2014

Aiming Attention Using Selective Stimulation of Locus Coeruleus and Its Medial Prefrontal Efferents

Zackary A. Cope
Medical University of South Carolina

Aiming Attention Using Selective Stimulation of Locus Coeruleus and Its Medial Prefrontal Efferents

by

Zackary A. Cope

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Neurosciences

2014

Approved by:

Gary S. Aston Jones

Advisory Committee Chairman _____

Lawrence J. Chandler _____

Thomas C. Jhou _____

Mark A. Eckert _____

Stan B. Floresco _____

ABSTRACT

Appropriate modification of behavior in response to dynamic environmental conditions is essential for the adaptation and survival of most biological organisims. This adaptability allows for organisms to maximize the benefit of behavior related energy expenditure (utility) while minimizing cost. Modern theories of locus coeruleus (LC) function implicate a pivotal role for the noradrenergic (NA) nucleus in mediating switches between focused behavior during periods of high utility (exploit) versus disengagement of behavior and exploration of other, more rewarding opportunities. Two modes of activity in LC neurons have been well characterized. During periods of accurate and focused behavior, LC neurons exhibit supressed baseline activity and task-related phasic bursts. However, as focus and accuracy wanes, phasic activity is supressed and baseline (tonic) impulse activity is elevated. These experiments sought to exogenously induce a tonic pattern of activity in LC neurons and their medial prefrontal cortical (mPFC) efferents to test the tenets of adaptive gain theory. This theory posits that phasic activity facilitates focused task performance whereas tonic activity promotes disengagement from ongiong behaviors. Thus, tonic activation immediately following a rule chage should be sufficient to improve performance on a set-shifitng task. Indeed, DREADD mediated stimulation of LC terminals within the mPFC decreased trials to reach criterion. However, this effect appears to result from improved application of the new rule rather than an induction of a behaviorally flexible phenotype. Further, these results were not seen for manipulations administered within the LC. These findings may reflect a new understanding of the role of LC in set-shifting and flexible behavior.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

LC: Locus Coeruleus

NA: Noradrenaline, Noradrenergic

NE: Norepinepherine

mPFC: Medial Prefrontal Cortex

DREADD: Designer Receptors Exclusively Activated by Designer Drugs

aSST: Automated Strategy Set-Shifting Task

WCST: Wisconsin Card Sorting Task

ADHD: Attention Deficit Hyperactivity Disorder

OCD: Obsessive Compulsive Disorder

PTSD: Post-Traumatic Stress Disorder

ASST: Attention Set-Shifting Task

ACC: Anterior Cingulate Cortex

6-OHDA: 6-hydroxy Dopamine

DNAB: Dorsal Noradrenergic Bundle

CNO: Clozapine n-Oxide

ChR2: Channelrhodopsin 2

PRSx8: Phox-2 Responsive Synthetic x8

AP: Anterior/Posterior

ML: Medial/Lateral

DV: Dorsal/Ventral

CxEEG: Cortical Electroencephalogram

HpEEG: Hippocampal Electroencephalogram

PBS: Phosphate Buffered Saline

PBST: PBS with Triton

TH: Tyrosine hydroxylase

EDS: Extradimensional shift

REV: Reversal

TTC: Trials to Reach Criterion

hM3Dq: Gq-coupled Human Muscarinic Designer Receptor 3

PL: Prelimbic cortex

IL: Infralimbic Cortex

hM4Di: Gi-coupled Human Muscarinic Designer Receptor 4

IP3: Inositol Triphosphate

DA: Dopamine

ACKNOWLEDGEMENTS

I would like to begin by thanking my graduate mentor Gary Aston-Jones. It is his prodigous body of work and talent that laid the foundation for this research. By his guidance and that of the members of my dissertation committee, Judson Chandler, Stan Floresco, Thomas Jhou, and Mark Eckert, this otherwise unwieldy project was expertly steered into port. I would also like to extend thanks to every member of the Aston-Jones laboratory that has come and gone during my time at MUSC. Special recognition is deserved by Elena Vazey, David Moorman, Steven Mahler, Matthew Riedy, Brittney Cox, and Michael Smith who all directly contributed to the success (and none of the failures) of this project. Most of all, I want to thank Emily Ball for her love, understanding, tolerance, and unwaivering faith throughout this tribulation. It is her constant support that has sustained me throughout.

1 INTRODUCTION

In order for any organism to survive and thrive in a constantly changing environment, the ability to adapt is essential. Adaptability allows an organism to maximize benefit while minimizing cost when faced with an array of opportunities. Inherent in this ability is the capacity to identify when utility (effectiveness of an ongoing task in obtaining reward) is waning so that one may disengage from ongoing goals to seek other, more rewarding opportunities.

Clinically, this ability is often measured with tests of behavioral flexibility and set shifting, such as the Wisconsin Card Sorting Task (WCST). Such tests have revealed that impairments in behavioral flexibility are prominent in several mental illnesses. Perhaps the most readily identifiable example, ADHD, is characterized as an inability to appropriately allocate attention when it is necessary (Seidman et al., 1997). An inability to ignore ambient or predictable environmental stimuli is a key feature in schizophrenia (Braff et al., 2001). In stark contrast, other mental illnesses such as addiction, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), and autism are frequently characterized by inabilities to disengage attention from a particular stimulus to attend to more pertinent concerns (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force., 2013).

Adaptive flexibility may rely on striking a proper balance between inattention and focus. Frequently, inattention is characterized as a qualitatively bad trait and focus as a good. However, the extremes of this continuum both serve a specific function in

exploring and exploiting our environment and being overly biased to either side of that continuum could be detrimental. Understanding how this balance is maintained and finding ways to intervene on this system when the balance has become inordinately tipped may greatly enhance the treatments available for these disorders.

The Locus Coeruleus and Cognitive Control

Mounting evidence, including many studies from our laboratory, implicates the locus coeruleus NE (LC-NE) system as an integral component in the ability to behave flexibly (Usher et al., 1999; Aston-Jones et al., 2000; Aston-Jones and Cohen, 2005a, b; Bouret and Sara, 2005; Corbetta et al., 2008). Although the prevailing theories of LC function (adaptive gain, reorienting system, network reset) may differ in their interpretation of how this system drives behavioral flexibility, all propose that this system is critical for the adaptive control of behavior (Aston-Jones and Cohen, 2005a; Bouret and Sara, 2005; Corbetta et al., 2008).

The locus coeruleus is a relatively small nucleus located in the dorsorostral pons. Efferent LC projections are highly divergent and course throughout the CNS. The LC was long thought to be the only source of NE innervation in many telencephalic areas, including hippocampus

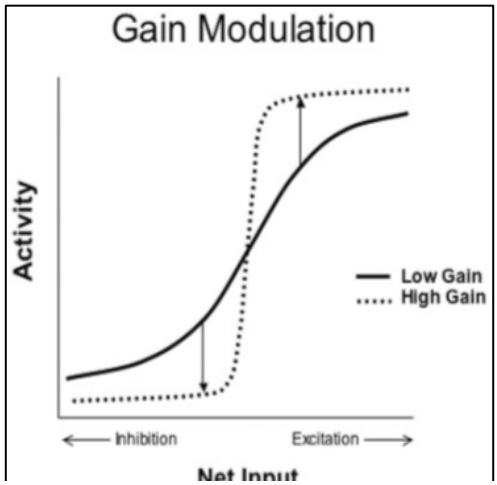

Figure 1.1: Unitary action of LC increases signal to noise ratio, facilitating signal throughput related to task relevant stimuli. (Servan-Schreiber et al., 1990)

and cerebral cortex (Berridge and Waterhouse, 2003). Although, a recent study reported that a very small but consistent percentage (<1%) of noradrenergic axons in the mouse somatosensory cortex derive from a different rhobomeric origin than LC (Robertson et al., 2013). Regardless, in terms of prefrontal executive functions such as cognitive flexibility, LC is the predominant source of adrenergic modulation of these behaviors.

Depending on the receptor subtypes present on target neurons, actions of LC projections can impose temporally specific and opposing excitatory (α_1, β receptors) or inhibitory (α_2 receptors) modulation in separate targets. This modulatory input from LC is thought to increase the gain (synaptic responsiveness) of target cell activity, a property that has been modeled mathematically (Fig 1.1)(Servan-Schreiber et al., 1990).

Early investigation into afferent control of the LC focused predominantly on sub-cortical structures describing only two major afferent projections to the LC, the nuclei paragigantocellularis lateralis and prepositus hypoglossi (Aston-Jones et al., 1986). More recently, it has become clear that LC also receives important inputs from frontal cortex. Neurophysiological studies have demonstrated that microstimulation of the mPFC exerted strong excitatory influence on LC (Jodo et al., 1998). Later, retrograde tracing analysis from the LC demonstrated strong afferent connectivity to the LC from the orbitofrontal cortex (OFC) and ACC in monkeys (Aston-Jones and Cohen, 2005a, b). Due to the known roles of these cortical regions in cognitive functioning, discussed below, they may represent highly attractive targets for future study into the afferent cortical control of LC and subsequent influence on attentional task performance.

Early recording studies in LC revealed phasic activation of neurons following salient stimuli, which provoked an orienting response (Aston-Jones et al., 1986). However, subsequent study revealed a considerably more complex cognitive role for the LC-NE system (Aston-Jones and Cohen, 2005a, b; Bouret and Sara, 2005; Corbetta et al., 2008). Prior research by Aston-Jones and colleagues outlined two modes of activity in LC neurons, termed phasic and tonic. The phasic mode is characterized as an overall decrease in baseline firing coupled with a burst of activity following presentation of a target stimulus but preceding a behavioral response. In contrast, tonic mode is associated with elevated baseline LC impulse activity but little to no phasic response to stimuli or events. During the phasic mode of LC firing, performance on various cognitive tasks that require focused attention was facilitated in both monkeys (Usher et al., 1999; Rajkowski et al., 2004) and rats (Bouret and Sara, 2004). This phasic firing tightly precedes, and is thought to represent commitment to a behavioral response, rather than simply representing sensory components of stimulus detection (Clayton et al., 2004; Aston-Jones and Cohen, 2005a). Conversely, high levels of tonic firing without phasic activations (i.e., tonic mode) are related to increased errors on tasks that require focused attention (Usher et al., 1999; Rajkowski et al., 2004) as well as decreased foveation of a target stimulus used to measure readiness and task attentiveness (Aston-Jones et al., 1996).

These patterns of LC activity are thought to represent a mechanism by which an organism can appropriately attribute attention. Phasic mode would facilitate sustained attention to maximize reward in a period of high opportunity (exploit). In contrast, tonic mode would result in disengagement from a task as reward and utility wanes in order to

facilitate exploration of more rewarding options (explore). The AGT represents this relationship as an inverted U-shaped curve resembling a classic Yerkes-Dodson relationship between firing mode and task performance (Fig. 1.2)(Aston-Jones and Cohen, 2005a).

Other theories have also described a role of the LC in behavioral flexibility. One such theory, the network reset theory, posits that, upon detection of a target stimulus, phasic LC activity facilitates a redistribution of activity between target neural networks, which is needed in order to execute the cognitive shifts required for accommodation to a dynamic environment (Bouret and Sara, 2005). Additionally, the reorienting system theory interprets behavioral flexibility in terms of an interaction between ventral and dorsal neural networks in frontoparietal cortices. In this framework, dorsal networks form associations between environmental stimuli and adaptive responses. Ventral networks interrupt ongoing behavior to allow more adaptive responses to take place. This theory posits that LC phasic input facilitates continuation of a behavior whereas high tonic input results in an interruption of this behavior to allow a shift to take place (Corbetta et al., 2008). Although these three prevailing theories differ somewhat in terms of their theoretical predictions and interpretation of the current data, all three posit that LC-NA transitions

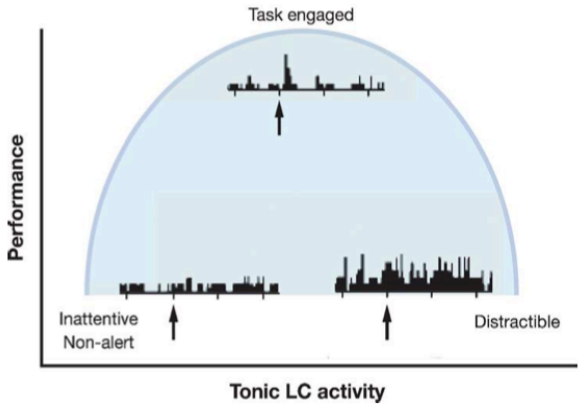

Figure 1.2: Yerkes-Dodson relationship of tonic LC activity to task performance (Aston-Jones and Cohen, 2005a).

between phasic and tonic firing are essential to behavioral flexibility. However, to verify this relationship it is important to demonstrate that direct manipulation of LC firing can influence attentional control.

A Clinical Profile of Behavioral Flexibility

In the clinical setting, the Wisconsin Card Sorting Task (WCST) is a standard test for assessing frontal lobe function, specifically as it pertains to cognitive flexibility. In the WCST, behavioral flexibility is assessed by asking the patient to sort a deck of cards by the stimuli shown on the cards according to a set of unknown rules that can change at any time. The stimulus dimensions by which the cards can be sorted include suit, number, or color, of stimuli, all of which have an array of forms. During the test, the facilitator only indicates that sorting is correct or incorrect. When the test administrator indicates that sorting is being done incorrectly, the patient must adopt a new sorting strategy until the facilitator indicates they are doing so correctly.

Many patients with mental illnesses that include impairments in attention demonstrate deficits when performing this task. Schizoaffective children (Schneider and Asarnow, 1987) and schizophrenic adults (Choi and Kurtz, 2009) both commit frequent preservative errors, or sorting according to old rules indicating an impairment in the ability to drop an old strategy. Severity of opioid dependence is also predictive of and associated with preservative errors on the WCST (Lyvers and Yakimoff, 2003; Pirastu et al., 2006) indicating behavioral inflexibility that may result from an inability to transition from phasic to tonic LC mode when faced with stimuli related to the drug. WCST

impairments have also been reported in obsessive-compulsive disorder (Lacerda et al., 2003; Shin et al., 2008) as well as autism (Kaland et al., 2008).

Of the disorders associated with impaired behavioral flexibility, ADHD may be the most obviously related to this function. Studies assessing ADHD in children using WCST have produced mixed results, failing to define a clear profile of cognitive flexibility in ADHD. However, it is clear that these individuals are impaired in performing the WCST. In a meta-analysis of child psychiatric studies using the WCST, children with ADHD demonstrated a lower percent of trials correct, increased error commission, completion of fewer categories, and increased perseveration (Romine et al., 2004). Perseveration is seen in adolescents with ADHD as well (Reeve and Schandler, 2001). Thus it is noteworthy that low doses of methylphenidate (Ritalin), a drug commonly prescribed to treat ADHD, has been found to suppress tonic LC firing while enhancing the signal to noise ratio of the phasic inhibitory response (Devilbiss and Berridge, 2006). These results indicate that the efficacy of methylphenidate to facilitate task performance may rely on an overall suppression of tonic LC-NA activity that results in decreased distractibility rather than enhanced focus.

An Experimental Profile of Behavioral Flexibility

The attention set shifting task (ASST) was designed as a laboratory analog of the Wisconsin Card Sorting Task (Birrell and Brown, 2000). In the conventional ASST, rats are presented with a choice of two dishes filled with two different scented digging media. In one example, there are 2 stimulus dimensions, digging media and scent, with 2 forms

of each stimulus dimension presented in every trial. Only one of the two pots is baited with a reward on any given trial; the bait corresponds to either a specific scent or a specific digging media. The rat must learn to discriminate which one of the 4 presented stimuli (2 media X 2 scents) predicts the food reward and dig in the corresponding pot to receive the reward. When their performance on the task reaches criterion, manipulations can be performed to assess behavioral flexibility. For instance, without presenting any novel forms within the previously rewarded stimulus dimension, the rat must learn that the opposite form within the same stimulus dimension is predictive of the food reward on subsequent trials. This is termed reversal. If 2 novel stimuli are introduced within the same discriminative dimension, it is termed an intradimensional set-shift. As a final challenge, one can require the rat to learn that the previously rewarded dimension is no longer predictive of reward but, rather, the other dimension is then relevant for receipt of the food reward. This is termed an extradimensional shift. This paradigm quantifies behavioral flexibility in terms of how many trials are required to learn that a different stimulus or dimension is relevant once the rules of that task switch. This form of the ASST has been used successfully in many experiments to test how different manipulations can interfere with behavioral flexibility (Birrell and Brown, 2000; Tait et al., 2007; McGaughy et al., 2008). The following studies use a simplified version of this task, requiring lever responding according to relevant stimuli (Darrah et al., 2008; Floresco et al., 2008; Haluk and Floresco, 2009). This type of set-shifting task lends more temporal precision of stimulus presentation, increased throughput, and richer trial-by-trial error analysis.

The Automated Strategy Set-Shifting Task (aSST)

In order to make claims as to what temporally precise manipulations are doing to behavior, the task used to measure behavior must be also be temporally precise. The traditional ASST presents many challenges to this end. Foremost, the stimuli used can be quite messy and imprecise. Digging media can become scattered throughout the testing chamber and scents may be perceptible throughout the entire testing session, rather than only when a trial commences. Additionally, this task requires setup and cleaning between trials, which introduces a high degree of temporal variability during the inter-trial interval. Lastly, due to how the stimulus exemplars are presented, it is not possible to analyze the exact type of errors the rat is making on each given trial.

The following research utilizes an automated version of this task, which overcomes many of these challenges. This specific task, developed by Floresco and colleagues, has been used effectively to examine the role of mPFC (Floresco et al., 2008) and ventral striatum (Haluk and Floresco, 2009) in set-shifting behaviors. In this task, two cue lights are each positioned above a retractable operant lever. At the beginning of the trial, one of the two cue lights, pseudo-randomly chosen on every trial, is turned on for three seconds before the levers are inserted into the behavioral chamber. The rat must press one of the levers to receive a sucrose reward (see figure 3.3). Two stimulus dimensions present in the chamber can indicate to the rat which lever will result in the reward on every trial. The rat can either press the lever corresponding to the illuminated cue light or the rat can use a spatial strategy, pressing only the left or right lever on every trial. Animals are initially

trained to press the lever under the illuminated cue light. Once they are able to do so for a streak of 20 correct trials, they are returned to the chamber on the following day to undergo an EDS. The first 20 trials of the EDS follow the known light rule. However,

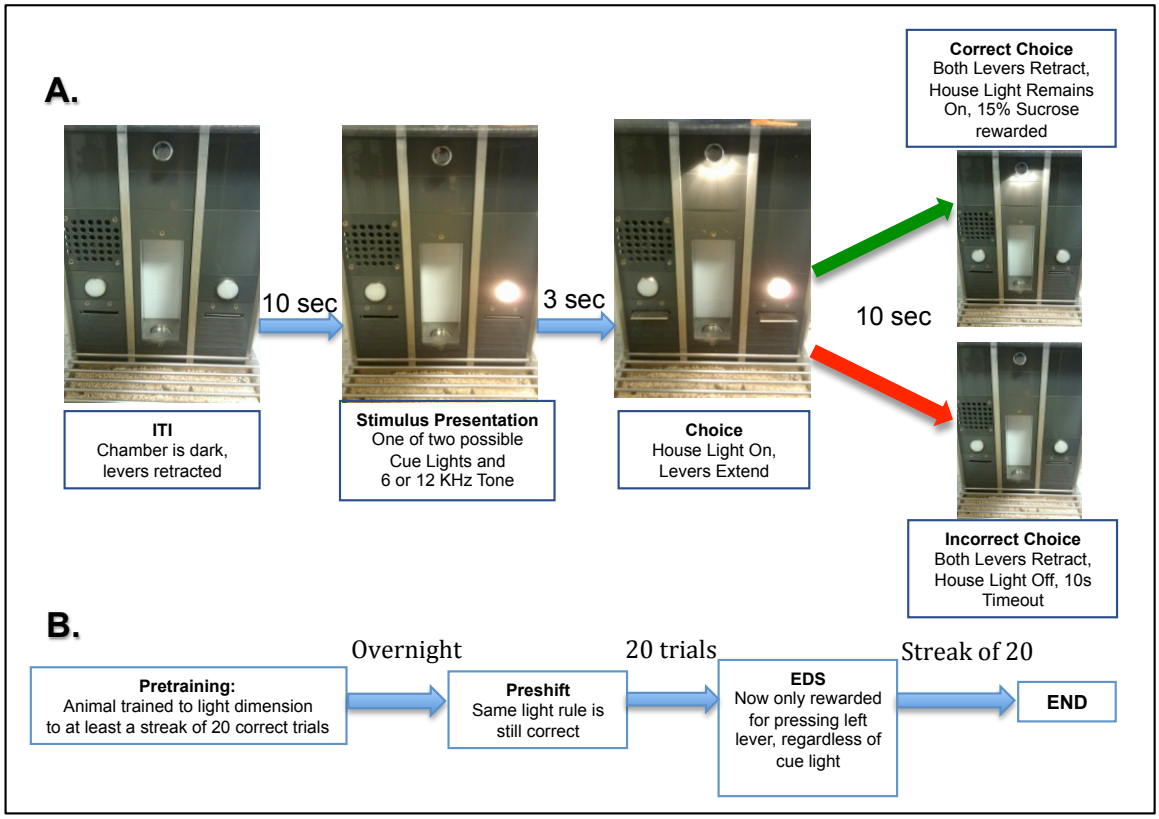

Figure 1.3 A depiction of the automated strategy set-shifting task (A). Rats are pre-trained to follow the light cue (or tone on ChR2 experiments only) to receive a sucrose reward. Once able to execute this rule correctly for a streak of 20 trials the rats will be returned to the testing chamber to perform the EDS (B).

without cueing the rat in any way, the rule switches so that the rat must press according to the spatial position of the lever, regardless of the position of the cue light. The rat must learn that the old rule is no longer discriminative of the reward, disengage from that strategy, and learn to follow the new rule. According to AGT, tonic stimulation of LC

immediately following the rule change should facilitate disengagement from the old strategy evident by a decrease in perseverative behavior. If the rat ceases perseveration sooner, it should take the rat fewer trials to reach criterion performance on the new rule.

