nicotine and scopolamine to mice prior to behavioral testing.

The VTA sends dopaminergic projections to several brain regions receiving input from the dSUB, including the mPFC and Nacc. ICSS activation of DA projection neurons from the VTA to Nacc is sufficient to drive reinforcement of operant behavior (Steinberg et al, 2014), thus demonstrating a critical role in DA signaling in driving ICSS behavior. ICSS of dSUB projections to the Nacc is also sufficient to reinforce nose poking, indicating a possible role for DA in dSUB ICSS despite the lack of evidence for a direct connection to the VTA. Stimulation
of the vSUB does lead to increases in DA levels in targets of the VTA, including the Nacc, mPFC, hippocampus, and amygdala (Blaha et al, 1997, Cooper et al, 2006, Floresco et al, 2001). Activation of the Nacc through analogous dSUB circuits may also lead to increases in DA levels in the Nacc, thus driving ICSS behavior. To determine whether DA systems affect optogenetic ICSS of dSUB neurons, we tested the effects of systemic DA agonism and antagonism on ICSS behavior by administering acute cocaine, chronic cocaine, and D₁ antagonist SCH-23390 to mice prior to testing. Cocaine was chosen for its role as a potent activator of DA signaling in the brain as well as its status as a drug of abuse. D₁ receptor antagonism was chosen for its ability to block ICSS behaviors (Steinberg et al, 2014).

Materials and Methods

Animals

Wild-type male C57Bl/6 mice were bred at the Institute for Behavioral Genetics, University of Colorado at Boulder, Boulder, CO, and group housed until surgical procedures occurring postnatal day 30-60. Animals were given food and water ad libitum. All experiments were conducted during the dark period of a reverse (12:12) light/dark cycle. All procedures were completed in accordance with the Guide for the Care and Use of Animals and approved the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

Effects of acute nicotinic agonism on optogenetic ICSS

Mice were injected in the dSUB with a virus containing the ChR2 gene and cannulae placed in the dSUB as described in the Chapter III methods section. Following cannula implantation and recovery, mice were placed in operant chambers and allowed to self-administer 20 Hz laser light to the dSUB daily for 30 minute sessions for 3 days to establish baseline
responding. On the fourth day, mice were administered either nicotinic receptor agonist nicotine (0.2 mg/kg, ip) or saline (ip) immediately prior to being placed in the operant chamber and allowed to self-stimulate as normal. Sessions were shortened to 30 minutes to prevent complete metabolism of the drug prior to the end of the session.

**Effects of acute muscarinic antagonism on optogenetic ICSS**

Mice were injected in the dSUB with a virus containing the ChR2 gene and cannulae placed in the dSUB as described in the Chapter III methods section. Following cannula implantation and recovery, mice were placed in operant chambers and allowed to self-administer 20 Hz laser light to the dSUB daily for 30 minute sessions for 3 days to establish baseline responding. On the fourth day, mice were administered either muscarinic acetylcholine receptor antagonist scopolamine (1 mg/kg, ip) or saline (ip) immediately prior to being placed in the operant chamber and allowed to self-stimulate as normal. Sessions were shortened to 30 minutes to prevent complete metabolism of the drug prior to the end of the session.

**Effects of acute dopaminergic agonism on optogenetic ICSS**

Mice were injected in the dSUB with a virus containing the ChR2 gene and cannulae placed in the dSUB as described in the Chapter III methods section. Following cannula implantation and recovery, mice were placed in operant chambers and allowed to self-administer 5 or 20 Hz laser light to the dSUB daily for 3 hour sessions for 3 days to establish baseline responding. On the fourth day, mice received either saline or cocaine (10 mg/kg, ip) immediately prior to being placed in the operant chamber and allowed to self-stimulate as normal.