A Simple Neural Circuit for Behavioral Flexibility

As previously mentioned, LC receives considerable afferent drive from the frontal cortex (Jodo et al., 1998). Retrograde tracer analysis has determined that ACC projects to LC (Aston-Jones and Cohen, 2005a, b). When engagement in an ongoing task becomes ineffective for attaining a reward, it would be adaptive for the LC to transition from phasic to tonic firing to facilitate task disengagement and exploration for other possibly rewarding opportunities. Due to its widely theorized role as a monitor of conflict in task performance, it is likely ACC may be driving this transition. This theory has been largely derived from electrophysiological and imaging studies in humans. These data have shown that the ACC responds to a broad spectrum of negative stimuli related to monetary loss (Williams et al., 2004), social rejection (Eisenberger et al., 2003), and error commission (Brázdil et al., 2002; Fitzgerald et al., 2005; Yeung and Cohen, 2006). Additionally, task difficulty (Barch et al., 1997) and tasks imposing conflicting choices such as overriding a prepotent response (Barch et al., 2001), or choosing between two overlapping choices (Barch et al., 2000), have been shown to modulate activity in the ACC. However, it remains to be determined how this role of the ACC is functionally incorporated into more complex behaviors. There is ongoing controversy as to exactly what information is being encoded by the ACC. Some reports indicate that ACC in

primates does not respond simply to conflict inducing stimuli (Nakamura et al., 2005; Emeric et al., 2008), but is modulated by error commission, reinforcement, and conflict related to opposing responses (Ito et al., 2003). It has also been demonstrated that activity in rat ACC increases during periods of attention preceding stimulus presentation as well as immediately following an incorrect trial. This may indicate that the ACC is determining probability of error commission for possible behaviors by comparing against the success of prior strategies. Within the context of the this study, we hypothesize that ACC monitors conflict in ongoing performance, and that, when probability for conflict outweighs the cost of performing the task, the ACC helps to drive the transition from phasic to tonic firing. This would, in turn, facilitate task disengagement and increased behavioral flexibility.

The role of LC input to the cortex in behavioral flexibility has been more clearly delineated. In rodents, lesions of the mPFC and its subfields were shown to impair behavioral flexibility on strategy-based tasks such as various radial arm mazes (Joel et al., 1997) or place-response cross maze tasks (Ragozzino et al., 1999). Additionally, mPFC ibotenic acid lesions selectively impaired extradimensional shifting on the ASST (Birrell and Brown, 2000). Additional data suggest that it is the LC-NA inputs into these regions that modulate flexibility in this task. 6-OHDA lesions of the dorsal noradrenergic bundle (DNAB) of ascending NE fibers from LC (the input of NE to mPFC) were found to also selectively impair extradimensional shifting. Similar results were found for selective noradrenergic de-afferentation, of the mPFC (McGaughy et al., 2008). Together, these results consistently reveal that LC-NA innervation of mPFC is necessary for behavioral

flexibility measured by the ASST. However, sufficiency of LC-NA input into the mPFC for mediating behavioral flexibility has yet to be demonstrated due to methodological challenges that have only recently been overcome.

Emerging Tools for Cell Specific Neural Control

Until recently, methods for activating or inhibiting neurons have been unable to unify cell-type specificity with precise temporal control. Pharmacological methods, although somewhat specific, are unable to reproduce the rich, temporally precise, and dynamic neural signals conveyed by neural units. On the other hand, electrical methods can endow temporally precise control of neural units, but lack specificity as nearby units of all types are affected. Newly developed tools using microbial opsins provide a means to achieve both criteria. Type I opsins are a class of light sensitive proteins that have been isolated from algae and fungi. These proteins bind the photopigment retinal in all-*trans* conformation, which photoisomerizes to the 13-*cis* conformation while remaining bound to the opsin protein. This conformational change in the photopigment induces a conformation change in the opsin that will produce changes in local ion-flux. Depending on the nature of the opsin, the resulting ion flux can be either excitatory or inhibitory and can be triggered by specific wavelengths of light. These proteins can be delivered to cells by viral vectors with cell-type specific promoters and stimulated by small, implantable optical fibers connected to a laser of appropriate wavelength (Boyden et al., 2005; Fenno et al., 2011).

Using these techniques, researchers have demonstrated unprecedented precision and control, both excitatory and inhibitory, of brain neurons (Boyden et al., 2005; Stuber, 2010; Stuber et al., 2010; Tye et al., 2011). Results preceding this study have achieved this degree of control in rat LC-NE neurons *in-vivo* using the excitatory cation channel opsin channelrhodopsin-2 (ChR2) under control of a artificial dopamine β-hydroxylase (DBH) promoter PRSx8 (Vazey et al., 2011a; Vazey et al., 2011b). These types of manipulations are sufficient to drive modulations in target tissues (Boyden et al., 2005; Kanbar et al., 2010; Stuber, 2010; Stuber et al., 2010; Tye et al., 2011) and have produced measurable changes in behaviors of rats (see below) and mice such as sleep to wake transitions and behavioral arrest (Carter et al., 2010) and bi-directional control of anxiety (Tye et al., 2011). Although expression of these proteins can be more easily accomplished by transgenic manipulations in mice, the limited behavioral range of these animals impairs study of complex cognitive behaviors.

Another emerging tool, pharmacosynthetic DREADDs, also confer unprecedented cell specificity of stimulation or inhibition while also preserving typical receptor kinetics of neural systems. DREADDs are muscarinic g-protein coupled receptors that activate endogenous Gs, Gi, or Gq cell signaling pathways. However, through a process of controlled selection, these particular variants have evolved in a directed manner so that they bind no endogenous ligand. Instead, these receptors bind and are activated by the biologically inert designer drug CNO. When bound, these receptors activate their corresponding g-protein pathway. Studies have shown DREADDs to be sufficient for activating (Alexander et al., 2009; Krashes et al., 2011) and inhibiting (Armbruster et al.,

2007; Nair et al., 2013) target cells. Experiments using DREADDs have shown them to be sufficient to modulate depression like behaviors (Nair et al., 2013), reward learning (Ferguson and Neumaier, 2012), feeding (Krashes et al., 2011), locomotion, and seizure activity (Alexander et al., 2009).

The following studies seek to employ these novel tools for specific neural control in the LC of rats performing the aSST. These new optogenetic and pharmacosynthetic techniques could extend these results and directly answer theoretical questions posed by AGT. In doing so, these experiments reveal that cell specific stimulation of LC efferents in PFC is sufficient to improve set-shifting performance, whereas stimulation within LC, either by optogenetic or DREADD based means, failed to improve set-shifting performance. Further, improved performance on the task does not appear to result from a reduction of perseverative behavior but, rather, a reduction in regressive responding. That is, the animals receiving this stimulation are better able to consistently utilize a new strategy once it has been discriminated.

2. TOWARDS A FRAMEWORK FOR OPTIMIZED SET-SHIFTING

According to adaptive gain theory, induction of a tonic pattern of activity in LC cells should result in a flexible behavioral phenotype that will allow for exploration of environmental contingencies and modification of ongoing behavior. Oppositely, induction of phasic activity should result in a focused behavioral phenotype, fortifying ongoing behavior. Thus, the delivered stimuli could have either beneficial or detrimental

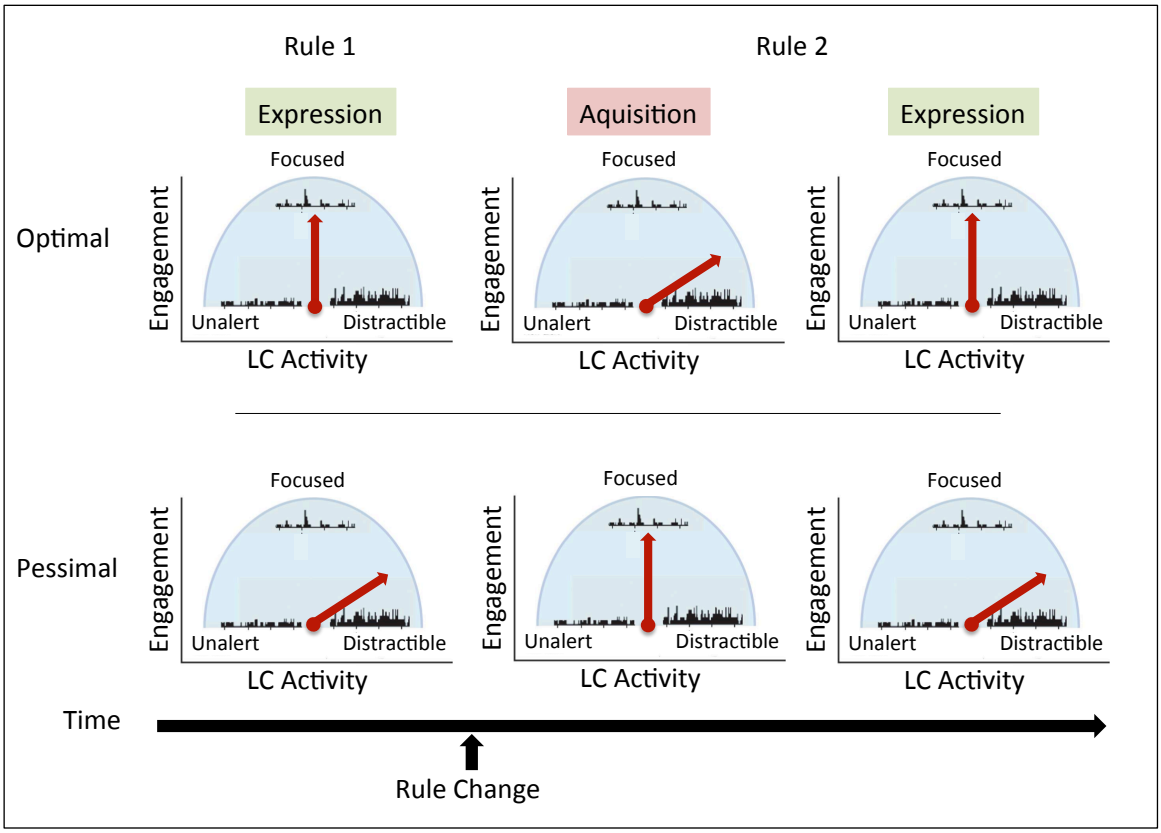

Figure 2.1 Varying patterned activity of LC during different epochs of a set-shifting procedure could produce differing behavioral phenotypes that could either be advantageous or detrimental to overall task performance.

effects on shifting behavior depending on the timing and pattern of the stimulation delivered (see figure 2.1). Optimal performance would initially require focused performance on the known rule before the shift. However, following the rule change, LC would optimally switch into a pattern of elevated tonic activity to facilitate exploration of new task contengencies. Then, as the animal began to acquire the new rule, LC would transition back into a phasic pattern to facilitate consistient expression of the newly learned behavior.

In theory, temporally inverting these patterns should lead to the worst behavioral outcome on a set-shifting task. Tonic activity during the pre-shift epoch could impair consistient execution of the known rule. Phasic activity immediately following the rule change would prevent the animal from disengaging from the behavior that is no longer beneficial. Finally, as the animal began to discriminate the new rule, sustained tonic activity might prevent its consistient application on every trial.

Accounting for the assumptions of adaptive gain theory, it is clear that the stimulation delivered to LC throughout these procedures would need to be as temporally precise as possible both in terms of duration and timing. These sets of experiments aimed to induce a pattern of tonic activation immediately following the rule change to induce a flexible behavioral phenotype during the acquisition phase of the new rule. The behavioral outcome of this manipulation should manifest as a decrease in perseverative behavior, which should allow the rat to discriminate the new rule sooner. This would result in an overall decrease in the trails taken to reach criterion. This chapter will outline a number of preliminary experiments undertaken, which served to optimize the method of

stimulation and behavioral parameters to selectively stimulate LC and its PFC efferents within the aSST.

Verification of Optogenetic LC Stimulation

Before attempts to employ optogenetic methods within a complex behavioral paradigm, intitial efforts focused on developing and verifying techniques to administer these manipulations in the awake, behaving rat. First, the small size of LC and its location directly adjacent to the 4th ventricle required determination of surgical methods for consistiently accurate viral injection and implantation. Second, the viral construct used to transduce ChR2 in LC required vetting for specificity and potency. Finally, sufficiency of these methods to produce quantifiable behavioral and physiological changes that are consistient with an LC elicited increase in arousal needed to be demonstrated.

Methods: animal care and surgery. All methods used were in compliance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee. Animals were all housed in a temperature and humidity-controlled room under a reverse 12 h light/dark cycle (lights on at 6pm) with food and water available *ad libitum*. Animals were allowed 5 days for acclimatization and handling before any experimental procedures commenced.

The lentiviral vector used in this initial trial study was cloned and packaged by the University of Pennsylvania Viral Vector Core. LC specific expression of the mCherry reporter tagged ChR2 was achieved using the synthetic dopamine beta-hydroxylase

promoter PRSx8 (Hwang et al., 2001). For surgical procedures, rats were anesthetized and maintained at a therapeudic plane of anesthesia with isoflurane. They were then placed into a stereotaxic frame and their nose was tiled downward so that bregma measured 2 mm below true lambda. This was done so that LC targeted implements would avoid the transverse sinus. A craniotomy was then drilled at AP -3.7 mm, ML ± 1.2 mm as measured from true lambda. Bone fragments and dura mater was cleared from the surface of the cerebellum to ensure clear entry of glass pipettes into tissue. A chloridated silver wire was inserted into a pontamine blue solution filling a glass pipette with a tip precisely broken at broken at 2.5 µm to produce a high impedance recording electrode (~20MΩ). This electrode was attaced to a hydraulic micromanipulator (Stoelting) and lowered ventrally at AP -3.7 mm, ML ±1.2 mm. Recordings were made as the pipette was lowered in this vicinity until single LC units could be discriminated based on their waveform components, slow basal rate (2-3 Hz), and burst pause activity resulting from a brief toe pinch (Aston-Jones and Bloom, 1981). When LC had been identified, a double barrel pipette consisting of an identically pulled recording pipette precisely glued 200 µm below the tip of an angled injector pipette with a 40 µm opening was filled with 3 µl of the viral construct and attached to the micromanipulator. This double pipette was then lowered through the same track until LC cells were again located. The virus was unilaterally delivered with brief pheumatic pulses (Picospritzer III, Parker Instruments) over a 100 µm ventral to dorsal extent within the identified LC area. The injector then remained in place for 15 minutes to allow for diffusion of the virus before it was

withdrawn. A 22G guide canula (Plastics One) was then lowered 1.5 mm above the dorsal most identified LC cell and secured in place with dental cement.

Four skull screws with tightly wound 0.010 inch diameter stripped stainless steel wire were affixed to the skull for recording of cortical (CxEEG) and hippocampal (HpEEG) electroencephalographic activity. Each wire end was soldered to a female Amphenol pin. CxEEG leads were placed at AP -1.0 mm, ML ±3.0 mm (positive, measured from bregma) and AP +1.0 mm, ML ±4.0 mm (negative, measured from lambda). HpEEG leads were placed at AP +4.0 mm, ML ±1.0 mm (positive, mesured from lambda) and AP +3.0 mm, ML ±1.0 (negative, measured from bregma). In addition, two partially stripped wires were threaded under each of the animals' trapezius muscles to record electromyographic (EMG) activity of the head and neck. The female amphenol leads were then plugged into a six-pin plastic coupler (Plastics One) and the entire apparatus was secured to the skull with dental acrylic. Rats were then returned to their homecage and allowed two weeks for recovery and viral expression.

Methods: optogenetic LC stimulation and EEG recording. Experiments were performed a minimum of one hour into the animals' light cycle. Rats were acclimated over the course of one week to fall asleep in their homecage placed inside a soundproofed chamber. On stimulation and recording sessions, rats were lightly anesthetized to allow for insertion of the optical fiber and attachment to the EEG recording plug. A 200 μM diameter optical fiber (Thorlabs) was glued inside a 28 gauge injector canula (Plastics One) and precision cut with a diamond knife to protrude 0.5 mm from the end of the injector, which protruded 1 mm from the guide canula when inserted and secured. A six-

channel cable fitting the coupler (Plastics One) was plugged in and secured. The optical fiber was then attached to a 473λ solid-state laser (OEM Laser Systems) and the EEG cable through a bioamplifier (CWE Inc.) to a data acquisition interface (CED Micro 1401). The CED was also used to deliver TTL pulses to drive the laser and to process EEG recordings. Spike 2 software (CED) was used to acquire EEG/EMG traces in real time and to simultaneously deliver trains of stimulation with the laser.

The rat was allowed to wake from anesthesia and was then placed into the soundproofed chamber to sleep. Stimulation was delivered during non-REM sleep as verified by the presence of slow-wave CxEEG activity and lack of EMG activity. Five minutes of continuous of slow-wave activity was recorded before stimulation was delivered. The laser was calibrated to deliver 15 ms pulses of light at intensities of 2.5, 5, 7.5, and 10 mW and frequencies of 1.5, 3, 5, 10, and 15 Hz. Each frequency was delivered for a single intensity before laser output was increased. Each train of stimulation was delivered for 20 seconds or until the animal woke from sleep. If the stimulus train was insufficient to wake the animal, the next highest frequency was delivered following a one-minute wait. If EEG showed a change in slow wave activity of if the animal woke, another 5-minute baseline of SWS was taken before the next stimulation was delivered. If the animal woke, the same stimulus train was delivered. If the animals' SWS was disrupted, but the animal did not show a behavioral waking response the next highest stimulus train was delivered.

Methods: verification of viral expression and implant accuracy. Animals were deeply anesthetized with an overdose of Ketamine/Xylazine and perfused through the

heart for 30 seconds with saline and then 5 minutes with cold 4% paraformaldehyde for tissue fixation. The brain was then removed and placed in 4% paraformaldehyde overnight for post-fixation and transferred the following day into 20% sucrose for cryoprotection and kept in this solution until buoyancy was lost. Before slicing, the brain was frozen on dry ice and mounted on a Leica cryostat for sectioning. Four sets of 40 μm sections were taken through LC and stored in phosphate buffered saline (PBS) with 0.1% sodium azide until staining. One set of LC sections was washed 3x5 minutes with PBS and then 3x5 minutes in phosphate buffered saline with 0.3% Triton X-1000 (PBST). Sections were then incubated in 3% normal donkey serum (NDS, Jackson ImmunoResearch) for 60 minutes. Sections were simultaneously incubated overnight in mouse anti-TH (1:1000, Immunostar 22941) and rabbit anti-DS Red (1:500, Clontech 632496) in PBST and 3% NDS. The following day, the tissue was washed 3x5 minutes in PBST and then transferred into flourophore-conjugated secondary antibodies for 3 hours. Donkey anti-mouse Alexa Fluor 488 (1:500, Invitrogen) was used to visualize TH positive LC neurons; and donkey anti-rabbit Alexa Fluor 594 (1:500, Invitrogen) to visualize mCherry tagged ChR2 expression. Slices were washed 3x5 minutes in PBS, mounted, and coverslipped with Citiflour mounting medium. Sections were imaged and captured using a confocal laser-scanning microscope (Leica TCS SP5) with argon, argon/krypton, and helium/neon lasers. Damage resulting from the guide and injection canulae was tracked throughout the tissue. Since injection of dyes such as pontamine blue would disrupt visualization of florescent labeling, the most ventral point of tissue damage along the canulae tracks was found and compared to the Paxinos and Watson rat brain

atlas (Paxinos and Watson, 2008) to localize position of injection canulae carrying the optical fiber.

Results. Animals injected with the PRSx8 lentiviral vector under these surgical parameters demonstrated sufficient expression of mCherry tagged ChR2. Expression of ChR2 was highly selective for TH-positive LC cells. Further, canula implants were effectively targeted at LC (Figure 2.2).

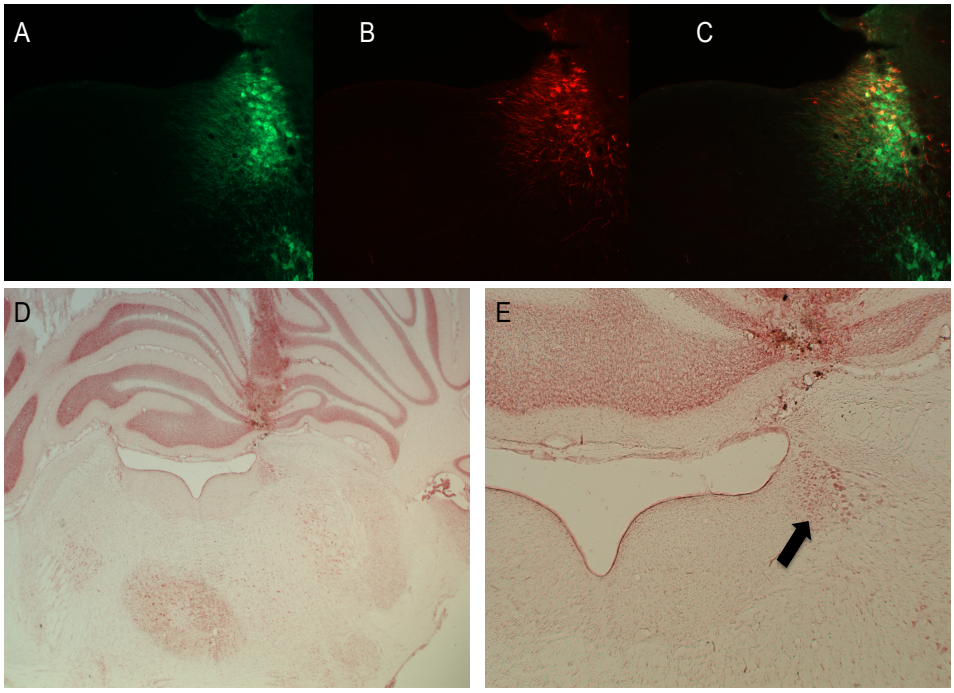

Figure 2.2 TH-positive LC cells (A) express mCherry fused ChR2 (B) selectively within the LC (images merged in C). Canulae tracks (D) accurately target LC (E, black arrow).

In a ChR2 expressing animal with cannulae accurately placed immediately dorsal to LC, a 3 Hz train of 15 ms, 5 mW light pulses was sufficient to reduce slow-wave

activity during non-REM sleep (example shown in Figure 2.3). Immediately prior to the delivery of stimulation, CxEEG displayed slow-wave activity associated to non-REM sleep. Shortly after initiating the train of stimulation, low frequency CxEEG activity was eliminated, consisitent with a predicted increase in arousal. This was consistiently followed closely by a waking resonse. A power analysis of the trace shown in Figure 2.3A reveals a dramatic decrease in power at low frequencies during the stimulation period compared to the preceeding epoch of SWS.

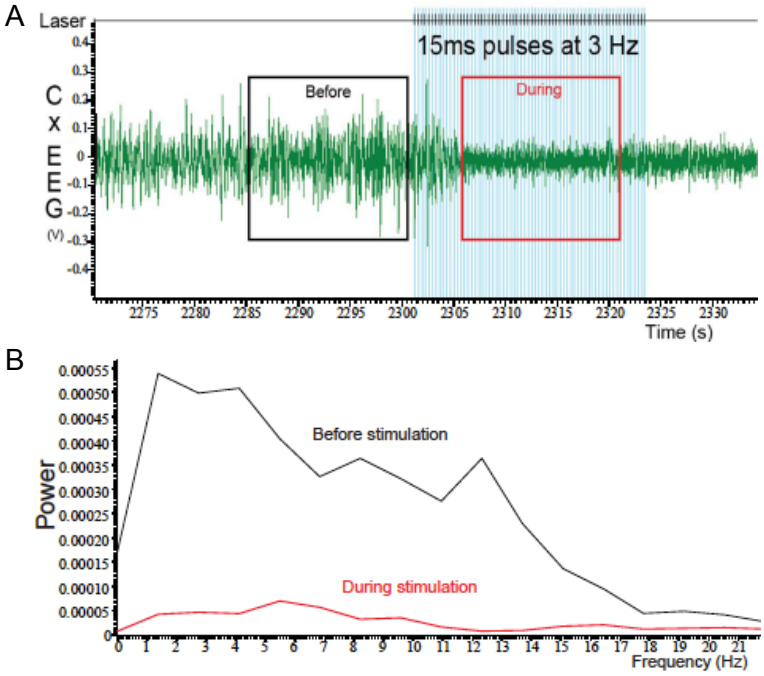

Figure 2.3 An example of ChR2 mediated LC stimulation producing a decrease in SWS associated low-frequency CxEEG activity consistent with an increase in arousal. A raw CxEEG trace (A) is shown before, during, and after stimulation was delivered. A power analysis (B) for the boxed regions in A is reveals a sharp decrease in power for all frequencies below 18 Hz.

Discussion. The results of this set of pilot experiments provide proof-of-concept for ChR2 mediated stimulation of locus coerulus in an awake, behaving animal.

Consistient, LC specific expression of ChR2 was achieved through electrophysiologically targeted injections of the lentiviral vector under control of the PRSx8 promoter. Further, delivery of light pulses was able to elicit a quantifiable increase in arousal and a behavioral waking response. One limitation to this approach was the fact that the animal needed to be anesthetized in order for the optical fiber to be inserted without breakage. For cognitive behavioral testing, anesthesia immediately preceeding an EDS could potentially interfere with the animals' ability to perform the required behavior. To adress this issue, subsequent experiments incorporated durable, chronically implantable optical fibers that could be easily attached to fiber optic cables without the need to sedate the animal.

Optogenetic Stimulation of LC and Optimization of Set-Shifting Procedures

Initial attempts were made early on in the development of these procedures to further the capabilities of the task put forth by Floresco, et al. (Floresco et al., 2008). Using the more tradtional digging set-shift task, it was clear that noradrenergic manipulations produced deficits that were specific to extradimensional shifts (Tait et al., 2007; McGaughy et al., 2008). In this task, it is possible to perform intradimensional shifts (IDS) in which new forms of stimuli within the same discriminatory dimension are introduced, requiring the rat to learn a new discriminator while staying within the same dimension. This type of shift shows that the rat can form specific attentional sets. Additionally, this task allowed for reversals following every shift type. In a reversal, none of the stimuli are changed, but the previously ignored stimulus within the relevant

dimension becomes the discriminator. Reversals are possible within the operant based task, but until now had only been performed within the spatial lever dimension. These tasks, however, do not allow for parsing of different error types. Incorporation of new forms of stimuli that could allow for IDS or cue-based REV could strenghten potiental finding by allowing closer comparision to the published literature.

To this end, early optogenetic set-shifting experiments sought, largely without success, to incorporate new types of stimuli and behavioral strategies that could allow for IDS and multi-dimensional reversals. Some of those approaches are briefly summarized here. Strobing lights were introduced within the light dimension. Instead of only one light being presented to the rat, one of the lights remained on constantly while the other strobed. Here the rat could choose to press the solid or the strobing light. However, this stimulus required a considerable deal of extra training and rats varied greatly in their tolerance to perform this discrimination. Many rats would simply omit trials. Also within the light dimension, we attempted to assess whether a rat could learn to press the lever opposite the illuminated cue light. Rats were considerably better at performing discriminations for the lit cue light, so this difference in stimulus salience did not make it a good candidate for set-shifts. Colored lights were also considered. However, rats have extremely limited color vision and even that is restricted to the green to ultraviolet wavelengths, with maximum sensitivity to blue (Jacobs et al., 2001). It was concluded that modifications of the light dimension may prove too difficult to employ.

Another option was the addition of auditory stimuli. One way this was attempted was to maintain a spatial approach similar to the cue light. In this arrangement, one

speaker was positioned above each cue light. However, only one speaker played a tone on a given trial. This discrimination too also proved too difficult for the rat to incorporate into this task. This may be due in part to the animal being able to move about freely when the tone was presented, as their position within the behavioral chamber could change their perception of the source of the tone. Textured tones that either rose or fell, broken vs. solid tones, and clicks were also tried with varying, but inconsistient success.

One approach that did achieve a measure of success was presentation of a single tone. This single tone was either a high or low frenquency. Rats were required to press a lever that corresponded to either the high or low tone. By presenting either a 6 or 12 KHz tone simultaneously with the cue light, rats most rats were eventually able to execute a streak of 20 correct trials performing a tone discrimination. However, it was rare that a rat was able to execute a shift to this discrimination within a single session. Nonetheless, it did provide an additional stimulus domain to which the rat could be initially trained and shifted from.

The following experiments incorporated this tone training into the operant set-shift task. Rats performed EDSs and reversals while optogenetic stimulation was delivered to LC. It was hypothesized that a continuous 3 Hz train of 15 ms light pulses delivered for 15 trials immediately following the rule change would induce a behavoirally flexible phenotype that would decrease perseverative behavior and allow the animals to learn the new rule sooner. Indeed, unilateral stimulation of LC was able to produce a decrease in perseverative responding when than animal was shifted from the light or tone

dimension. However, this effect was transient, at best, and did not result in a significant overall improvement in the rats performance of the EDS.

Methods: behavioral pretraining. Rats were allowed one week for acclimation and and handling before starting experimental procedures. During training, rats were fed a maximum 5 chow pellets daily and were restricted to no greater than 20% less than their free-feeding weight. All operant training took place in a MedAssocitates chamber placed inside a sound attenuating box. The behavioral apparatus consisted of a centrally positioned fluid reward well flanked on either side by a retractable lever and cue light and above by a house light. The fluid reward well was connected to a 20 ml syringe in a syringe pump (MedAssociates) that would deliver 0.05 ml of 15% sucrose on a rewarded trial.

All rats began on FR1 training. During an FR1 session, the house light would remain illuminated and one of the two levers would be inserted into the box for the entire session. Any press of the lever that did not take place when the reward pump was already activated would result in delivery of the reward. Rats underwent one 30 minute session for each lever daily until they were able to attain 50 rewards on each lever on a single day. Once this phase of training was completed, rats were required to press a presented lever within 10 seconds. In this phase, a trial would begin with the house light off and both levers retracted. The house light would turn on and one of the two levers would be extended into the chamber. The rat was required to press the lever within 10 seconds to receive the reward. Each lever was presented 45 times during a session and the rat was required to omit no more than 5 trials in a given session after a minimum of 4 total

sessions before proceeding to the next phase of training. On the last day of this phase, the rats' side bias was assessed. Here, at the beginning of a trial both levers were inserted into the chamber. Pressing either of the levers (free choice) resulted in delivery of the reward. The next trial was a forced choice requiring the rat to choose the opposite lever from the free choice. This was repeated until the rat made the correct forced choice and reset the next trial to a free-choice. This continued until the rat had carried out 9 free choice sessions and the lever most often pressed during the free-choice was determined to be the rats' side bias.

The final phase of pretraining for these experiments required the rat to disciminate a tone stimulus amid distractor stimuli. Again, before a trial, all lights were extinguished and levers retracted. The trial began with a pseudorandom simultaneous presentation of either a 6 or 12 KHz pure tone and illumination of one of the two cue lights. These stimuli were presented for three seconds before both levers were inserted into the chamber. If the 6 KHz tone was played, the rat was required to press the left lever, or the right lever for the 12 KHz tone. The cue light was not predictive of the rewarded lever. Rats carried out 300 trials daily, 6 days a week, until they were able to execute a streak of 20 correct trials in a given session.

Methods: injection of viral vectors and implatation of optical fibers. With a few notable departures, the surgical procedures used herein follow closely with those described in the previous section. EEG and EMG leads were not required for these procedures, so two skull screws were affixed to each of the parietal bones to anchor the implant to the skull. Initially, LC virus injection and optic implantation was performed

unilaterally as before. Later experiments attempted to transduce virus and implant optics bilaterally. In these procedures, LC was electrophysiologically located and injected as before. The injector was then raised out of the brain and cleared by puffing air through the injector into a small volume of saline. The injector was then refilled with another 1.5 µl aliquot of the lentiviral vector which, which was injected at the same depth and ML ±2.5 from the localized LC. Also new to these procedures was the incorporation of a control viral vector. As with the ChR2 vector, translation of the viral product was controlled by the PRSx8 promotor that is specific to Phox-2B expressing cells like the LC. However, transduction with this virus only produced the reporter protien mCherry and not ChR2. Methods for histological verification of viral transduction follows from the previous section.

These experiments also incorporated chronically inplanted fiber optics that did not require sedation of the animal to connect the light source. Unilateral implants consisted of a precisely polished 200 µm optic core epoxyed inside a 2.5 mm stainless steel lucent connector ferrule (Precision Fiber Products). Bilateral implants used the same core, but were made using individual 1.25 mm ceramic or stainless steel ferrules precisely secured to a common base so that the ferrules measured 2.4 mm center-to-center. Just before implantation, the end of the optic fiber was cut with a diamond knife and the implant was connected to the laser. For each implant the settings for the laser resulting in 5 mW output was recorded for calibration during stimulated test sessions. These implants were then attached to a stereotaxic holder and lowered the same LC coordinates just above the

most dorsally located LC cell. Following surgery, animals were allowed 2 weeks for recovery and viral transduction before resuming behavioral testing.

Methods: operant set-shifting procedures. Following the two week recoevery, animals were returned to the behavioral apparatus and underwent a retraining session on the tone stimulus before EDS testing began. During this retraining session and throughout EDS testing, the rats' implant was connected, via a ceramic split sleeve, to a custom FC/PC patch cable terminating with a lucent connector of the same size as the implant. Once the animal was able to adequately recall the tone discrimination they could begin performing EDSs and REVs. The first EDS the rat performed proceeded from the tone dimension. The initial 20 trials of the first EDS were performed identically as in the initial tone discrimination. On the 21st trial the relevant stimulus dimension was switched without any additional cueing to the animal other than the loss of reward for performance of the incorrect behavior and a 10s timeout. On the first EDS the animal had to switch from the known tone discrimination to adopt a spatial strategy whereby they were to press only the lever opposite their side bias on a given trial. On EDS sessions where the animal received optogenetic stimulation, 5mW, 15 ms light pulses were continuously delivered at 3 Hz throughout the first 15 trials following the rule change. Following the rule change, rats continued performing trials until they could execute a streak of 20 correct on the new rule or until they had recieved 520 total trial presentations.

Next rats performed a reversal within the spatial dimension. Stimulus presentation throughout all trials remained consistient regardless of which type of shift was performed. As with EDS, the first 20 trials required the rat to press the lever opposite

their side bias, the same rule they had previously learned in the first EDS. However, on the 21st trial, the rat was then required to press the opposite lever to receive the reward. The progession through subsequent shifts is shown in figure 2.4. To counterbalance for order effects, rats received optogenetic stimulation either on the first 3 or final 3 shifts performed.

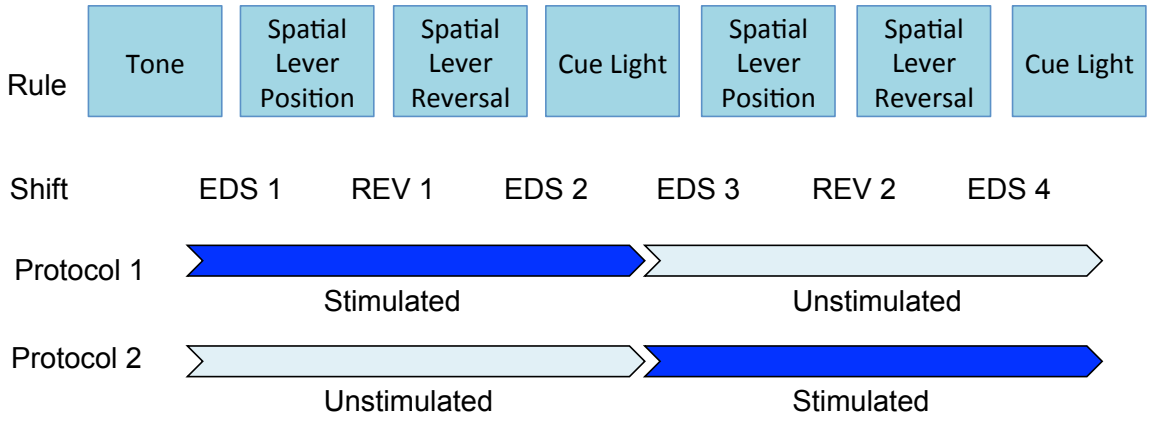

Figure 2.4 The progression of optogenetic set-shifting procedures. The progression through each rule is shown on the top row. A change between rules is denoted by the proper shift-type in the second row. Animals were tonically stimulated either on the first or final 3 shifts to counterbalance order effects.

Methods: dependant variables and statistical analysis. This operant procedure produces a rich set of dependant variables that can provide a great deal of insight into the animals progress throughout the set shift. Pre-shift percent correct is calculated for choice trials during the 20 trial epoch preceeding the rule change. If the animal failed to reach at least 65% correct during the pre-shift period, that shift was not used in the analysis. Total correct and incorrect trials during the post-rule change epoch until the point the animals reaches a streak of 20 correct trials are added to calculate trials to reach criterion (TTC).

Incorrect trials can be split into 3 error types. Perseverative and regressive errors both denote the same error behavior, an incorrect choice that follows the old rule. The difference in these two types of errors are the frequency and the point at which they occur during the set-shift session. To calculate, trials are divided into bins of 8 in which it was possible to make this sort of error. After the first bin following the rule change, the first bin in which the animal commits fewer than 6 of 8 errors of this type is the last bin that perseverative errors are counted. The remaining errors of this type are all termed regressive errors. This differentiation is thought to reflect persistience on an unrewarding behavior (perseveration) versus a failure to consistiently maintain a rewarding behavior (regressive). On reversals, bins of 16 with a cutoff of 12 are used since the animal can make a perseverative response on every trial. On a small number of EDS trials, animals may make a choice that would not have been correct under the old rule or the new rule. These errors are termed non-perseverative errors.

These behavioral data include within-subject comparisons for animals over multiple EDSs and reversals as well as between-subjects comparisons for animals who receive different viral injections. Further, analysis of binned perseverative errors may have different numbers of observations depending on when the animal met criteria. As such, data in these experiments were analyzed using linear mixed models. A diagonal repeated covariance structure was used as large differences in variance over multiple observations was not a likely concern.

Results: unilateral optogenetic stimulation during the EDS. A total of 15 animals with accurate implant placement and unilateral virus expression in LC were used for this

study. Of those 15, 11 animals were transduced with ChR2, and 4 with the mCherry control virus. A total of 31 EDS observations were made from ChR2 animals, 22 from unstimulated EDS and 19 from stimulated EDS. 16 total observations were made for control animals, 8 from stimulated EDS, 8 unstimulated EDS. On reversals, 5 observations were made for control animals (2 unstimulated, 3 stimulated) and 17 for ChR2 animals (8 unstimulated, 9 stimulated).

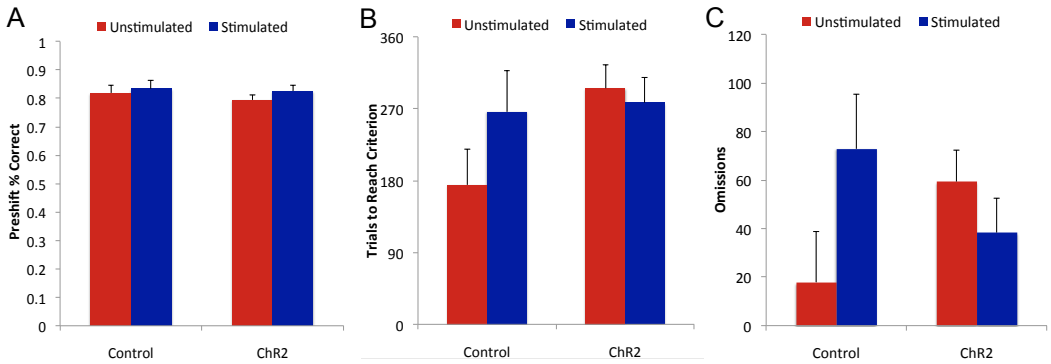

Figure 2.5 Overall performance on EDS is unaffected by ChR2 mediated stimulation of LC. Within subjects comparisons are represented by clustered bars. Unstimulated trials are shown in red and stimulated in blue. Percent accuracy on the 20 pre-shift trials is shown in A, TTC in B, and omissions in C.

Considering all shifts together, there were no baseline differences in the animals' ability to recall the relevant rule as pre-shift accuracy did not differ for any group ($F_{LMM(1, 38.143)}$=0.131, p=0.720, figure 2.5A). Likewise, LC stimulation produced no change in the animals' overall TTC ($F_{LMM(1, 34.090)}$=1.866, p=0.181, figure 2.5B). There were, however, significant differences in the number of omissions ($F_{LMM(1,41.063)}$=4.330, p=0.044, figure 2.5C). This difference was found only in control animals and resulted from a decrease in omissions on unstimulated trials.

Error analysis reveals no significant interactions of virus*stimulation for any error type. There was no significant difference in the comission of perseverative errors ($F_{LMM(1, 18.928)}$=1.348, p=0.260, figure 2.6A), regressive errors ($F_{LMM(1, 23.684)}$=0.733, p=0.400, figure 2.6B), or non-perseverative errors ($F_{LMM(1, 33.380)}$=0.001, p=0.981, figure 2.6C).

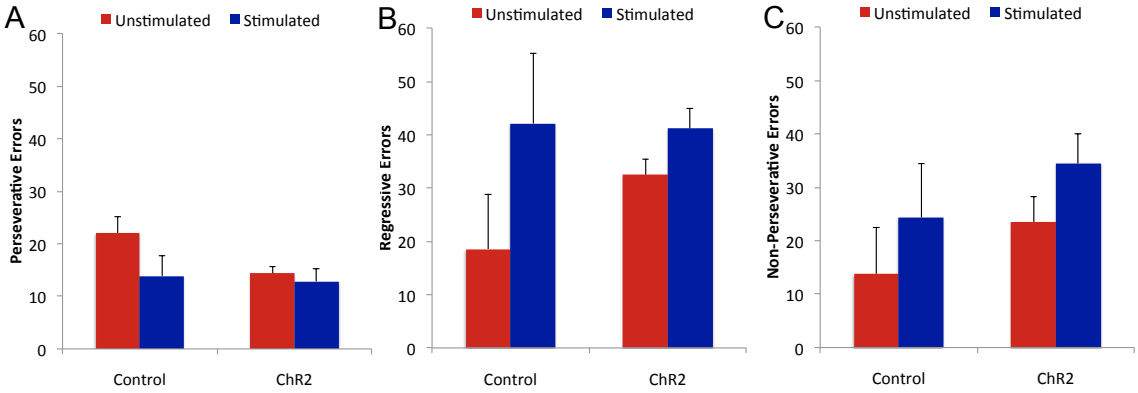

Figure 2.6 Analysis of error types show no significant effects as a result of ChR2 mediated stimulation of LC. Clustered bars represent within subjects comparisons. Unstimulated EDSs are shown in red, stimulated in blue. Perseverative errors are shown in A, regressive in B, and non-perseverative in C.

A closer analysis of timcourse of perseverative error comission is shown in figures 2.7 and 2.8. The first 6 bins used in the calculation of perseverative and regressive errors are plotted. It was hypothesized that optogenetic stimulation of LC will decrease perseverative errors. However, when not accounting for the type of shift the animal was performing (virus*stimulation*bin), no effect of optogenetic stimulation was seen ($F_{LMM(5, 73.235)}$=0.769, p=0.287, figure 2.7). However, there were different types of shift the animal could have executed. When we compare shifts from an unpredictable dimension (light or tone) to shifts from the predictable spatial that does not require a trial-by-trial cue presentation, we find a main effect of shift type ($F_{LMM(1, 219.049)}$=69.163,

p<0.001) and a significant four-way interation of virus*stimulation*shift type*bin ($F_{LMM(5, 73.235)}$=1.935, p=0.049).