**Effects of chronic dopaminergic agonism on optogenetic ICSS**
Mice were injected in the dSUB with a virus containing the ChR2 gene and cannulae placed in the dSUB as described in the Chapter III methods section. Following cannula implantation and recovery, mice were placed in operant chambers and allowed to self-administer 7.5 Hz laser light to the dSUB daily for 3 hour sessions for 7 days. 7.5 Hz was chosen as an intermediate frequency following the frequency-response curve experiment that would allow us to identify either an upward or downward shift in responding rates. Prior to each session, mice were administered cocaine (15 mg/kg, ip) and allowed to self-stimulate as normal. After 7 sessions, mice were again placed in the operant chambers for 3 hour sessions for 5 days and allowed to self-stimulate as normal but received no cocaine. After the abstinence period, mice received a cocaine challenge (15 mg/kg, ip) prior to being placed in the operant chambers and allowed to self-stimulate for one final 3 hour session.

**Effects of acute dopaminergic antagonism on optogenetic ICSS**

Mice were injected in the dSUB with a virus containing the ChR2 gene and cannulae placed in the dSUB as described in the Chapter III methods section. Following cannula implantation and recovery, mice were placed in operant chambers and allowed to self-administer 20 Hz laser light to the dSUB daily for 30 minute sessions for 3 days to establish baseline responding. On the fourth day, mice received either saline or D₁ receptor antagonist SCH-23390

![Figure IV-1. Effect of acute nicotine on dorsal subiculum self-stimulation.](image)

Mice did not significantly alter responding rates at the active hole when administered saline or acute nicotine (0.2 mg/kg, p=0.19, n=7).
(0.01 or 0.05 mg/kg, ip) immediately prior to being placed in the operant chamber and allowed to self-stimulate as normal.

Statistical Analysis

Statistical analysis for groups was performed using two-tailed student’s paired t-tests for normally distributed data. The threshold for statistical significance was set at p < 0.05.

All values are the mean ± SEM unless otherwise stated.

Results

Effects of acute nicotinic agonism on dSUB ICSS

Given the role of cholinergic signaling in hippocampal and subicular function (Hasselmo et al, 2006, Yakel et al, 2012), we first investigated whether acute systemic nicotinic agonism affected the expression of dSUB ICSS. Compared to saline controls, administration of nicotine (0.2 mg/kg, ip) had no significant effect on nose poking rates (Figure IV-1) ($t_6=0.91$, $p=0.19$, $n=7$). The 0.2 mg/kg dose was chosen as an intermediate dose to activate nicotinic signaling without causing significant locomotor effects (Matta et al, 2007).

Effects of acute muscarinic antagonism on dSUB ICSS

Mice administered acute scopolamine (1 mg/kg, ip) significantly decreased self-stimulation rates compared to saline controls ($p<0.019$, $n=7$).
Muscarinic activity can also influence hippocampal and subicular function (Hasselmo et al., 2006). To test the effects of muscarinic blockade, mice were administered the muscarinic acetylcholine receptor antagonist scopolamine prior to ICSS testing. Compared to saline controls, 1 mg/kg scopolamine significantly decreased poking at the active hole (Figure IV-2) \( (t_6 = 2.63, p<0.019, n=7) \), suggesting that muscarinic activity plays a necessary role in the reinforcing effects of dSUB laser self-stimulation.

**Effects of acute dopaminergic agonism on dSUB ICSS**

We next explored whether acute dopamine agonism increased responding rates by administering acute cocaine to mice prior to behavioral testing. Mice administered acute cocaine (10 mg/kg, ip) and allowed to self-stimulate at 5 or 20 Hz showed no significant difference in poking rates compared to saline controls (Figure IV-3) \( (t_7 = 1.35 \text{ and } 0.02, p=0.22 \text{ and } 0.99, \text{ respectively}) \).