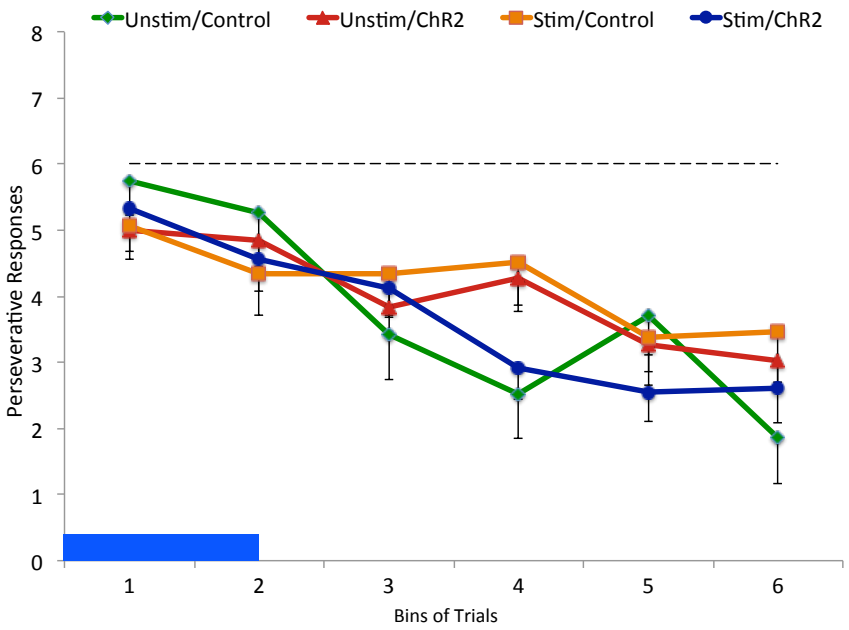

Figure 2.7 A time course analysis of perseverative behavior irrespective of the type of shift the animal performed. The dotted line represents the cut-off used to determine when perseverative behavior has ceased. Unstimulated (green) and stimulated (orange) shifts by mCherry animals are shown with unstimulated (red) and stimulated (blue) EDSs by ChR2 animals.

To attempt to parse the four-way interaction, each shift type was considered separately. On an EDS from the spatial dimension, the previous rule did not require the rat to wait and receive a cue to perform the correct behavior. On these types of shifts, tonic stimulation in ChR2 animals appears to have no effect on perseverative behavior (figure 2.8A). However, on EDSs from an unpredictable dimension, the rat had to withhold responding to receive either a light or tone cue that dictated which lever was to be pressed. On these types of shifts, it appears that ChR2 rats perseverate less when receiving stimulation compared to unstimulated EDS and stimulated mCherry controls.

Indeed, statistical comparison of bin 1 for ChR2 animals on unpredictable shifts shows that this difference is significant (t=11.184, p=0.018).

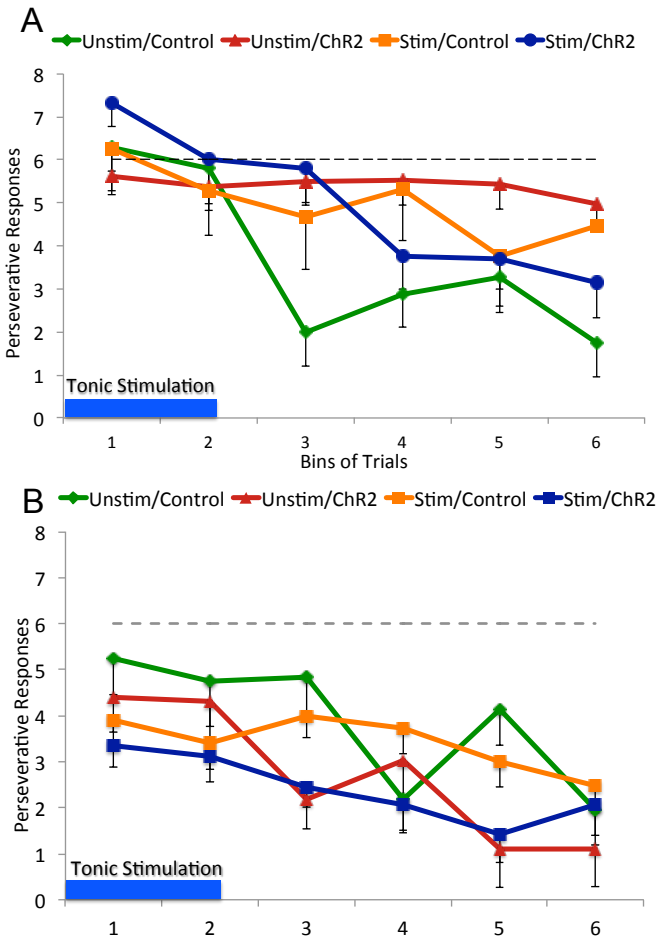

Figure 2.8 Shifts from a predictable spatial rule (A) are compared separately for shifts from an unpredictable rule (B). ChR2 animals appear to make fewer perseverative responses on stimulated shifts (blue) from an unpredictable dimension compared to unstimulated EDSs (red) and unstimulated mCherry controls (green). The dashed line represents the criteria level for classification of perseverative errors.

Results: unilateral optogenetic stimulation during reversals. As with EDS, optogenetic stimulation during reversals also failed to produce significant changes in measures of overall task performance. Groups did not differ on baseline performance

before the rule change ($F_{LMM(1, 16.370)}$=0.508, p=0.486, figure 2.9A). Following the rule change, there were no significant differences in TTC ($F_{LMM(1, 17.659)}$=0.036, p=0.851, figure 2.9B) or omissions ($F_{LMM(1, 16.871)}$=1.375, p=0.257, figure 2.9C). Analysis of error commission also reveals no differences in perseverative ($F_{LMM(1, 16.647)}$=3.811, p=0.068, figure 2.9D) or regressive errors ($F_{LMM(1, 15.414)}$=1.653, p=0.217, figure 2.9E). Time-course analysis of perseverative behavior, as was done for EDS, does not reveal any significant differences due to LC stimulation ($F_{LMM(1,12.265)}$=1.361, p=0.268, figure 2.10).

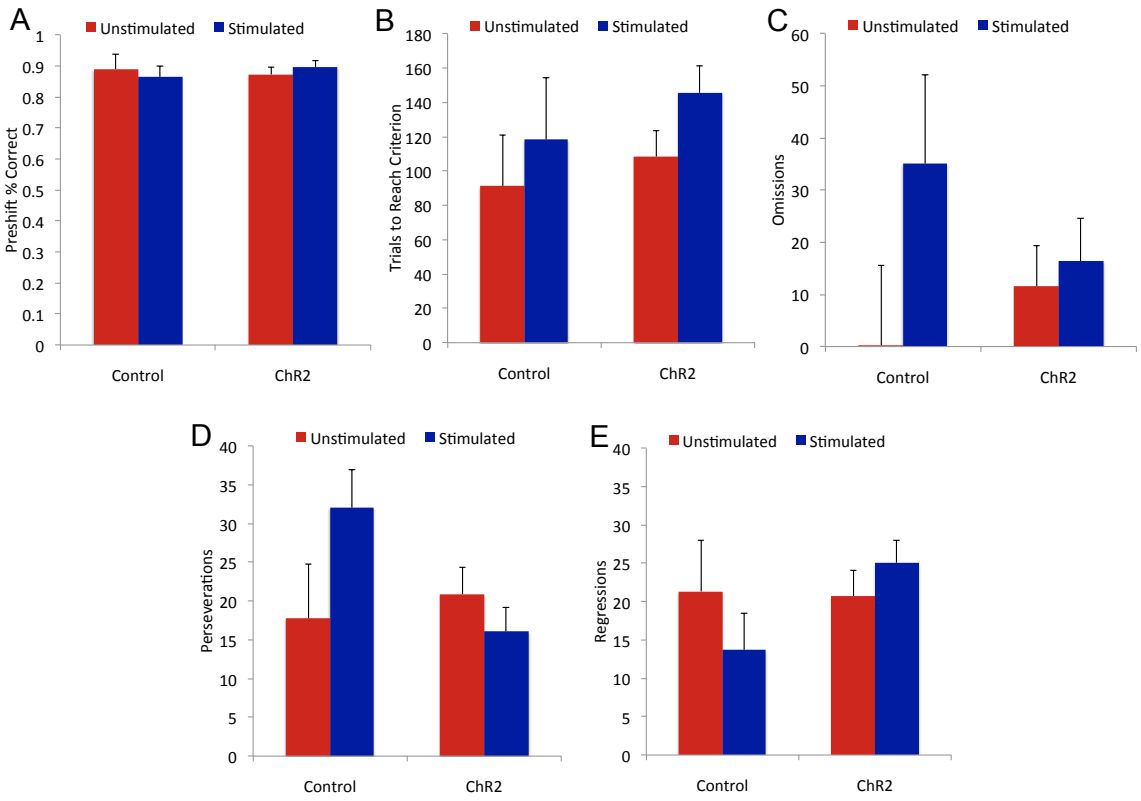

Figure 2.9 Analysis of reversal performance reveals no effects due to optogenetic stimulation. Within subjects comparisons are represented by clustered bars. Unstimulated REVs are shown in red, stimulated in blue. Variables shown include pre-shift accuracy (A), TTC (B), omissions (C), perseverations (D), and regressive errors (E).

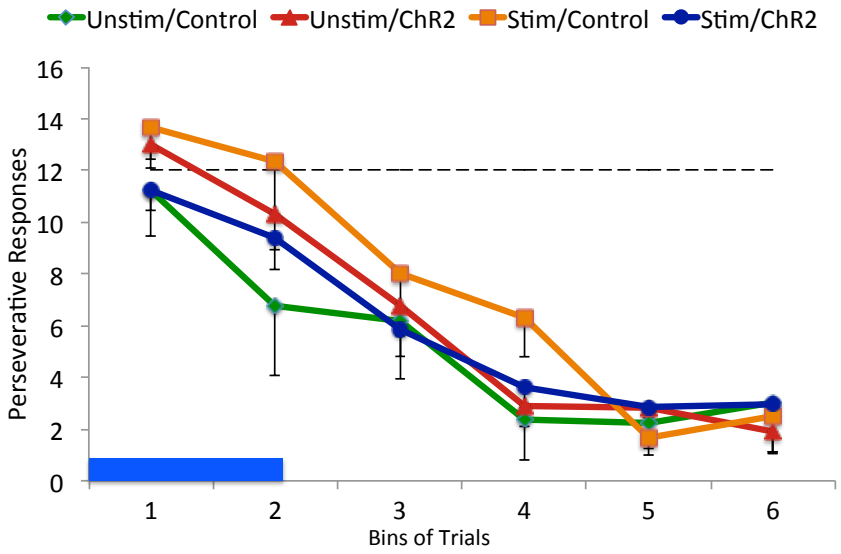

Figure 2.10 Binned analysis of perseverative behavior on reversals reveals no changes resulting from optogenetic stimulation of LC. The dashed line represents the cutoff for errors to be counted perseverative. Groups shown include stimulated (orange) and unstimulated (green) REV in control animals and stimulated (blue) and unstimulated (red) REVs in ChR2 animals.

Results: bilateral optogenetic stimulation in EDS. Failing to observe any changes in overall task performance as a result of optogenetic stimulation of LC, a pilot experiment attempted to bilaterally stimulate LC in hopes that exerting tonic activity in both nuclei might produce the hypothesized effects. Of the animals with bilateral virus expression and accurate bilateral fiber optic implantation in LC, 4 animals were transduced with ChR2 and 3 with mCherry. A total of 10 EDS observations were included for each group. Again, bilateral ChR2 stimulation failed to produce any differences in the overall performance on any of the EDS variables of interest (figure 2.11). There were no significant differences in animals' ability to recall the previous rule ($F_{LMM(1, 9.926)}$=0.333, p=0.577, figure 2.11) or TTC ($F_{LMM(1, 8.700)}$=4.514, 0.064, figure 2.11B). Analysis of perseverative ($F_{LMM(1, 6.784}$=0.583, p=0.471, figure 2.11C) and

regressive errors ($F_{LMM(1, 5.725)}$=0.682, p=0.442) also fails to reveal an effect of LC stimulation on overall EDS performance. Although there was a significant main effect of stimulation in decreasing regressive errors ($F_{LMM(1, 5.725)}$=7.080, p=0.035), stimulation did so regardless of whether the animal was expressing ChR2 or control virus.

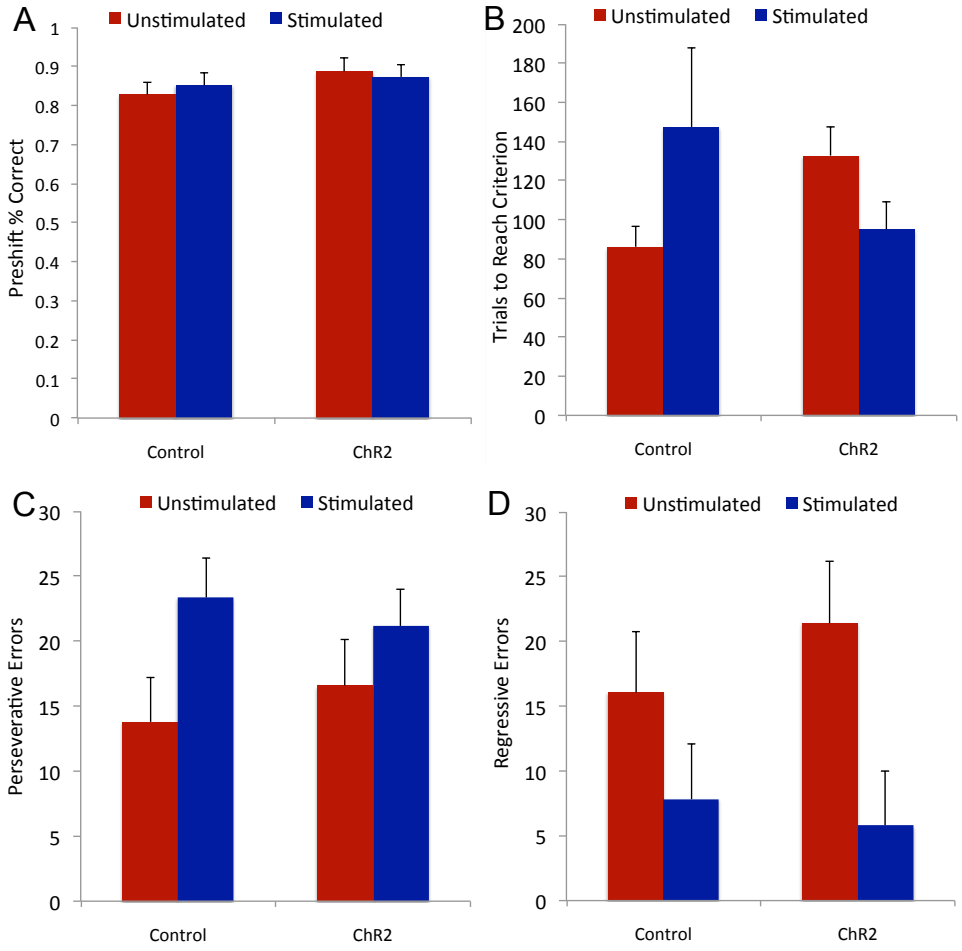

Figure 2.11 Bilateral stimulation of LC fails to produce any statistically significant differences in animals' overall task performance. Clustered bars represent within subjects comparisons. Stimulated shifts are represented in blue and unstimulated in red. Preshift accuracy is shown in (A), TTC (B), perseverative errors (C), and regressive errors (D)

Verification of Induced Activity by ChR2 and Gq-DREADDs

Failing to see any considerable behavioral effects resulting from optogenetic LC stimulation during the aSST, additional studies were done to assess the extent to which these stimulation parameters were able to induce activity in LC. The immediate early gene c-fos has been extensively characterized due to its elevated translation following periods of neuronal activation. Levels of its protein product Fos are found to be elevated to peak levels 90-120 minutes after a period of elevated activity (Dragunow and Faull, 1989; Bullitt, 1990). If ChR2 is producing a sustained period of elevated tonic activity when stimulation is administered, we would expect to see elevated levels of Fos protein in tissue fixed 90-120 minutes following the stimulation. The following experiments attempted to verify the efficacy of ChR2 to elicit activation of c-fos following a 15-minute period of 3 Hz stimulation. These experiments also assessed the ability of Gq coupled Human Muscarinic 3 Designer Receptor Exclusively Activated By Designer Drugs (hM3Dq, DREADD) to elicit Fos in LC. Comparison of the two methods, albeit using different histological techniques, indicates the hM3Dq produces a much clearer picture of induced activity compared to ChR2 in a way that is consistent with a period of neuronal activation.

Methods: optogenetically stimulated c-fos activation. Following conclusion of behavioral testing for the aSST rats, they entered a 3 day period of acclimation that included daily 15 minute sessions in their inactive operant box connected to an FO patch cable. After 15 minutes they were returned to their homecage. This was done to avoid non-specific activation of Fos due to novel inactivity of the behavioral apparatus. On the

fourth day, the rat returned to the operant chamber, and received 15 ms, 5 mW pulses of light delivered at 3 Hz for 15 minutes. They were then returned to their homecage. These animals were deeply anesthetized and perfused intracardially with 4% PFA as previously described 90 after the end of the stimulation session. The brains were post-fixed, cryoprotected, and sectioned as in previous experiments. One set of sections through LC was used for each rat.

Laboratory procedures for Fos visualization have been extensively described (Delfs et al., 1998; Harris et al., 2005; Harris et al., 2007; Mahler and Aston-Jones, 2012; Sartor and Aston-Jones, 2012). Briefly, Fos was visualized by incubating sections in rabbit anti-fos (1:5000, Santa Cruz Biotechnology) overnight, then in biotinylated donkey anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch Laboratories) for 2 h, and in avidin-biotin complex (ABC, 1:500) for 1.5 hrs. Finally, sections were incubated in 3,3'-diaminobenzidine (DAB, Sigma) and nickel ammonium sulfate, producing a purple-black reaction product in the nucleus. These sections were then co-stained with neutral red to distinguish LC cell bodies from neighboring nuclei. Slides were then coverslipped and allowed to dry before being photographed at 20X magnification.

Two bilateral sections through LC were used to quantify LC-Fos activation resulting from optogenetic stimulation. LC cell bodies, which were readily distinguishable from surrounding nuclei, were counted on each side. Then purple black nuclei within those cell bodies were counted and Fos was quantified as a percentage of Fos positive nuclei per LC cell. In animals expressing ChR2 unilaterally, the side receiving ChR2 stimulation was compared to the non-stimulated side as well as to LC of

animals who were not expressing detectable levels of ChR2. Bilaterally stimulated animals were compared to mCherry controls.

Results: ChR2 elicited Fos expression in LC. Samples from animals in the unilateral stimulation set-shifting were used in this analysis. Histological samples and quantification of unilateral ChR2 elicited Fos expression in LC is shown in figure 2.12. Unilateral stimulation failed to significantly increase Fos relative to either the unstimulated side or to controls not expressing ChR2 ($F_{ANOVA(1,52)}$=0.016, p=0.899). Histological samples and quantification of bilaterally elicited Fos expression is shown in figure 2.13. Two of the animals from bilateral aSST experiments were excluded from this analysis due to loss of implants before the final stimulation could be delivered. Although group sizes are too small to be properly quantified, it appears that bilateral stimulation did not increase Fos expression relative to controls.

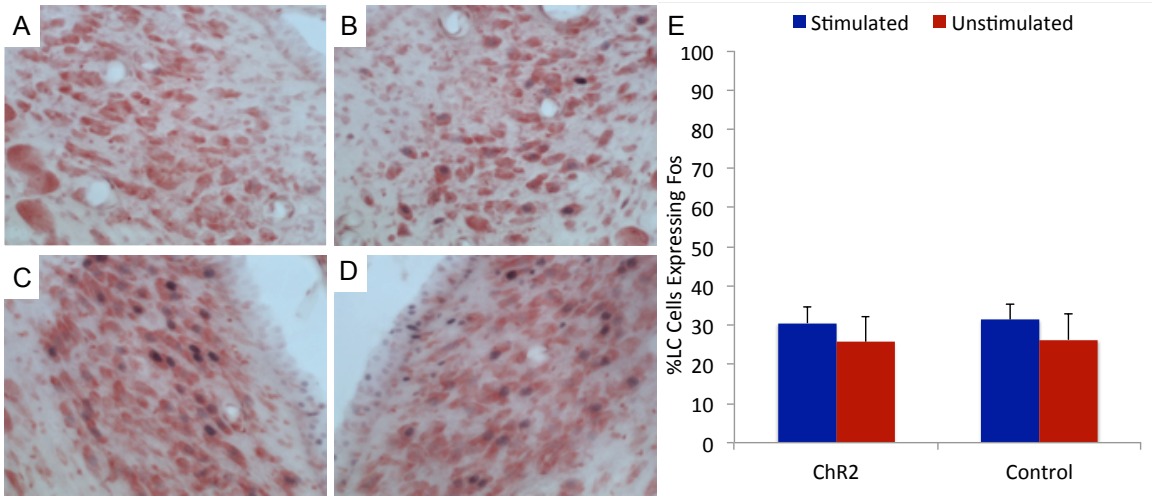

Figure 2.12 Unilateral optogenetic stimulation fails to elicit Fos expression in LC. Unstimlated (A) and stimulated (B) ChR2 animals are shown across the top, and unstimulated (C) and stimulated (D) control animals across the bottom. Fos expression for all unilateral animals is quantified in E.

Having failed to see any robust modulation of Fos expression due to optogenetic stimulation, subsequent experiments sought to validate hM3Dq as a means to deliver cell-type specific stimulation to LC. These Gq coupled receptors can be delivered to LC cells with viral vectors using the exact methodology previously verified for ChR2 and can be selectively activated by the otherwise inert ligand clozapine n-oxide (Armbruster et al., 2007). Using these receptors, the following experiments demonstrate a robust, dose dependent induction of Fos in LC cells and a significantly correlated induction of Fos in mPFC.

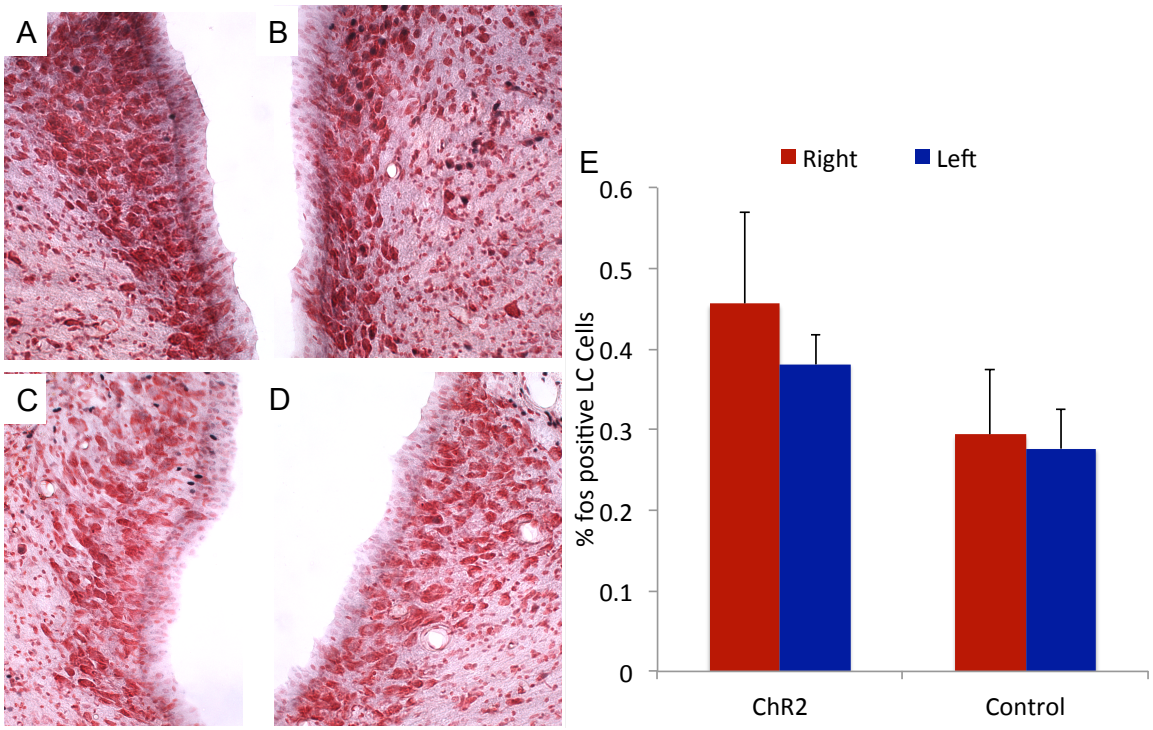

Figure 2.13 Bilateral optogenetic stimulation fails to elicit Fos expression in LC. Stimulated left (A) and right (B) LC of ChR2 animals are shown across the top, and Stimulated left (C) and right (D) LC of control animals across the bottom. Fos expression for all bilateral animals is quantified in E.

Methods: surgical injection of hM3Dq vectors. Surgical methods follow closely for those previously described for optogenetics experiments. Notable departures from the established protocol include differences in the vectors used to deliver hM3Dq to LC cells as well implanted implements for microinjection of CNO. hM3Dq was delivered using an adeno-associated virus (AAV) serotype 2/9, expressing the DREADD receptor fused to a hemagglutanin (HA) tag under the control of the PRSx8 promoter. The control virus was identical, but only expressed the mCherry reporter protein. Bilateral 28G canulae were also implanted 1 mm dorsal to the dorsal 1/3 of electrophysiologically localized LC. These canulae were used in experiments that will be explained in later chapters.

Methods: histological verification of viral expression and Fos induction. Procedures for tissue fixation and preparation follow from those previously described. For hM3Dq animals, sections were simultaneously incubated overnight in mouse anti-TH (1:1000, Immunostar 22941) and rabbit anti-HA (1:1000, Cell Signaling C29F4) in PBST and 3% NDS. Sections for control animals expressing the mCherry protein were incubated with the same mouse anti-TH and rabbit anti-DS red (1:500, Clontech 632496). Donkey anti-mouse Alexa Fluor 488 (1:500, Invitrogen) was used to visualize TH positive LC neurons; and donkey anti-rabbit Alexa Fluor 594 (1:500, Invitrogen) to visualize HA-tagged HM3D DREADD receptor expression or control mCherry reporter.

Two-hours and twenty minutes before animals were sacrificed they received a systemic IP injection of either 1 mg/Kg (hM3Dq n=9) or 5 mg/Kg CNO (hM3Dq n=12, mCherry n=9) dissolved in saline vehicle with 5% dimethyl sulfoxide for induction of Fos. Sectioning, washing, blocking, and visualization procedures remained the same as

previously described. LC sections were incubated in mouse anti-TH (1:1000, Immunostar 22941) and rabbit anti-cfos (1:1000 Calbiochem 0148958 or 1:5000 Calbiochem 2441976) in PBST and 3% NDS overnight. Donkey anti-mouse 594 Alexa Fluor 594 (1:500, Invitrogen) was used to visualize TH-positive LC (TH-LC) cells and Donkey anti-rabbit Alexa Fluor 488 for fos. Fos positive TH-LC cells were counted and quantified as a percentage of all TH-LC cells. PFC sections were incubated overnight in rabbit anti-cfos (1:1000 Calbiochem 0148958 or 1:5000 Calbiochem 2441976) in PBST and 3% NDS. Donkey anti-rabbit Alexa Fluor 488 was used to visualize fos staining in PFC. Fos was quantified as Fos particles/µm^2 of PL and IL cortex.

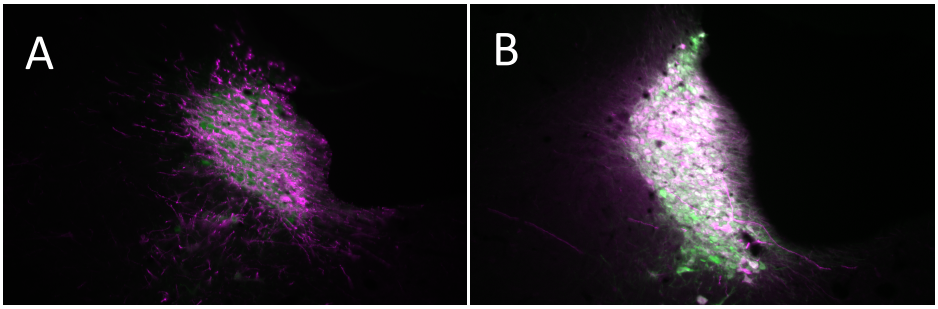

Figure 2.14 Immunohistological verification of cell-specific viral expression. A, Expression of PRSx-8 regulated hM3Dq receptors (magenta) in TH-positive LC cells (green). B, Expression of PRSx-8 regulated mCherry reporter protein (magenta) in TH-positive LC cells (green).

Results: verification of hM3Dq and mCherry transduction. Selective expression of hM3Dq (Figure 1A) and control mCherry reporter protein (Figure 2.14) in LC was achieved with PRSX8 regulated viral vectors. Co-expression of TH and HA tagged hM3Dq is shown in A and mCherry in B. Bilateral expression was achieved for all animals used in this analysis.

Results: hM3Dq mediated induction of Fos in LC and mPFC. CNO selectively and dose dependently increased Fos in LC of hM3Dq animals (Figure 2.14A). In hM3Dq animals CNO elicited a significant increase in Fos positive LC cells compared to mCherry controls ($F_{ANOVA(1)}$=52.155, p<0.001). Within-subjects, CNO dose dependently increased Fos positive LC cells in hM3Dq animals ($t_{(20)}$=3.448, p=0.002). In PFC, CNO

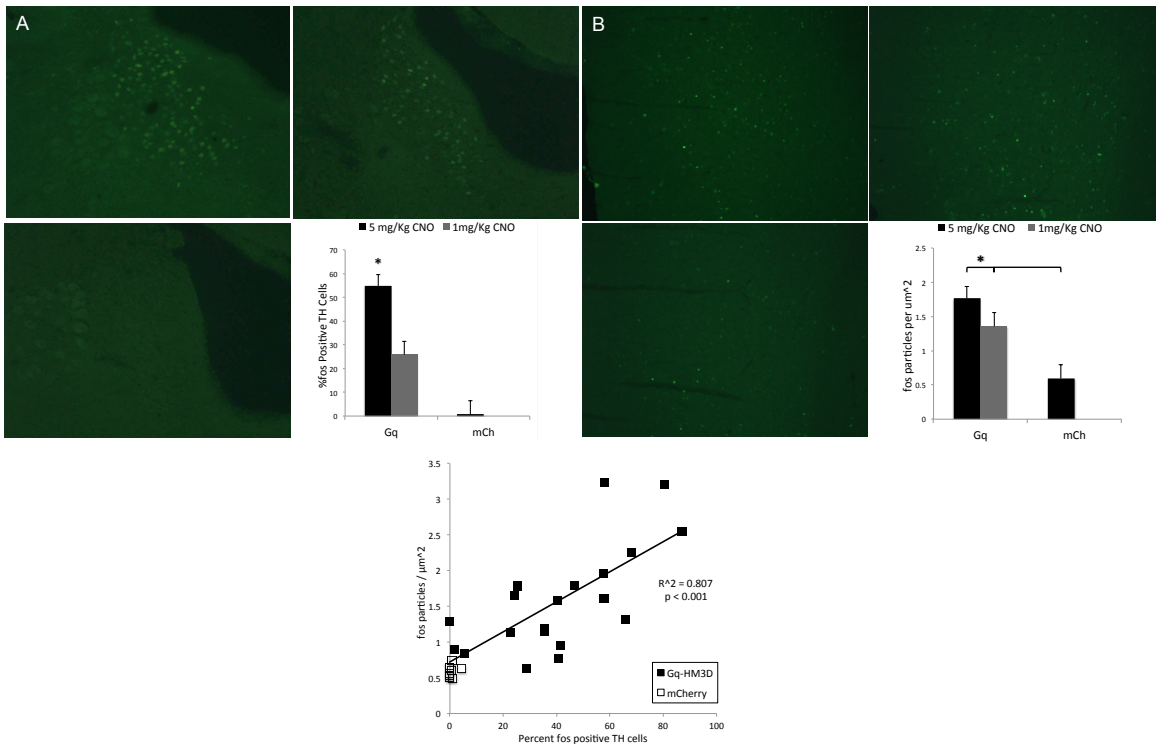

Figure 2.15 HM3Dq mediated induction of fos in LC and PFC by CNO. A, Fos expression (green) in hM3Dq expressing LC by 5 mg/Kg (n=12, top left) and 1 mg/Kg (n=9, top right) CNO i.p. and by 5 mg/Kg CNO i.p. in mCherry expressing LC (n=9,bottom left). Quantified in lower right (*p=0.004 compared to 1 mg/Kg hM3Dq). B, fos induction in PFC of LC hM3Dq animals by 5 mg/Kg (n=12, top left) and 1 mg/Kg (n=9, top right) i.p. CNO compared to 5 mg/Kg CNO in LC-mCherry animals (n=9, lower left). Quantified in lower right (*p<0.001, hM3Dq compared to mCherry). C, correlation of Fos expression in PFC vs. LC in hM3Dq animals (black squares) and mCherry animals (open squares).

increased Fos density in hM3Dq animals ($F_{ANOVA(1)}$=19.370, p<0.001) compared to controls (Figure 1B). However, Fos expression in hM3Dq animals did not differ by dose (equal variance not assumed, $t_{(19)}$=1.446, p=0.168). One 5mg/Kg animal was omitted from this analysis due to PFC tissue damage during brain removal. The proportion of Fos positive LC cells was closely correlated to the density of Fos expression in PFC (Figure 2C, R^2=0.81, p<0.001).

Discussion

According to adaptive gain theory, optimized set-shifting would be attained with tonic stimulation immediately following the rule change and properly timed phasic stimulation as the animal begins to learn and consistently use the new discrimination. Given these parameters, optogenetic stimulation of LC appeared to be aptly suited to achieve these requirements. Stimulation could be properly confined to the relevant behavioral epochs in ways that are unprecedented compared to pharmacological means. The results of these studies, however, give reasons to reconsider.

Initial proof-of-concept studies showed promise in that ChR2 mediated stimulation was sufficient to produce a waking response from SWS. However, it was insufficient to improve performance on the aSST. The only condition in which optogenetic stimulation did produce an effect was on perseverative errors, within-subjects, in a single bin of trials, and only on shifts from an unpredictable dimension. Additionally, the effect size was very small and only barely significant given a one-tailed prediction. Given the number of comparisons needed to reach this result and the very small effect size, it is not a finding that elicits a great deal of confidence to inform future study.

Given that this effect was witnessed with unilateral stimulation of LC, it followed that bilateral stimulation of LC might bolster and strengthen results going forward. For instance, if ChR2 stimulation were only exerting tonic control on one LC, the other LC could potentially remain in the phasic pattern that would be predicted for perseverative behavior. In this instance, the endogenously active LC could be sustaining the behavior that ChR2 stimulation is trying to reduce. It was thought that exerting control over both nuclei might produce the expected effects.

However, the data from the bilateral stimulation experiments do not appear to support this notion either. Although, this finding may have more to do with practical concerns related to delivering bilateral stimulation to LC rather than a critical gap in our

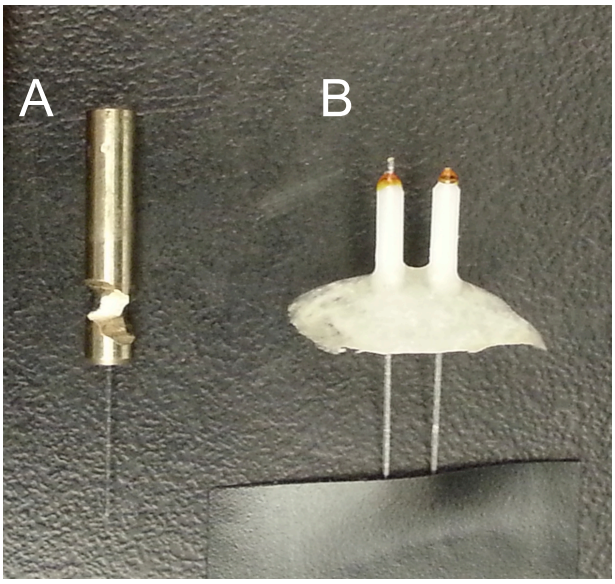

Figure 2.16 Optical implants used for ChR2 experiments. 2.5 mm stainless steel single ferrules (A) and custom bilateral 1.25 mm ceramic optical ferrules with a 2.4 mm center-to-center distance (B) are shown. Single ferrules required a ML an AP angle for implantation, considerably decreasing implant accuracy. Bilateral ferrules allowed for implantation without a ML angle.

understanding of LC function. The 2.5 mm optical ferrules used for the unilateral experiments were too large to be implanted parallel to one another as LC is only about 2.4 mm apart. Initial attempts were made to implant these ferrules at a 15° ML angle in addition to the AP angle used to avoid the transverse sinus. This method proved too inaccurate to consistently, bilaterally hit LC with these implants. So, a custom bilateral implant using 1.25 mm diameter ferrules was developed that would not require angled implantation. These ferules are shown in figure 2.16.

Although considerably more accurate compared to two separate implants aimed at AP and ML angles, the custom bilateral implants posed their own challenges that may have contributed to the lack of effects witnessed in the behavioral experiments. The first design of these implants utilized ceramic ferrules, as seen in figure 2.16B. These ferrules broke frequently, resulting in loss of animals from experimental procedures. The next design used stainless steel ferrules that frequently detached from the patch cable over the course of an aSST session, distracting the animal from the task. All countermeasures to prevent detachment proved too bulky or were otherwise not well tolerated by the animal. Generally, tethering to a rat's head over the course of a sensitive and challenging behavioral task of long duration may not be a viable strategy with the currently available hardware.

Implant issues may also be contributing to the lack of effects witnessed for optogenetic stimulation of Fos production. Even a slight loss of coupling between the patch cable and optical ferrule over the course of the 15-minute stimulation procedure would result in a severe attenuation of light output in the LC area. Further, despite taking

steps to individually calibrate the light source each implant in every animal, there is no way to verify the appropriate light output over the course of the stimulation session or behavioral task. Further, any slight variation in the position of the implants, or variation in the cut of the optical fiber could change the amount of light delivered to the cells. In this case, activity of a ChR2 expressing cell may go unaffected even though it would appear that the implant was accurately positioned. Taken together, there is reason for skepticism that LC was actually receiving the type of stimulation these experiments attempted to deliver. Verifying light output in the awake, behaving animal either by combined photonic detection or simultaneous electrophysiological recording could overcome these practical concerns. However, these approaches would require an even greater leap in the sophistication of tools currently available for these types of experiments. A simpler approach could rely on calibrating the light output according to an independent behavioral outcome, such as waking, to verify what amount of light would need to be administered to induce the expected levels of activity in LC cells.

Considering electrophysiological response of LC neurons resulting from optogenetic stimulation raises an additional theoretical concern of whether light pulses from a single light source is truly mimicking the intended increased tonic activity. Photons from a single light source would cause any nearby neuron sufficiently expressing ChR2 to depolarize and fire. This would likely produce a synchronous, pulsatile pattern in LC neurons superimposed over their baseline activity, which is not the sort of activity predicted for a tonically active LC nucleus.

These results are in stark contrast with the ability of CNO activated hM3Dq to elicit Fos production in LC and a commensurate Fos response in mPFC. These differences in Fos were very clear, dose dependent, and proportionate, at least insofar as mPFC is considered. Although, attempts to measure Fos elicited by a microinjection of CNO in mPFC proved difficult. In such experiments, Fos expression varied widely throughout mPFC, even across hemispheres in the same animal, in ways that were not consistent with the treatment given. This may reflect a non-specific activation of this region resulting simply from the microinjection itself. However, within subject aCSF treatment would account for any behavioral effects resulting from the microinjection procedure.

In addition, DREADDs do not require tethering of the animals' heads to the behavioral apparatus. This manipulation also preserves typical receptor-ligand pharmacology that could overcome the synchronous activity possibly brought about by light based stimulation methods. It is for these reasons that continued experiments sought to employ DREADD based methods to stimulate LC and its projections to mPFC.

3. DREADD MEDIATED STIMULATION OF LC IN SET-SHIFTING

Designer receptors exclusively activated by designer drugs (DREADDs) are a relatively new research tool developed to provide stimulation, inhibition, or other modulation to selective neuronal populations. Unlike ChR2, these receptors, having been modified from endogenous muscarinic receptors, are able to stimulate native G-protien pathways to produce differing physiological effects within the neuron. This is also done in a way that preserves typical stochastic receptor/ligand dynamics. This has the advantage of producing a physiological response that more closely mimics what is observed in neuronal systems, while still preserving the unmatched specificity confered by the opsins. This advantage, however, currently comes at the cost of temporal precision. The experiments that follow in subsequent chapters attempt to confer a small degree of temporal precision by microinjecting the activating ligand CNO within LC and mPFC to stimulate NA cells and efferent projections in those areas. This was done with hopes of confining the period of LC activation to the epoch in which a cognitively flexible behavioral phenotype would be advantageous on the aSST.

DREADDs were developed with the goal of creating G-protien coupled receptors (GPCRs) with no demonstrable constituative activity that, when bound by an otherwise inert small molecule, could activate endogenous signaling pathways. These engineered GPCRs could then be sequenced, cloned, and packaged into viral vectors for cell selective expression in neural systems. This would allow for control of G-protein activity within specific cell types with little to no off-target effects. This goal was achieved

through a process of directed mutagenesis developed by Armbruster and collegues (Armbruster et al., 2007; Alexander et al., 2009).

To accomplish these goals, mutants of the human muscarinic-3 receptor (HM3) were grown in yeast and selected for their ability to bind CNO and activate the Gq signalling pathway. When activated, Gq signaling will result in the production of inositol triphosphate (IP3). Mutants that produced IP3 in response to CNO were selected and then subsequently screened for low levels of constituative activity in the absence of CNO. The mutant Y149C, A239G M3-muscarinic receptor was the first DREADD receptor to fulfill these criteria. This receptor is simply known as hM3Dq (Armbruster et al., 2007). Other variants have been developed to directly inhibit (hM4Di) or stimulate (GsD) cyclic-AMP (cAMP) production and even to interfere with β-arrestin regulation of GPCR trafficking (Armbruster et al., 2007; Alexander et al., 2009; Sternson and Roth, 2014).

Use of these receptors has lead to a number of high impact confirmatory and novel findings that were not possible using traditional methods. These include discovery and identification of feeding circuitry(Aponte et al., 2011; Krashes et al., 2011; Stachniak et al., 2014), neural substrates of memory encoding (Garner et al., 2012), mechanisms of synaptic plasticity (Kozorovitskiy et al., 2012; Stachniak et al., 2014), as well as the neural circuitry of reward-seeking and addiction behavior (Ferguson et al., 2013; Mahler et al., 2014) to name a few. Most notable for the purposes of the current study has to do the ability of DREADD mediated Gq signaling to increase activity of LC cells, elicit changes in arousal as measured by EEG, and facilitate emergence from anesthesia (Vazey and Aston-Jones, 2014).