**Effects of chronic dopaminergic agonism on dSUB ICSS**

When acute cocaine had no effects on ICSS responding, we tested whether chronic cocaine administration affected responding by giving mice cocaine daily for 7 days, followed by a 5 day abstinence period and a final day of acute cocaine challenge. Repeated cocaine administration (15 mg/kg, ip) for 7 days had no effect on active hole poking rates comparing the
first to the last day (Figure IV-4) \((t_6= 1.51, p=0.18, n=7)\). Responding at the active hole remained unchanged during the 5 day abstinence period following the chronic cocaine administration and when mice were administered a priming dose of cocaine (15 mg/kg, ip) after the abstinence period \((t_6=0.73 \text{ and } 0.50, p=0.49 \text{ and } 0.63, n=7)\).

**Effects of acute dopaminergic antagonism on dSUB ICSS**

To test whether systemic dopaminergic antagonism can significantly decrease responding, mice were administered high and low doses of D\(_1\) receptor antagonist SCH-23390 prior to testing. At the lower dose \((0.01 \text{ mg/kg, ip})\), responding in the active hole was no different between saline controls and drug treated mice (Figure IV-5) \((t_5=1.12, p=0.31, n=6)\). However, mice receiving a higher dose \((0.05 \text{ mg/kg, ip})\) did significantly reduce poking rates \((t_5= 2.95, p<0.016, n=6)\). It is possible though unlikely that this was due to a locomotor effect, as responding at the inactive hole remained unchanged.

**Discussion**
The present series of experiments tested the effects of systemic cholinergic and dopaminergic activity on dSUB ICSS. We tested the effect of cholinergic signaling on dSUB ICSS by administering nicotine or mAChR antagonist scopolamine prior to testing. Cholinergic signaling has been shown to be important in hippocampal function, as in vitro administration of acetylcholine alters long term plasticity (Shor et al, 2009), and systemic administration of scopolamine impairs success in hippocampal-dependent tasks (Deiana et al, 2011).

Administration of nicotine had no significant effect on poking rates at 0.2 mg/kg, a dose shown to be pharmacologically relevant while not affecting locomotor performance (Matta et al, 2007). However, scopolamine administration significantly lowered ICSS rates, suggesting that mAChRs play a role in dSUB ICSS. Interestingly, systemic administration of scopolamine does not block ICSS in the VTA, suggesting that intact hippocampal function is necessary for optogenetic ICSS of the dSUB (Agars and Kokkinidis, 1992). Alterations in subicular neuron bursting rates may also explain scopolamine’s effects on dSUB ICSS, as mAChR blockade is shown to decrease LTP at CA1 synapses and lower bursting plasticity in subicular neurons (Moore et al, 2009).

However, if scopolamine’s effect on ICSS is due to decreased plasticity in burst firing, it is possible that optogenetic stimulation could occlude these effects by forcing subicular neurons to fire in a specific pattern similar to in vivo bursting.
Surprisingly, neither acute nor chronic administration of cocaine altered self-stimulation rates in mice. Previous work using the same drug stock supported cocaine self-administration, demonstrating that the drugs had proper physiological effects and were not expired or ineffective (Pomrenze et al, 2013). A potent activator of dopamine signaling, we hypothesized that cocaine would increase ICSS responding by acting on similar systems to the ICSS itself, that is, by increasing DA levels in the Nacc. A few mechanisms could explain this discrepancy. One, cocaine’s inability to increase responding may be due to an occlusion or ceiling effect, whereby optogenetic stimulation of the dSUB results in similar levels of neurotransmitter release as cocaine or keeps release near a maximal level. Unfortunately, while chemical stimulation of the ventral subiculum can increase DA levels in the Nacc up to 150% (Legault et al 2000) it is not known whether optogenetic stimulation of dSUB increases DA levels in targets of the VTA or by what magnitude. Future studies utilizing microdialysis in conjunction with optogenetic stimulation would answer the question of whether dSUB stimulation results in increased DA release and whether optogenetic stimulation occludes DA release in response to cocaine.