In that study, rat LC was transduced with hM3Dq. Single cell and network activity was then measured in response to small, microinjected doses of CNO. These experiments found that in anesthetized animals, a 60 nL dose of CNO increased baseline discharge and decreased interspike intervals of LC neurons that were expressing hM3Dq for up to 6 minutes. Additionally, microinjection of 5 μM CNO in LC was sufficient to increase overall cortical EEG power, reduce burst suppression, and increase theta-band frequency. Unfortunately, these studies failed to capture a return to baseline following microinjection of CNO in LC. Nonetheless, both of these results are consistent with an increase in arousal. Indeed, a systemic dose of CNO was sufficient to decrease latency to emerge from anesthesia in these animals. This study suggests that microinjected CNO in LC is sufficient to increase tonic activity of LC cells in a way that is consistent with an increase in arousal as measured by cortical EEG (Vazey and Aston-Jones, 2014).

The following experiment attempted use microinjected doses of CNO in LC to transiently increase tonic activity during the behavioral epoch where a flexible behavioral phenotype would be advantageous in the aSST. This was done under the hypothesis that a behaviorally flexible state during this epoch would decrease perseverative errors and improve overall task performance as measured by TTC. However, microinjections of behaviorally efficacious doses of CNO did not improve overall task performance. Rather, the high dose produced an increase in regressive responding, suggesting the animal was unable to consistently follow the new rule.

Methods

Animal Care and Surgery. All methods used were in compliance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee. Animals were all housed in a temperature and humidity-controlled room under a reverse 12 h light/dark cycle (lights on at 6pm) with water available *ad libitum*. Animals were allowed 5 days for acclimatization and handling before any experimental procedures commenced. Following surgical procedures, food was available *ad libitum* for 10 d. During behavioral training and testing, rats were limited to 5 chow pellets (Harlan Teklad 8656 Sterilizable Rodent Diet) daily given following their training or testing session. Of animals bilaterally expressing hM3Dq, canulae were placed within or just lateral to LC in 22 animals, and in 9 animals expressing mCherry (figure 3.1).

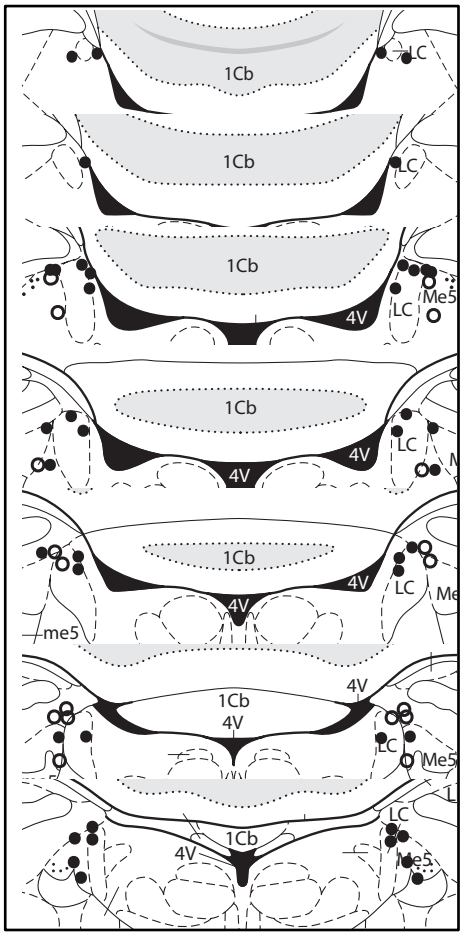

Figure 3.1 Accurate canulations in hM3Dq (closed circles) and mCherry (open circles) animals

Operant pretraining. Pretraining procedures follow from the previous chapter through side-bias testing. The tone stimulus was not used in the following experiments. Eliminating the tones considerably decreased the amount of

pretraining required for each animal and increased baseline levels of perseveration during EDS testing. This allowed a lower floor for decreases in perseveration to exert a significant effect. Following side bias testing rats were initially trained to discriminate the light stimulus dimension. On these trials, all chamber lights are initially off and the levers are retracted during a 10 s intertrial interval. Then one of the two cue lights, randomly chosen on every trial, was illuminated for three seconds and remained on until the rat pressed a lever or the trial timed out after 10 seconds. Once the cue light was on for 3 seconds, the house light was illuminated and both levers were extended into the chamber. The rat was required to press the lever that was positioned beneath the cue light within 10 seconds to attain reward. If the wrong lever was pressed, the house and cue lights were extinguished and the levers retracted; this was followed by a 10 second timeout. Rats were given 200 trials per training session until they were able to reach a streak of 20 correct trials.

Viral Vectors. The two vectors used in this study were cloned and packaged by the University of Pennsylvania Viral Vector Core. LC specific expression of the HA reporter tagged Gq coupled HM3D was achieved using the synthetic dopamine beta-hydroxylase promoter PRSx8 (Abbott et al., 2009). The control vector was regulated using the same PRSx8 promoter, but only expressed mCherry reporter protein.

Surgical Injection of Viral Vectors and Guide Canula Implantation. Once rats had completed pretraining procedures, rats underwent surgical procedures for injection of virus and bilateral LC canulation. These procedures follow closely from surgical procedures previously described. Only notable departures follow in this description. Four

skull screws were affixed to the parietal plates and secured with adhesive luting cement (C&B Metabond, Parkell). 1.5 µl of the HM3D or control vector was bilaterally infused over a 100 µm DV extent with brief pneumatic pulses (Picospritzer III, Parker Instruments) at the physiologically localized LC coordinates and allowed to diffuse throughout the tissue over 15 minutes before the injector was withdrawn. For LC microinjection procedures, a 26 gauge double guide canula was lowered 1 mm dorsal from the DV center of localized LC. One cohort was implanted with 2.4 mm center-to-center double canula. To allay concerns over leakage of the microinjection into the 4th ventricle, later cohorts were implanted with 3 mm center-to-center double canula aimed just lateral to LC. There was no significant interaction on trials to reach criterion between canula width and treatment (linear mixed model implant width*treatment $F=0.063$, $p=0.60$). The rats were allowed 2 weeks for recovery and virus transduction before testing resumed.

Extradimensional Strategy Set-Shifting and Microinjection Procedures. Trials during EDS testing proceeded identically to those in visual cue training. Two types of EDSs were performed, visual cue shift to spatial response or spatial response shift to visual cue. REVs were not performed in the following experiments. This was done to avoid potential confounds resulting from damage due to multiple microinjections. So, to minimize the number of total microinjections, rats performed only one stimulated and one unstimulated EDS from the both the unpredictable light dimension and the spatial dimension for a total of 4 separate EDSs. Only 1 EDS was performed per daily session (figure 3.2).

All rats were first trained on the light dimension and then performed an EDS to the spatial response to the lever opposite their side bias. On the following day, they performed a spatial response to visual cue EDS. This sequence was repeated on the 2

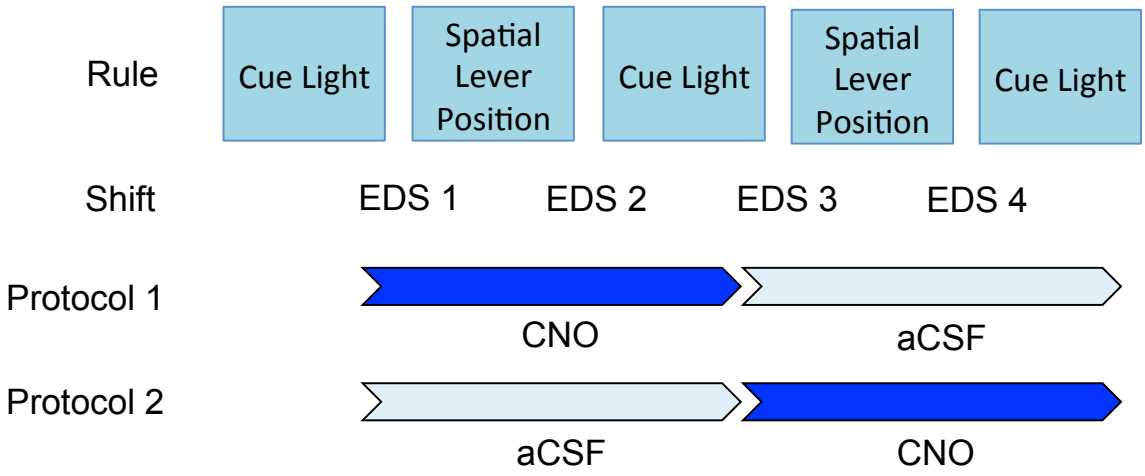

Figure 3.2 DREADD set-shifting protocols were altered from ChR2 experiments. Rats performed 4 EDS, 2 from the light dimension and 2 from the spatial dimension. They received CNO or aCSF according to one of the two protocols to balance possible order effects.

subsequent testing days (see figure 3.2). Rats received a 300 nl microinjection of either one dose of CNO (0.1 mM or 0.5 mM in artificial cerebrospinal fluid (aCSF), received from NIH-NCI NS064882-01) or aCSF counterbalanced over the first two or final two EDSs. These doses of CNO were determined in a small pilot experiment, similar to that performed for ChR2 experiments, to be the lowest tested doses sufficient to elicit a waking response.

On an EDS, the first 20 trials were carried out according to the last rule performed to criterion. The animal was then removed from the chamber, obturators were removed from the guide canula, and a 33 G double injector was inserted through and secured onto

the guide canula so that the injector protruded 1 mm below the guide. Each injector was connected to a 10 µl Hamilton syringe through polyethylene tubing (PE 20, Becton Dickinson). The infusion was delivered over 50 seconds and the injectors remained in place for one minute afterwards. The injector was then removed, the obturators replaced, and the animal was returned to the behavioral chamber. The initial 10 trials before the rule change again followed the last known rule. On the 11th trial, without any cueing to the animal, the rule was switched to the other possible strategy. The animal proceeded to carry out trials until they were able to execute a streak of 20 correct trials on the new strategy or until they reached 320 trials. EDSs in which the animal failed to meet criteria by 320 trials were not included in the statistical analyses. The dependent measures and statistical analysis thereof follow from the previous section. DREADD animals in these data were run across three separate cohorts. MCherry animals were run in a separate cohort and are compared separately.

Results

For the first 20 trials of a set-shifting session, the rat was required to follow the last rule previously performed with all rats being initially trained to accurately perform the visual cue rule. If the rat had last performed a visual cue shift to spatial response EDS, the following EDS would begin with 20 trials on same spatial response rule and vice-versa. There were no within or between-subjects baseline differences on this first 20 trial block (Figure 3.3A, $F_{LMM(3,54.870)}$=0.877, p=0.459). Following this first 20 trial block, rats were removed from the behavioral chamber and given microinjections of CNO (0.5 mM

or 0.1 mM) or aCSF and then carried out another 10 trials on the initial rule. Again, there were no between or within-group differences in the animals ability to continue to utilize the known rule following microinjection (Figure 3.3B, $F_{LMM(3, 41.308)}$=2.686, p=0.059). On the 11th trial following microinjection, the strategy-rule changes and the rat must shift its strategy to continue to receive reward on every trial and trials continue under the new rule until the rat reaches a streak of 20 correct trials. Trials to criterion were significantly

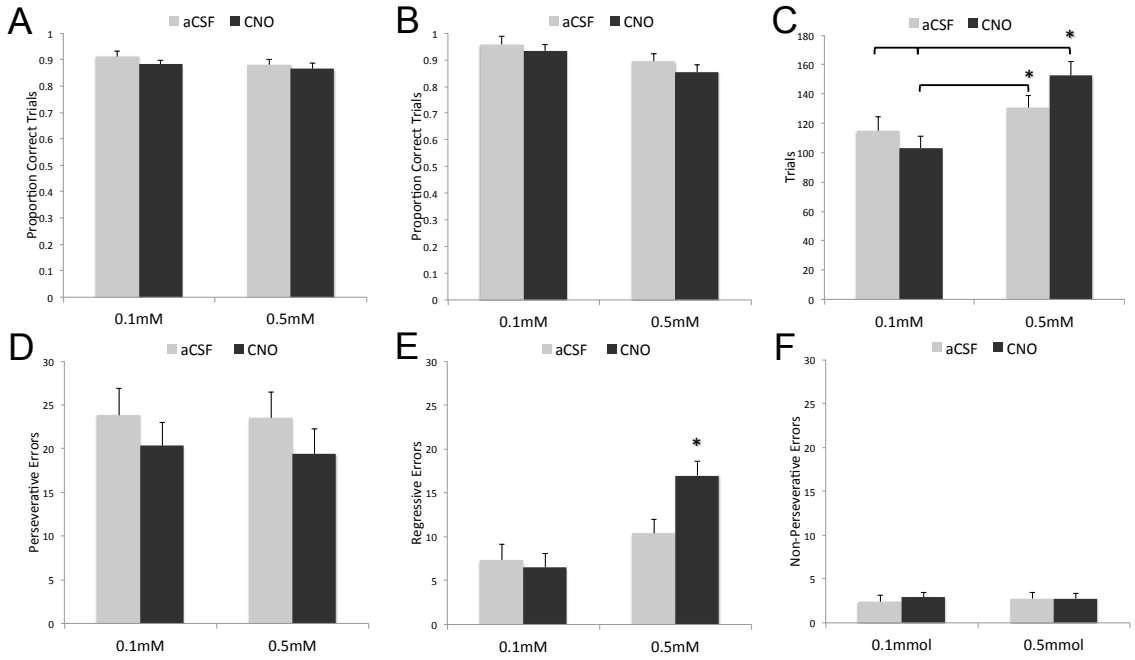

Figure 3.3 Strategy set-shifting results indicate hM3Dq mediated stimulation from a 0.5 mM injection of CNO in LC increases regressive responding within subjects (clustered bars). Proportion of correct trials preceding rule change before (A) and following (B) microinjection of aCSF (grey bars) or CNO (black bars). (C), total trials to reach criterion following rule change (*p=0.003). Breakdown of errors by perseverative (D), regressive (E, *p=0.001), or non-perseverative (F) error commission.

increased between subjects in the 0.5 mM group compared to the 0.1 mM group (Figure 3.3C, $F_{LMM(3,35.337)}$=5.553, p=0.003). However, no significant within-subjects differences were seen to result in this group from treatment with CNO ($LSD_{(38.545)}$=21.747 ± 12.859,

p=0.099). Error analysis revealed no significant main effects on perseverative (Figure 3.3D, $F_{LMM(3, 41.908)}$=0.571, (E) (*p=0.001), and non-perseverative (F). However, regressive responding was significantly increased by 0.5 mM CNO (Figure 3.3E, $F_{LMM(3, 33.930)}$=6.397, p=0.001) within-subjects ($LSD_{(37.538)}$=6.609 ± 2.571, p=0.014). Omissions did noti differ significantly as a result of CNO treatment ($F_{LMM(3, 47.350)}$=1.621, p=0.197, Figure 3.5A)

The mCherry control group was tested as an entirely separate cohort so is, thus,

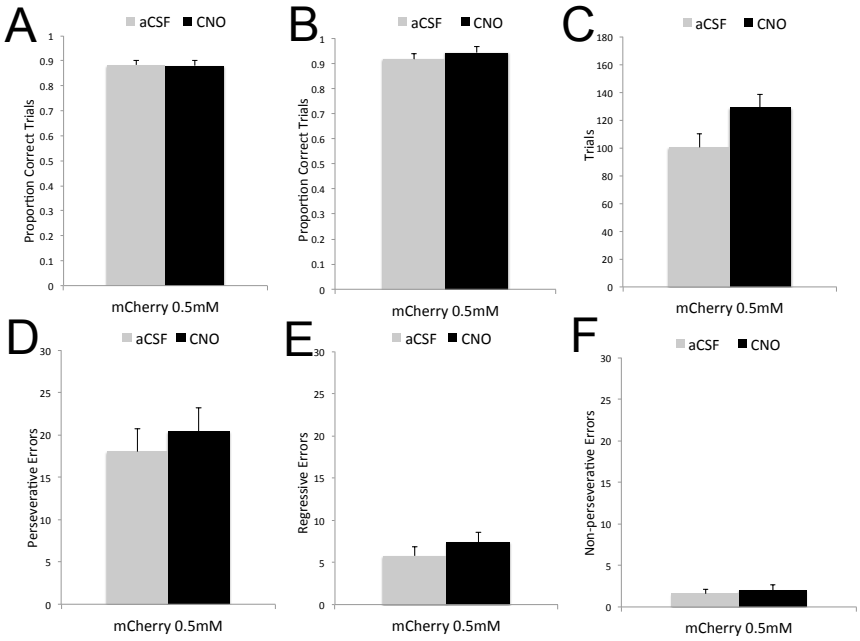

Figure 3.4 Microinjections of 0.5 mM CNO in LC produce no effects in mCherry controls. Proportion of correct trials preceding rule change before (A) and following (B) microinjection of aCSF (grey bars) or CNO (black bars). (C), total trials to reach criterion following rule change. Breakdown of errors by perseverative (D), regressive (E), and non-perseverative (F).

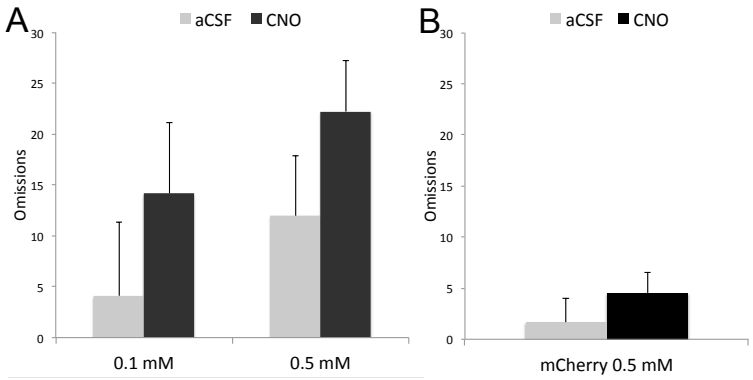

Figure 3.5 EDS omissions in hM3Dq (A) and mCherry (B) animals do not differ as a result of CNO treatment.

statistically compared independently from hM3Dq animals. Treatment with the 0.5 mM dose of CNO seen to be effective to increase regressive responding in hM3Dq animals produced no significant differences on any of the same measures. Accuracy prior to (Figure 3.4A, $F_{LMM(1, 23.559)}$=0.000, p=0.983) and following (Figure 3.4B, $F_{LMM(1, 23.107)}$=0.665, p=0.423) microinjection, and trials to criterion (Figure 3.4C, $F_{LMM(1, 14.379)}$=4.333, p=0.056) did not statistically differ within subjects. Error commission was also statistically equal for perseverative (Figure 3.4D, $F_{LMM(1, 15.212)}$=0.373, p=0.550), regressive (Figure 3.4E, $F_{LMM(1, 15.954)}$=0.892, p=0.359), and non-perseverative (Figure 3.4F, $F_{LMM(1, 19.125)}$=0.328, 0.573) error types. Omissions did not differ significantly as a result of CNO treatment ($F_{LMM(3, 5.346)}$=0.822, p=0.404, Figure 3.5)

To determine if the lack or presence of treatment effects in hM3Dq animals may have been due to order effects, that is that EDS performance may only be influenced by CNO treatment on the first EDS performed, the data from the first EDS performed was analyzed separately (Figure 3.6). Of the 10 total animals in the 0.1mM dose group, 4

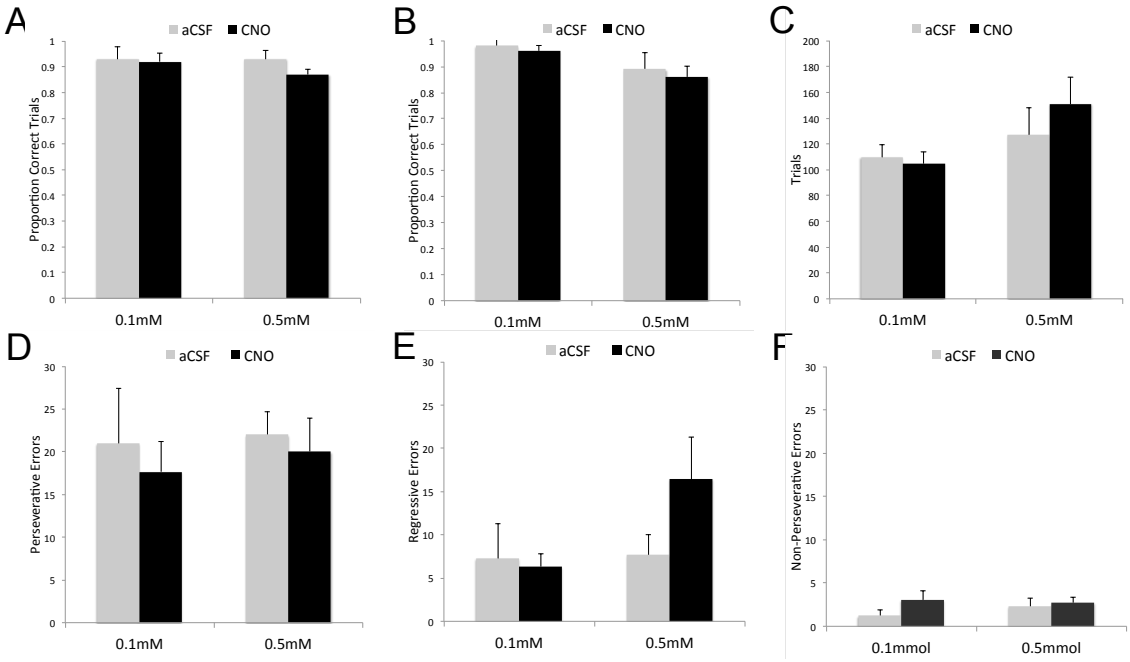

Figure 3.6 No significant differences in performance result from CNO treatment when only the first EDS are compared. Proportion of correct trials preceding rule change before (A) and following (B) microinjection of aCSF (grey bars) or CNO (black bars). (C), total trials to reach criterion following rule change. Breakdown of errors by perseverative (D), regressive, or non-perseverative (F) error commission.

received aCSF and 6 CNO, while 3 and 7 animals in the high dose group recieved aCSF and CNO respectively. There were no signficant differences in this first shift analysis, although the trend toward increased regressive behavoir appears to be present as this initial timepoint. There were no differences in pre-shift performance prior to ($F_{ANOVA(3, 16)}$=0.884, p=0.470) or following ($F_{ANOVA(3, 16)}$=2.317, p=0.114) microinjection. TTC

($F_{ANOVA(3, 16)}$=1.677, p=0.212), perseverative errors ($F_{ANOVA(3,16)}$=0.165, p=0.918), regressive errors ($F_{ANOVA(3,16)}$=1.732, p=0.201) and non-regressive errors ($F_{ANOVA(3,16)}$=0.668, p=0.573) were also not significantly changed by treatment with CNO.

Discussion

HM3Dq mediated stimulation of LC failed to decrease perseverative errors and did not improve animals' overall performance on the aSST. This result fails to support the hypothesis that tonic LC activity actively induces a cognitively flexible state that would facilitate set-shifting behavior. What is witnessed is an increase in regressive responding, or failing to consistiently utilize the new behavior once it has been discriminated. These errors occur later in the set-shifting session, which may indicate that LC is remaining tonically active for a period that extends into the expression phase of the new rule. However, that it would affect late regressive behavior without affecting early perseverative behavior might give reason to question if this is the case.

Previous studies using this task have been able to induce increases in perseverative behavior with varying manipulations. For instance, blockade of PFC $GABA_A$ receptors with bicuculline selectively increased perseverative errors (Enomoto et al., 2010). Similarly, blockade of GluN2B NMDA receptors with Ro25-6981 did the same (Dalton et al., 2011). But, to date, no experiments have attempted to reduce perseverative behavior and facilitate performance on this task. So it is unclear whether it is possible to further reduce perseverative behavior below baseline levels typically seen

in this task. However, once perseveration has ceased, prolonged tonic activity may be impairing the animals' ability to determine and consistently utilize the new rule.

VTA dopamine (DA) neurons are also well known for patterned tonic and phasic activity that is thought to be related to set-shifting behavior. This relationship has been modeled as is relates to sharp changes in network activity in PFC neurons (Durstewitz and Seamans, 2008; Durstewitz et al., 2010). Since the manipulations in the present experiments are specific for LC, VTA would likely continue to operate in accordance with the task demands. Therefore, even though hM3Dq stimulation is able to induce a tonic mode of LC activity, phasic release of dopamine may continue in a task-relevant way beyond the rule change trial. This could continue to support perseverative behavior until VTA neurons endogenously switch their pattern of activity. It may be necessary to exert a measure of control over both nuclei in order to fully optimize behavior on this task.

Stimulation of LC by CNO did induce an increase in regressive behavior, a behavioral output that occurs later in an EDS session when CNO levels at the site of injection would be expected to diminish. Notably, this effect is apparent on the very first EDS when CNO is received, although between subjects comparisons at this time point likely have too few observations to achieve signficiance. It is noteworthy then that the full time course of microinjected CNO induced activity remains to be determined. Prior reports have shown that activity of LC cells is elevated for up to 6 minutes by a 60 nl microinfusion of CNO administered within the LC. Similarly, microinjection of 5 µl CNO into LC produced a rightward shift in cortical EEG and an increase in theta band

frequency. However, in addition to considerable differences in dosage, those data failed to capture a return to baseline (Vazey and Aston-Jones, 2014). An alternative explanation could be that increased tonic activity early in the EDS may be preventing updating of a new behavior. So, although the animal has realized the previous behavior is no longer relevant, alternative strategies have not been adequately formed. This could still manifest as an increase in regressive responding that lags behind the point at which CNO would be exerting its effects. Whatever the proper interpretation may be, a more thorough time course analysis of CNO elicited activity of cells in PFC resulting from stimulation of LC inputs would be required before direct comparisons between early and late session behavioral outputs could be made.

Another possible reason for the increase in regressive responding may result from stimulation of other LC pathways. It is worth noting that complete expression of hM3Dq was not achieved throughout LC in any animal. Under these conditions, it is almost certain that the extent and pattern of viral transduction differed in each animal. While LC projections to mPFC are necessary in set-shifting, limbic projections play a well-known role in memory retrieval and consolidation (Murchison et al., 2004; Sterpenich et al., 2006; Sara, 2009; Sara, 2010). Enhanced memory retrieval would likely favor the old strategy from which the animal is being shifted. It is possible that variation in viral expression patterns between animals is masking effects by exerting differing degrees of stimulation between prefrontal or limbic targets in each animal.

It follows then, that manipulations of NA activity outside of the context of behavioral inputs may need to be regionally selective in addition to cell specific and

temporally precise. Recent studies have demonstrated that CNO microinjected at terminal sites of infected nuclei is sufficient to modify activity within the target region and induce relevant behavioral outcomes (Mahler et al., 2014; Stachniak et al., 2014; Vazey and Aston-Jones, 2014). Therefore, it might follow that selective stimulation of LC efferents within the mPFC could induce the flexible behavioral phenotype sought in these experiments that would lead to enhanced set-shifting performance.

The experiments outlined in the following chapter sought to selectively stimulate hM3Dq expressing LC terminals in the PFC by microinjecting CNO directly at the intended site of action. In doing so, any potential interference resulting from stimulation of the whole LC nucleus, or different subsets thereof that would facilitate memory retrieval, would be eliminated. The resulting data should provide the clearest possible result to speak to the sufficiency of LC inputs into PFC and their role in set-shifting behaviors.

4. STIMULATION OF LC INPUTS TO PFC IMPROVES SET-SHIFTING

The LC-NE system sends broad projections throughout the telencephalon with differing efferent density and heterogeneous patterns of target receptor expression. Depending on the site of action, NA manipulation can play varying roles in an array of behavioral phenomena (Aston-Jones et al., 2000; Sara, 2009; George et al., 2012; Carter et al., 2013; Hickey et al., 2014). Also, within the LC itself there is a fair degree of heterogeneity such as in CRF innervation, kappa-opioid receptor expression (Van Bockstaele et al., 2001) and nociceptive properties (Hickey et al., 2014). So, perhaps it is not surprising that exogenous stimulation of this system may need to be regionally specific in order to elicit the behavior profile set forth by adaptive gain theory.

Recent studies have found that microinjections of CNO within distinct target regions are sufficient to elicit local modulation of activity and modify behavioral responses (Mahler et al., 2014; Stachniak et al., 2014). Stachniak et al., 2014 examined the synaptic activity of locally administered CNO on hM4Di expressing pyramidal cells also co-expressing ChR2. They reported that CNO effectively silenced synaptic release from these cells even though pulses of light were sufficient to elicit action potentials in the axon of the same cells. Extending this finding, they expressed hM4Di in the periventricular nucleus of the hypothalamus and administered CNO locally within target areas to map out the selective contributions of PVH inputs in order to assess the selective roles of these regions in mediating feeding behavior. Using a similar approach, Mahler, et al. 2014 expressed hM4Di in rostral ventral pallidum (RVP) neurons to assess their role in modulating VTA and cocaine self-administration. Local administration of CNO in the VTA of rats expressing hM4Di in RVP neurons revealed a divergent response of

VTA neurons with putative DA neurons becoming excited, while other faster spiking cells were inhibited. When CNO was locally administered before cocaine self-administration in these same animals, cue-induced reinstatement of cocaine seeking was blocked. Taken together, there is reasonable evidence that locally administered CNO would be efficacious to produce local stimulation of LC terminals within the PFC.

The experiments in this chapter used locally administered CNO to stimulate LC terminals in the mPFC to test their sufficiency to improve set-shifting performance on the aSST. This approach follows very closely to the methods put forth in chapter 3, with the only major exception of CNO administration within mPFC rather than LC. Using this approach, these experiments find that CNO indeed reduced TTC. However, this improvement was not associated with a reduction in perseverative errors. Rather, it was driven by a reduction in regressive errors. Further, this reduction in regressive errors appears to result from enhanced application of the new rule being discriminated rather than a precipitous shift away from the old rule.

Methods

Most experimental methods follow directly from chapter 3 with few exceptions. Notable differences are as follows. Following injection of viral vectors in LC, the craniotomy was filled with sterile Gelfoam (Pfizer). Two smaller holes were drilled for PFC canulae. Bilateral 26 gauge guide canula were implanted at AP +3.2, ML ±0.7, DV -3.2 as measured from bregma and top of skull. Although all implants were accurately located within prelimbic (PL) cortex, initial cohorts showed considerable anterior movement over the course of the 3 month post-surgical period. Later cohorts were implanted at AP 2.7

from bregma. All implants of animals included in these analyses were contained within PL or infralimbic (IL) cortex. 18 total animals, 9 hM3Dq and 9 mCherry animals with confirmed bilateral virus expression and accurate canulae within PL/IL were used for these procedures. HM3Dq and mCherry animals were each tested in separate cohorts, so are statistically compared separately.

Following surgery, animals were allowed 1 month for recovery and virus expression. Pretraining procedures began after this one-month period. EDS testing did not begin until two-months following virus injection to allow for trafficking of viral products to the PFC.

Verification of viral expression in LC follows directly from previous chapters. In addition to verifying expression at LC, expression of hM3Dq and mCherry was also verified in PFC sections. One set of 40 μm PFC sections was reacted in mouse anti-HA (1:1000) to label tagged hM3Dq or in rabbit anti-DSRed (1:1000) to label mCherry expression. hM3Dq expression was visualized with donkey anti-mouse Alexa Fluor 594 (1:500) and mCherry was visualized with donkey anti-rabbit Alexa Fluor 594 (1:500).

These procedures also incorporated a win/stay, lose/shift analysis to measure choice behavior following correct or incorrect trials. To do this, choice on a given trial was compared to the stimuli presented and outcome of the previous trial. If the animal chose the lever associated with the correct behavior on the previous trial, that trial was counted as a win/stay. If the animal chose the opposite lever from the one associated with the incorrect behavior on the previous trial, this was counted as a lose/shift. This was calculated as a proportion of win or loss trials, respectively. Win/stay and lose/shift behavior was analyzed for the first bin of 25 trials following the rule change as well as

the 25 trial bin preceding the bin that the animal achieved a streak of 20 correct trials.

Results

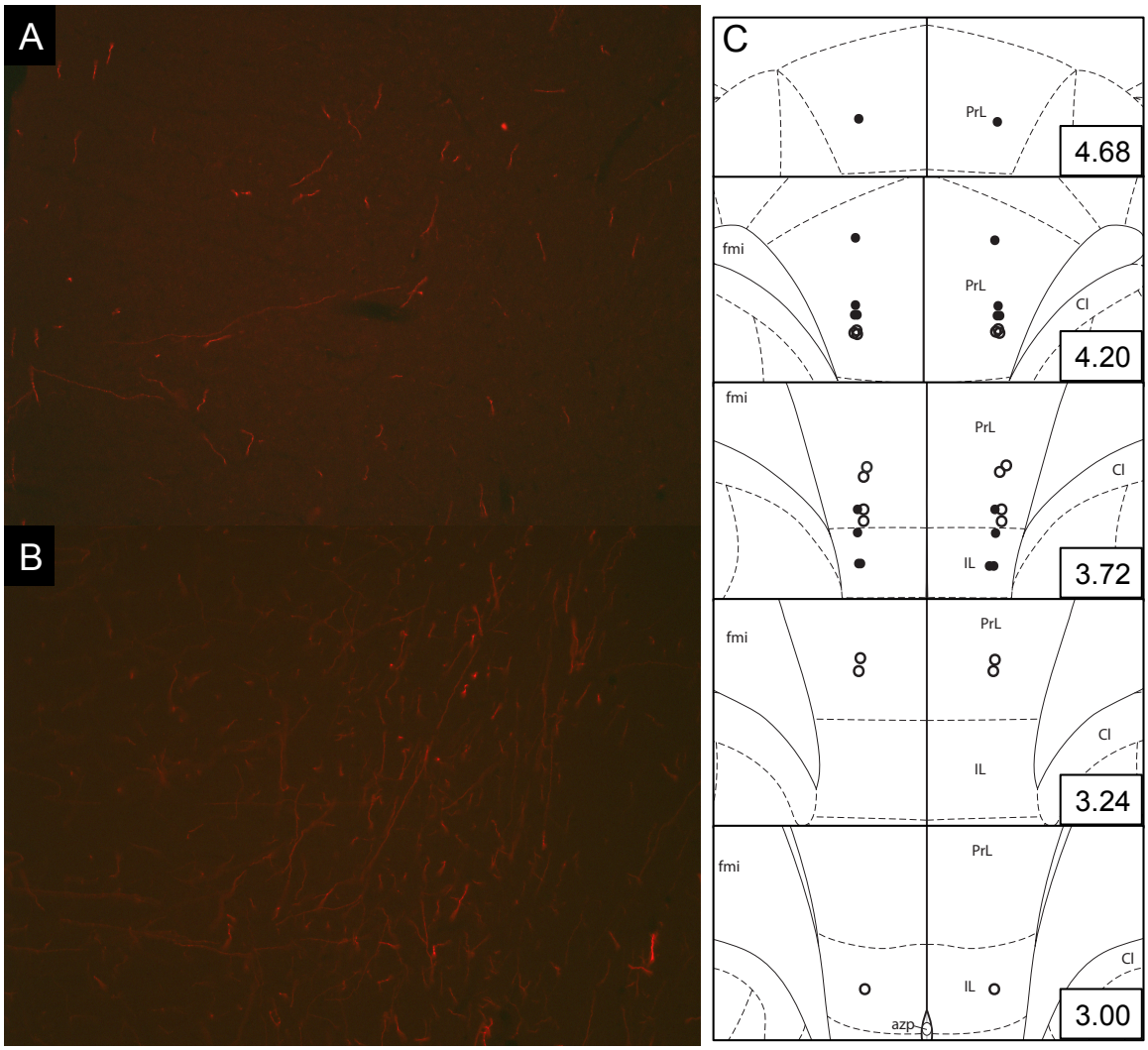

Figure 4.1 Expression of hM3Dq (A) and mCherry (B) is verified for PFC stimulation experiments. Placements of canulae tips for the 18 experimental animals are shown in C with closed circles representing hM3Dq animals (n=9) and open circles representing mCherry control animals (n=9).

Expression of hM3Dq (Figure 4.1A) and mCherry (Figure 4.1B) in mPFC was verified in animals with confirmed transduction of viral proteins in LC. Accurate bilateral canulae placements for the 18 animals utilized is shown in figure 4.1C. Performance of the known rule did not differ before (Figure 4.2A, $F_{LMM(1, 23.001)}$=1.812, p=.191) or following (Figure 4.2B, $F_{LMM(1, 14.996)}$=0.010, p=0.920) administration of aCSF or 0.5 mM CNO. Microinjection of CNO in PFC reduced TTC within-subjects (Figure 4.2C, $F_{LMM(1,}$

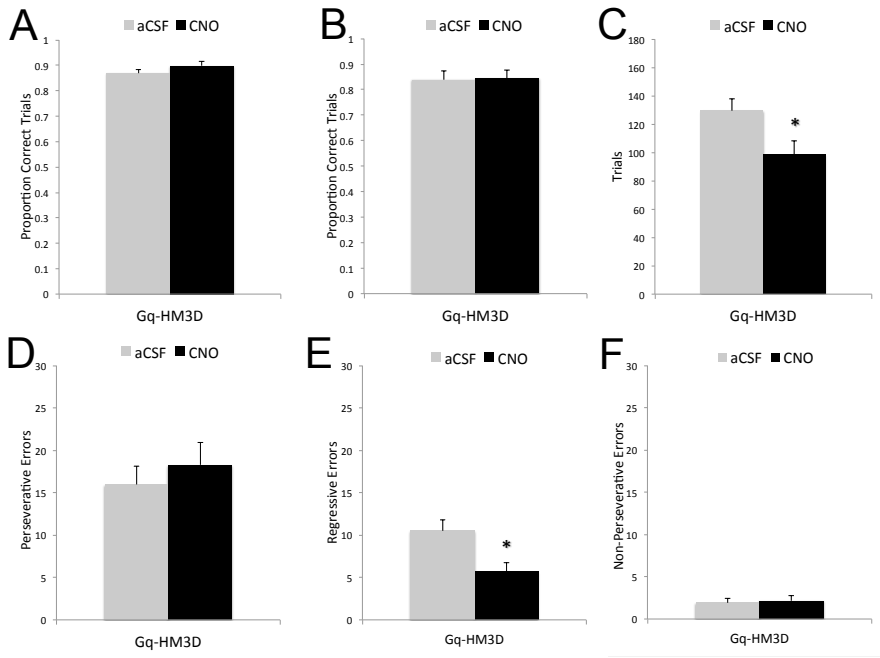

Figure 4.2 HM3Dq mediated stimulation of LC efferents in PFC resulting from microinjection of 0.5 mM CNO decreases TTC as a result of decreased regressive responding. Proportion of correct trials preceding rule change before (A) and following (B) microinjection of aCSF (grey) or CNO (black). C, total trials to reach criterion following rule change (*p=0.025). Breakdown of errors by perseverative (D), regressive (E) (*p=0.012), and non-perseverative (F).

$_{16.469)}$=6.128, p=0.025). Analysis of error types reveals that this reduction in TTC is associated with a decrease in regressive errors (Figure 4.2E, $F_{LMM(1, 20.583)}$=7.658,

p=0.012) while perseverative (Figure 4.2D, $F_{LMM(1, 15.263)}$=0.569, p=0.462) and non-perseverative error commission (Figure 4.2F, $F_{LMM(1, 17.770)}$=0.715, p=0.409) were unchanged. Omissions did not differ by CNO treatment ($F_{LMM(1,8.820)}$=1.050, p=0.753, figure 4.4).

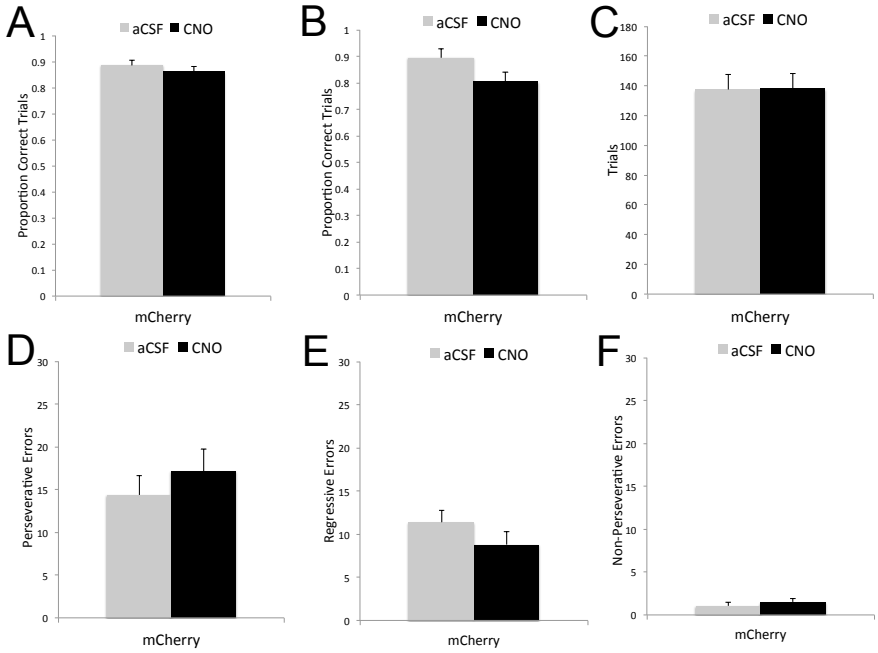

Figure 4.3 Microinjections of 0.5 mM CNO in PFC produce no effects in mCherry controls. Proportion of correct trials preceding rule change before (A) and following (B) microinjection of aCSF (grey bars) or CNO (black bars). C, total trials to reach criterion following rule change. Breakdown of errors by perseverative (D), regressive (E), and non-perseverative (F).

To attempt to determine the ways in which set-shifting performance was altered by CNO in the hM3Dq animals an analysis of win/stay and lose/shift performance was conducted. This measures how consistently the rat applies a successful strategy following rewarded trials (win/stay) or applies a different strategy following punished trials (lose/shift). At an early time point immediately following the rule change, there is no

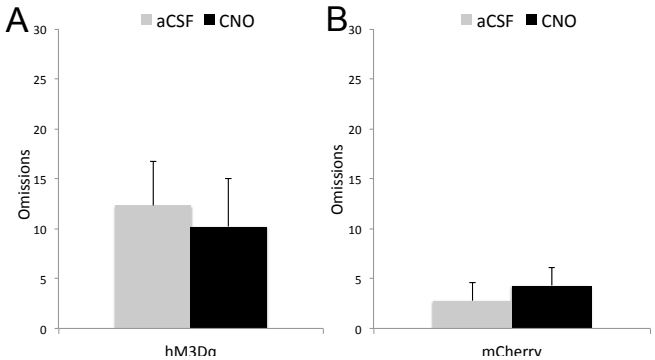

Figure 4.4 EDS omissions do not differ as a result of CNO treatment in hM3Dq (A) or mCherry (B) animals

Difference in win/stay performance (Figure 4.4A, $F_{LMM(1, 22.319)}$=1.1312, p=0.294) as a result of CNO treatment. However, lose/shift performance is slightly decreased (Figure 4.4B, $F_{LMM(1, 14.025)}$=4.643, p=0.049) at this same time point as a result of CNO treatment. At a later time point preceding the last bin of trials in which the animal meets criterion, win/stay performance is significantly increased by CNO (Figure 4.4C, $F_{LMM(1, 13.276)}$=6.427, p=0.025) while lose/shift performance is unaffected (Figure 4.4D, $F_{LMM(1, 11.657)}$=1.401, p=0.260).