Second, the lack of reinforcing effects of cocaine in dSUB ICSS may be due to the non-contingent delivery of the drug used in the current experiments. Non-contingent delivery of drugs, even drugs of abuse such as cocaine, is often less rewarding than self-administration or even aversive despite direct physiological effects on dopaminergic systems (Twining et al., 2009). One possible experiment to investigate the effects of the dSUB on cocaine self-administration would utilize halorhodopsin, an opsin protein that silences neural activity in response to yellow light, to inactive the dSUB during the acquisition, extinction, and reinstatement of cocaine self-administration behavior. This would determine more definitively
the effect of optogenetic stimulation/silencing on an already established reward-seeking paradigm.

Lastly, it is also possible that optogenetic stimulation of dSUB is occluding cocaine’s effects at the subiculum, as data has shown that bath applied cocaine in acute brain slices causes subicular neurons to switch action potential firing rates from a burst of spikes to single-spiking behavior (Cooper et al., 2006). Optogenetic stimulation at a specific frequency may force a neuron to fire at that frequency regardless of in vivo or cocaine treated firing rates, thus occluding cocaine’s direct effects on subicular neurons. Whole cell patch clamp studies have demonstrated that subicular neurons alter their firing patterns in response to bath applied cocaine (Cooper et al, 2006), thus future work could test whether optogenetic stimulation can occlude cocaine’s effects in the subiculum at a cellular level.

While cocaine administration had little effect on poking rates, administration of dopamine receptor D$_1$ antagonist SCH-23390 significantly reduced poking rates. The dose used is known to cause general locomotor effects (Grimm et al., 2012), however, poking rates at the inactive hole were not significantly different between drug and saline conditions. This may be due to a lack of locomotor effects, or it may be due to a lack of variability caused by the already low poking rates at the inactive hole. Due to experimental limitations, we were unable to directly inject SCH-23390 into the Nacc to specifically test whether Nacc DA drives the reinforcing effects of dSUB ICSS, though previous experiments have shown that intra-Nacc SCH-23390 is sufficient to block VTA DA neuron ICSS without causing locomotor deficits (Steinberg et al, 2014). Overall, it is likely that DA plays a role in dSUB ICSS, but the exact relationship remains unclear.
In summary, high doses of DA and muscarinic cholinergic antagonists are sufficient to block dSUB ICSS behavior, indicating a role for these systems in mediating the reinforcing effects of dSUB ICSS. However, agonists failed to change ICSS rates, suggesting a more complex role for these neurotransmitter systems in mediating optogenetic ICSS. It remains unknown why blockade of cholinergic and dopaminergic systems can reduce dSUB ICSS responding while activation of the same neurotransmitter systems fails to increase it, though it may be due to the artificial nature of optogenetic stimulation. Future studies utilizing microdialysis and direct injection of drugs into target regions may elucidate the role of dopamine and cholinergic systems on dSUB ICSS.
Chapter V: Summary and Discussion

Here we report the novel finding that optogenetic stimulation of dSUB neurons is sufficient to drive ICSS behavior in mice, demonstrating a causal role for the dSUB in modulating reinforcement of motivated behavior. dSUB projections to the Nacc are sufficient to drive this behavior, which we hypothesize drive dSUB ICSS by increasing DA levels in the Nacc. The reinforcing effects of stimulation effect are frequency-dependent, with increasing frequency of stimulation leading to increased reinforcement of ICSS behavior. Mice trained in dSUB ICSS can reinstate nose poking behavior on brief re-exposure to the ICSS paradigm, though the significant increases in responding are limited to re-exposure to ICSS conditions. Lastly, systemic D₁R and mAChR blockade can significantly block dSUB ICSS, indicating that these neurotransmitter systems play a role in mediating dSUB ICSS behavior.

Optogenetic stimulation of dSUB neurons is sufficient to drive increases in nose poking, demonstrating that dSUB neurons contribute to the reinforcement of operant behavior. Stimulation of dSUB projections to the Nacc is also sufficient to drive ICSS, indicating that these projections play a role in mediating the reinforcing effects of dSUB ICSS. We hypothesize that dSUB driven activation of the Nacc is reinforcing due to indirect activation of the VTA, which results in increased DA levels in the Nacc, thus attributing salience to the nose poking behavior or light from the laser stimulation. Non-contingent laser light delivery in previously trained mice did not reinstate ICSS, suggesting that stimulation of dSUB neurons was not acting by mediating feelings of reward.
Stimulation of dSUB neurons versus projections to the Nacc resulted in both larger increases in nose poking rates and higher rates of variability in the data. As suggested by Steinberg et al., (2014), this may be due to differences in the density of transfected light-reactive neurons or axons in each region, or it may suggest that other brain regions beyond the Nacc play a role in mediating the reinforcing effects of dSUB ICSS. Preliminary data suggests that stimulation of dSUB projections to the mPFC is not sufficient to drive ICSS behavior, but future experiments will address whether other projections such as those to the septal nuclei play a role.

We also found that optogenetic driven ICSS is frequency-dependent, with increases in stimulation frequency causing increases in behavioral responses in a sigmoidal fashion. This suggests that optogenetic stimulation of the dSUB drives behavior through increased firing in dSUB neurons and increased neurotransmission at targets rather than through activation of a specific frequency, such as those seen in the hippocampus and subiculum in vivo. Hippocampal theta rhythm oscillations occur at 6-10 Hz, while theta-burst stimulation (a train of five 100 Hz synaptic pulses occurring at 5 Hz for 3 seconds) is known to increase the occurrence of burst firing in subiculum slices. While we are unable to draw direct parallels between the optogenetic stimulation protocols used in the current study and in vivo activation of the subiculum, we hypothesize that the frequency-response curve would have taken an inverted-u shape should an individual frequency of stimulation have driven responding more than others.

Using extinction training and two reinstatement protocols, we demonstrated that contingent delivery of laser light in conjunction with nose poking is necessary for optogenetic ICSS reinforcement. Non-contingent light delivery failed to reinstate the behavior, indicating that light and stimulation itself is insufficient to drive the previously trained nose poke behavior. This suggests that stimulation during the operant behavior is reinforcing due to acting as a
predictive or salient signal in conjunction with that particular behavior rather than being inherently rewarding. Were stimulation of the dSUB inherently rewarding, it is likely that trained mice would have begun nose poking the active hole upon re-exposure to laser light or stimulation of the dSUB or continued to poke after the end of the 15 minute ICSS re-exposure period. This is in contrast to VTA optogenetic stimulation studies, which show that phasic optogenetic stimulation of VTA DA neurons can induce conditioned place preference in animals (Tsai et al, 2009), suggesting that animals can show a preference for an area in which they receive optogenetic stimulation.

Lastly, we investigated how cholinergic and dopaminergic neurotransmitter systems affect dSUB ICSS. Both systems either directly innervate the hippocampus and subiculum (cholinergic signaling) or direct targets of the subiculum (dopaminergic signaling). Thus, both are possible targets for pharmacological modulation of subicular function and ICSS behavior. Previous work using nicotinic and muscarinic antagonists have demonstrated inhibition of plasticity and behavior in the hippocampus (Deiana et al, 2011, Hasslemo, 2006, Power et al, 2003) as well as burst firing plasticity in the subiculum (Shor et al, 2009, Moore et al, 2009). We examined the role of nicotinic agonism and muscarinic antagonism in dSUB ICSS using nicotine and scopolamine, respectively. While nicotine had no significant effects on dSUB ICSS responding, muscarinic antagonism significantly reduced responding rates. This joins a large body of literature demonstrating scopolamine’s ability to interfere with hippocampal behaviors such as spatial navigation and memory formation and retrieval (Easton et al, 2012). However, the precise mechanisms behind scopolamine’s ability to inhibit dSUB ICSS are not clear. In the subiculum, administration of muscarinic antagonist atropine inhibits the induction of burst firing
plasticity (Moore et al, 2009). Thus, a decrease in burst firing mediated by scopolamine may result in significantly lower activity in target regions despite optogenetic stimulation.

DA plays a key role in mediating reinforcement and reward behaviors, and activation of the ventral subiculum leads to increases in DA levels in targets of the VTA (Blaha et al 1997), indicating a possible role for DA in mediating dSUB ICSS. Additionally, the dSUB and VTA both project to the mPFC and Nacc, and the VTA projects to the septal nuclei and hippocampus, providing several possible targets for DA to modulate dSUB ICSS behavior. Interestingly, acute and chronic agonism of dopaminergic systems using cocaine had no effect on dSUB ICSS. This suggests that DA release from the VTA may not be the primary driving force behind the reinforcement of dSUB ICSS. However, this lack of an effect may be due to a ceiling effect or an occlusion of the drug’s effects caused by optogenetic stimulation. Antagonism of DA signaling through systemic administration of D₁R antagonist SCH-23390 significantly reduced dSUB ICSS at higher doses, suggesting that DA does indeed still play a role in mediating dSUB ICSS. It is possible that this effect was due to locomotor inhibition, as the injection was systemic, though it is worth noting that poking rates at the inactive lever did not change significantly compared to saline controls.

Together, this data suggests that multiple neurotransmitter systems may play a role in mediating dSUB ICSS, though precisely how remains unclear. While the current experiments demonstrate the ability of systemic injections to inhibit dSUB ICSS responding, future experiments should use cannulae to deliver drugs to specific regions. This will determine exactly where neurotransmitters are acting to reinforce operant behavior in dSUB ICSS. For example, intra-Nacc infusions of SCH-23390 would specifically determine whether increases in Nacc DA occur in dSUB ICSS and whether they are necessary for driving responding. Prior work has
demonstrated the ability of intra-Nacc SCH-23390 to abolish VTA-mediated ICSS behavior (Steinberg 2014). A similar experiment using dSUB stimulation would further determine whether Nacc DA release is critical for driving dSUB ICSS, and that dSUB ICSS likely leads to indirect activation of the VTA.

Together, this data demonstrates a causal role for activation of the dSUB and subicular projections to the Nacc in reinforcing operant responding. However, the precise pathways mediating dSUB ICSS are not fully understood. The description of an indirect vSUB-VTA activation loop by Grace et al., (2010) provides a possible analogous mechanism through which dSUB stimulation can lead to increased DA levels in the Nacc and increase operant responding. (Figure V-1). The ability of dSUB to Nacc terminal stimulation to mediate ICSS and the ability of systemic SCH-23390 to abolish responding supports this hypothesis, though further experiments are needed to verify whether dSUB stimulation actually activates the VTA and leads to DA increases in targets. Additionally, stimulation of dSUB projections to the Nacc increased responding at a lower magnitude than direct dSUB stimulation. This could be due to a decrease in the density of

![Figure V-1. Proposed self-stimulation circuit diagram.](image)
light-sensitive tissue, or it could suggest that the Nacc only plays a partial role in mediating
dSUB ICSS, and that other regions contribute to the expression of dSUB ICSS. Another possible
region of interest is the septal nuclei. The initial experiments describing ICSS involved
stimulation of the septal area (Olds and Milner, 1954), demonstrating that stimulation of this
region can support ICSS. The dSUB sends projections to and receives input from the lateral
septum, thus stimulation of the dSUB stimulates the lateral septum, possibly contributing to the
reinforcing effects of dSUB stimulation.

The present findings describe a novel function of the dSUB: mediating reinforcement of
operant behavior. This adds to current literature investigating the importance of not only primary
reward regions such as the Nacc and VTA in mediating reinforcement and reward-seeking
behavior but also peripheral brain regions that project to these areas such as the hippocampus
and frontal cortex (Chen et al, 2013). New tools such as optogenetics make the dissociation of
neural circuits possible, allowing researchers to directly investigate the importance of individual
pathways and cell types in driving motivated behaviors. This will, in turn, facilitate better
understanding of the cellular and circuit-level mechanisms behind motivated behaviors and lead
to novel therapeutic targets for disorders of motivation such as addiction and obsessive-
compulsive disorder.
References