To determine if the lack or presence of treatment effects in hM3Dq animals may have been due to order effects, that is that EDS performance may only be influenced by CNO treatment on the first EDS performed, the data from the first EDS performed was analyzed separately (Figure 4.6). Of the 9 total animals in the 0.1mM dose group, 7 received aCSF and 2 CNO. On the very first shift, despite small group size, TTC was signficantly decreased at this timepoint when animals received CNO. Regressive errors were similarly decreased as in the full analysis, although not significantly. There were no differences in pre-shift performance prior to ($F_{ANOVA(1, 7)}$=0.003, p=0.956) or following

($F_{ANOVA(1, 7)}$=0.119, p=0.741) microinjection. TTC was signifcantly reduced as a result of CNO treatment ($F_{ANOVA(1, 7)}$ 8.437, p=0.023), while perseverative errors ($F_{ANOVA(1,7)}$=0.002, p=0.962), regressive errors ($F_{ANOVA(1,7)}$=2.772, p=0.140) and non-regressive errors ($F_{ANOVA(1,7)}$=0.5444, p=0.052) were not significantly changed by treatment with CNO.

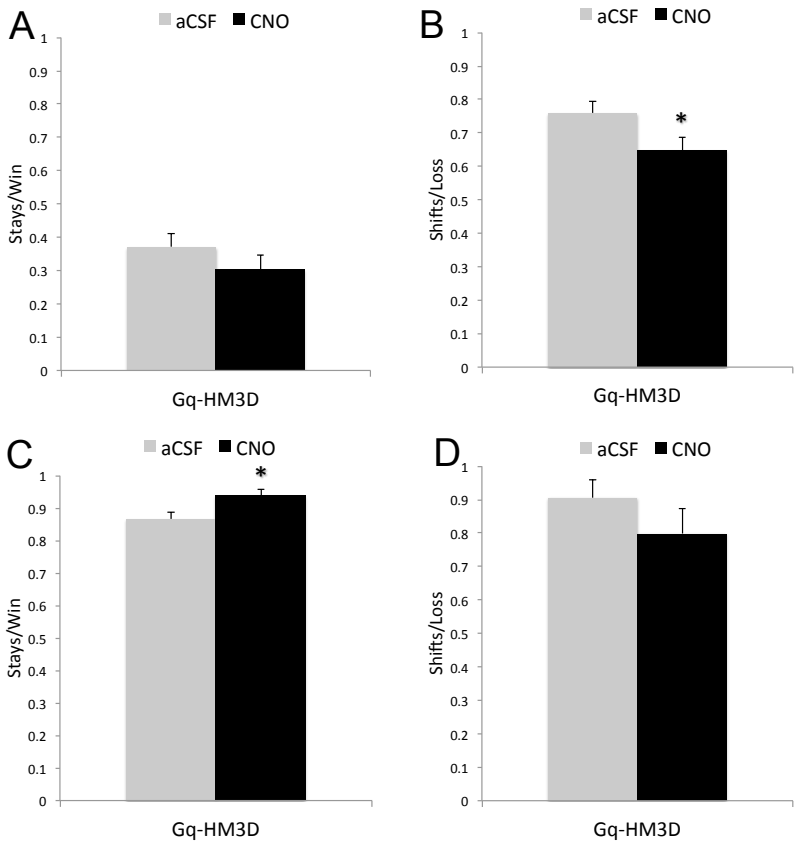

Figure 4.5 HM3Dq mediated stimulation of LC efferent terminals in PFC decreases lose/shift performance after the rule change and increases win/stay on later trials. Proportion of win/stay (A) and lose/shift (B, *p=0.049) performance for 25 trials immediately following the rule change. Proportion of win/stay (C, *p=0.025) and lose shift (D) performance on a 25 trial bin preceding the final bin in which criterion was met.

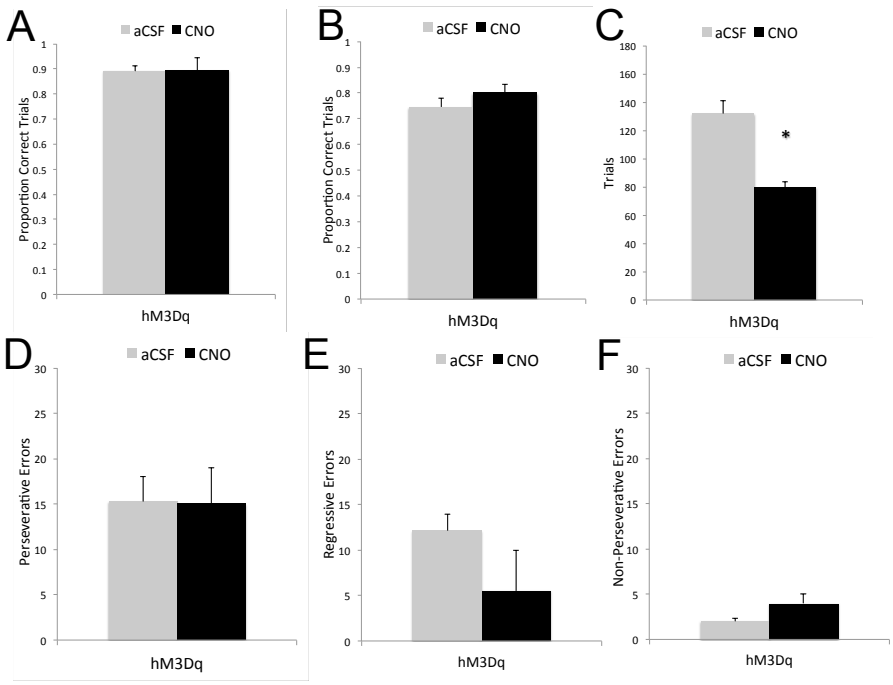

Figure 4.6 First EDS analysis reveals significant reduction in TTC resulting from treatment with CNO. Proportion of correct trials preceding rule change before (A) and following (B) microinjection of aCSF (grey bars) or CNO (black bars). (C), total trials to reach criterion following rule change (*p=0.023). Breakdown of errors by perseverative (D), regressive, or non-perseverative (F) error commission.

Discussion

DREADD mediated stimulation of LC efferent terminals in mPFC is sufficient to improve overall set-shifting performance. HM3Dq expressing rats required fewer trials to reach criterion on EDSs following an mPFC microinjection of CNO compared to EDSs where aCSF was administered. This effect is even evident when only data from the first EDS is compared. Control animals expressing mCherry required equivalent TTC regardless of the treatment received. Average TTC witnessed in control animals was similar to what was seen in hM3Dq animals receiving aCSF. The improved performance was associated with a significant decrease in regressive errors while none of the other

error types differed by treatment. Further, the decrease in regressive responding appears to arise from an enhancement of win/stay behavior late in the EDS session rather than an increase in lose/shift behavior. In other words, the decrease in regressive responding appears to result from more consistent application of the new rule once it has been discriminated rather than a more rapid abandonment of the old rule.

As in the previous chapter, these experiments find a significant effect of CNO on regressive responding rather than perseverative errors. However, instead of increasing regressive responding, as was the case for LC microinjection studies, CNO decreased regressive errors, resulting in a decrease in TTC. The lack of effect on perseverative behavior, again, might suggest that tonic stimulation of LC does not actively induce a cognitively flexible phenotype that would immediately shift responding from a learned behavior. However, this interpretation may be overly simplistic, relying on a number of assumptions that have not been tested in this framework.

First, this interpretation of the data presumes that NA is the only modulatory influence in play for the behavior being tested. As was established previously, patterned activity of DA neurons also exerts considerable influence on the persistence of reward-seeking behavior. By only exogenously controlling NA activity in mPFC, DA cells are left free to fire according to endogenous parameters. If this were the case, endogenous DA activity within reward circuits may be enough to sustain perseverative behavior regardless of the activity of NA terminals in mPFC. In order to induce the optimal state of cognitive flexibility that would further facilitate performance on this task, it may be necessary to exogenously control inputs from multiple areas within mPFC.

It is entirely possible that this test may not be sensitive to a reduction in perseveration due to a floor effect. Recall that previous studies using this task have been able to induce increases in perseverative behavior with varying manipulations (Floresco et al., 2008; Enomoto et al., 2010; Dalton et al., 2011) but have not reported reductions of this behavior within the same paradigm. Additionally, tasks probing stronger habitual behaviors have been able to reduce habitual responses by perturbing mPFC activity (Smith et al., 2012). In this experiment, rats were over trained on a T-maze task that required them to turn a particular direction in response to an auditory cue. Performance of the behavior persisted in control rats even following a period of reward devaluation, confirming the habitual nature of the learned response. By optically inhibiting IL cortical neurons, the experimenters were able to achieve a reduction in perseverative behavior (Smith et al., 2012). Overtraining was expressly avoided in the present study. So it may not be possible to further reduce perseverative behavior below baseline levels typically seen in this task without a incorporating a stronger habitual component.

What is clear is that performance on this set-shifting task is improved with stimulation of LC terminals in mPFC and that this improved performance is associated with a decrease in regressive responding. There are two possible behavioral outcomes that could produce this effect, 1) more rapid abandonment of the old rule and/or 2) more consistent application of the new rule once it has been discriminated. The win/stay lose/shift analysis speaks to this difference. Evidence for outcome 1 would manifest as an increase in lose/shift behavior, which is choosing the alternate strategy more frequently following an incorrect trial. Evidence for outcome 2 would manifest as an increase in

win/stay behavior, or choosing to respond with the same strategy following a successful strategy. What is witnessed is an increase in win/stay behavior in the 25 trails preceding the block of trials in which the animals achieved a 20 trial streak. An increase in lose/shift behavior is not seen at this time point. These results would suggest that the improved performance resulting from decreased regressive responding is due to an enhanced ability to consistently apply the new rule.

Set-shifting and behavioral flexibility are multi-faceted behaviors incorporating an array of cognitive abilities (Floresco and Jentsch, 2011). Facilitation of any one of these processes, such as response inhibition, extinction, or acquisition of the new response could improve overall performance on this task. Blockade of the norepinephrine transporter has been shown to improve inhibition of a pre-potent response (Bari et al., 2009). Likewise, there is a considerable literature on the role of PFC beta-adrenergic receptors in the facilitation of extinction (Mueller and Cahill, 2010). So, the behavioral facilitation seen here is likely to result from an enhancement of a set of inextricably linked cognitive processes. In addition to noradrenergic input, influence exerted by co-released dopamine (Devoto et al., 2001; Devoto et al., 2005), glutamate (Fung et al., 1994), or peptides such as galanin (Holets et al., 1988) cannot be ruled out. Co-infusion of CNO with antagonists for each synaptic target could parse the selective contributions, if any, of non-noradrenergic sources to this behavior.

Antidromic stimulation of LC cells projecting to mPFC could also contribute to this effect. Although the extent to which hM3Dq can produce antidromic depolarization remains to be established, it cannot be eliminated as a possible source of this response.

Within the PFC, there is evidence of selective innervation of discrete cortical sub-regions by individual LC cells (Chandler and Waterhouse, 2012). However, longstanding evidence suggests that a single branching LC cell can innervate multiple discrete targets throughout the forebrain (Moore and Bloom, 1979) in non-uniform patterns (Agster et al., 2013). So, although we can be reasonably confident that alternative afferent stimulation would be fairly limited within PFC, it cannot be ruled out for other known LC targets. However, due to the degree of specificity within PFC, it may be reasonable to assume that stimulation confined to PL/IL cortex would likely be stimulating and recruiting alternative afferents within the same network that contributes to improved performance on this task. Techniques combining terminal based stimulation and electrophysiology would be required to determine the degree to which antidromic activity could be contributing to this effect.

5. DISCUSSION

These experiments were designed to directly test specific tenets of adaptive gain theory. This theory posits that distractibility and focused attention are conserved behaviors that exist along the same continuum. Further, patterned tonic or phasic activity of the LC has a direct influence on the degree of distractibility or focus an individual is able to attribute at a given time. Specifically, when a behavior is of high utility, LC fires in a task-dependent phasic pattern that increases gain within the appropriate telencaphalic networks. This is thought to facilitate ongoing performance of the relevant behavior to exploit the rewarding opportunity. However, as the utility of a given behavior begins to wane, phasic activity becomes attenuated and baseline tonic activity is elevated. This switch is thought to bring about an adaptive disengagement from the ongoing behavior that would facilitate exploration of new, more rewarding opportunities. The experiments detailed herein sought to exogenously induce a pattern of tonic stimulation within LC and its targets in the mPFC to test if these manipulations would improve performance on a strategy set-shifting task.

There is a well-established literature, detailed previously, on the necessity of LC projections to the mPFC in set-shifting behavior (Birrell and Brown, 2000; Tait et al., 2007; McGaughy et al., 2008). The current experiments attempted to test the sufficiency of tonic stimulation within this circuit to improve performance on an operant set-shifting task (Floresco et al., 2008) by inducing a flexible behavioral phenotype that would disengage rats from a given behavior immediately following a rule change. Given these

assumptions, properly timed tonic stimulation of LC or its efferent inputs to mPFC should reduce overall TTC on this task. Further, if this manipulation is directly causing a disengagement from a behavior that is no longer relevant, a reduction in perseverative errors should be detected.

The primary findings of this study suggest that tonic stimulation of LC efferent inputs to mPFC is sufficient to reduce TTC and improve performance on the aSST. However, these manipulations were not sufficient to reduce perseverative errors. Rather, this manipulation produces the expected effect by reducing regressive responding. Analysis of trial outcome and subsequent choice behavior suggests that this effect results from an enhanced ability of the animal to consistently follow a new rule once it has been discriminated rather than by facilitating a more precipitous switch away from the old strategy. This effect was not witnessed when stimulation was confined within the LC nucleus itself, which may reflect differing functional roles of separate LC targets in this paradigm.

These experiments first required determination of an appropriate method to deliver cell-specific and temporally precise exogenous stimulation to LC. Given its unmatched temporal precision, initial efforts to carry out these experiments attempted to employ optogenetically-mediated stimulation to induce tonic firing in LC cells. Initial pilot results indicated that optogenetic stimulation of LC was efficacious in producing network wide changes in activity as well as an arousal and waking response in a sleeping animal. However, it does not appear that optogenetic stimulation was efficacious in modifying behavior in this task, except under highly specific circumstances and with very

small effect. Optogenetic stimulation also failed to produce any significant Fos induction compared to controls as it was administered here.

In contrast, hM3Dq mediated stimulation was able to produce a robust induction of Fos compared to mCherry controls. This effect was CNO dose dependent within the LC and positively correlated to Fos induction within the mPFC. Further, the two microinjected doses of CNO were sufficient to produce a similar waking response in hM3Dq pilot animals that was not seen when aCSF was administered. So, given that DREADD mediated stimulation was able to produce expected behavioral outcomes as well as an induction of an activity dependent molecular marker, it appeared to be a better candidate for these experiments.

These differences may reflect little other than practical issues with administering optogenetic stimulation within this framework. Canulae based methods have the considerable advantage of allowing for verification of light output both before and after a behavioral session. However, it is very difficult to insert these fibers through guide canulae without damaging them and any slight damage can result in a considerable loss of light output. Further it is not favorable to sedate the animal immediately prior to a challenging cognitive behavioral assay. Permanently implanted ferrule based systems subvert this latter concern, being easily attached without damage. However, it becomes impossible to verify light output at the source once the ferrule has been implanted. Future studies may do well to individually calibrate light output for each animal according to an independent behavioral measure, such as waking, rather than using a consistent output in

every animal. This could also help compensate for individual differences in levels of virus expression or slight variations in placement or angle of the implant.

One concern this approach would not address has to do with tethering of the animal by the head during the behavioral task. Often, FO cables would become detached from the animals implant, or otherwise distract the animal from the task. It was clear in observing animals' behavior throughout the task that they would often rear or reach back in order to grab or bite the cable. This behavior was occurring while the aSST was running. So, it is hard to conclude that data in those experiments provided a clear assessment of set-shifting behavior. Further, if the cable became detached, the animal would typically chew or otherwise attend to the cable rather than executing the task in a focused manner. This is compounded by the fact that even a small separation in coupling of the ferrule ends could severely attenuate light output of the implant.

Ultimately, all of these concerns are practical and should be addressable with advancements in the available technology. The countermeasures employed in these experiments proved either ineffective to combat these issues or were not well tolerated by the animals. Advancements in technology employing, ultra-lightweight, multi-parametric, wireless implants with multiple light outputs (Boyden, 2011; Kim et al., 2013) may help to overcome these issues. However, the experimental questions resulting from these techniques are currently outpacing the technology readily available to answer them.

Although lacking the temporal precision of ChR2, DREADD technology does not require any considerable technical advancement beyond basic tried-and-true behavioral pharmacology. It was for all of these reasons that this method seemed a viable alternative

for providing exogenous stimulation to LC. Indeed, this shift in methodology proved fruitful for answering our experimental questions.

Microinjections of CNO within hM3Dq expressing LC did not improve overall performance of the aSST as measured by TTC. The animals in the high dose group did require significantly more TTC, but this did not differ by the treatment received, either aCSF or CNO. So, this likely only reflects a difference in baseline performance of the task in these animals. However, when CNO was administered in the mPFC where LC terminals were expressing hM3Dq, EDS performance was improved within subjects compared to aCSF. Further, this effect was not witnessed in animals expressing only the mCherry reporter protein, whose TTC in both treatment conditions compares more closely to aCSF hM3Dq animals.

A similar site-specific divergent response was also witnessed on regressive errors. In hM3Dq animals receiving 0.5 mM CNO in LC, regressive errors were significantly increased compared to all other treatment conditions and groups. But, when the same dose of CNO was administered in the mPFC, regressive errors were decreased. Moreover, it is this decrease in regressive errors that underlies the improved overall performance seen in these animals.

These results would appear to indicate underlying qualitative differences in these two approaches, that is stimulation of the nucleus as a whole versus stimulation of a specific target area. One possible explanation for this divergent effect may have to do with the broad projection of LC processes throughout the telencephalon and the varying roles of the regions receiving this innervation. Within the mPFC, input from the LC is

necessary for (Birrell and Brown, 2000; Tait et al., 2007; McGaughy et al., 2008; Newman et al., 2008) and sufficient to improve (current data) set-shifting performance. However, within limbic circuitry, noradrenergic input plays a very well established role in the retrieval and reconsolidation of memories (Murchison et al., 2004; Sterpenich et al., 2006; Sara, 2010). Thus, when CNO is administered within the LC, it could be stimulating cells projecting to these or any other targets of the nucleus. Further, full bilateral transduction of virus was not achieved in any animal. So it is likely, if not certain, that different cells received different degrees of stimulation when CNO was administered locally in LC. This could result in a diversity of response in terms of performance on the aSST, potentially washing out any improvement in performance that may have been witnessed otherwise. The current results might even suggest that, in this group of animals, limbic projections were inordinately stimulated since an increase in regressive responding was measured. However, when CNO was administered only in mPFC, this competing drive was eliminated and the improvement in performance was realized.

It has also recently become clear that DREADDs may be working differently in the synapse than at the soma. Studies using the inhibitory Gi coupled human muscarinic-4 DREADD (hM4Di) have shown varying physiological effects depending on where CNO was administered. When administered at the soma of hM4Di expressing cells, CNO causes hyperpolarization but is unable to consistently prevent generation of action potentials (Ferguson et al., 2011; Krashes et al., 2011). However, when CNO is administered at hM4Di expressing terminals, it potently suppresses release of

neurotransmitter in an action potential independent manner (Stachniak et al., 2014). It is yet unclear whether hM3Dq would exert similar differing effects depending on the site of CNO administration. Perhaps CNO binding to hM3Dq receptors at mPFC LC terminals results in increased presynaptic calcium or activation of protein kinase C that would facilitate release of neurotransmitter regardless of action potential generation in the axon. In this case, tonic levels of synaptic NA would remain elevated, even if phasic activity persisted in LC in a task relevant pattern. In other words, NE release from these terminals onto mPFC synapses would more closely mimic a physiological tonic mode in the presence of CNO even if phasic impulse activity persisted in LC in response to the task conditions. These noted differences in site-specific actions of DREADD receptors could play a role in the divergent behavioral responses reported here.

There is also potential that this stimulatory effect may not remain isolated within the mPFC. If Gq activity in presynaptic terminals is increasing local calcium concentration, either by release of intracellular stores or PKC dependent regulation of ion channels, it follows that the resulting depolarization could activate voltage-gated channels leading to back-propagating action potentials. Although generation of back-propagating depolarization by hM3Dq mediated stimulation has yet to be demonstrated, it cannot be ruled out as a factor by these experiments. According to the published literature, all but a small percentage of LC cells will innervate a single discrete subregion of PFC (Chandler and Waterhouse, 2012). However, there is considerable evidence that a single LC cell can branch and innervate multiple subcortical structures (Moore and Bloom, 1979). These sites of subcortical innervation can vary greatly in the expression of synapse

forming varicosities (Agster et al., 2013). It follows then that alternative afferent stimulation would be limited within mPFC, so it is unlikely that other PFC sub-regions would be directly contributing to this effect unless directly stimulated by CNO. But, this assumption does not hold for other non-cortical LC targets that may share branching axons with terminals in mPFC. It may be the case that common subcortical targets recruited by this type of stimulation are part of a relevant behavioral network that mediates performance on this task. Therefore, although alternative afferent stimulation may be antidromically recruiting additional subcortical regions, it could be doing so in a network specific manner that enhances performance on this behavioral assay. However, when stimulation is delivered to somato-dendritic regions, the enhanced LC activity may modify activity across multiple networks, some of which could potentially support performance of the old rule even after the reward contingencies have changed.

If these effects do indeed result from action potential independent mechanisms, it calls into question how exactly Gq mediated stimulation is modifying activity of LC cells. Based on in-vivo recordings, these experiments assumed that CNO would simply increase of baseline activity of hM3Dq expressing LC cells (Vazey and Aston-Jones, 2014). Presuming that increased tonic activity would lead to a flexible behavioral phenotype, it was hypothesized that perseverative errors would be reduced as a result of an active disengagement from the previously relevant behavior. This was not witnessed for any experimental manipulation. A previous study examining the role of Gq mediated stimulation in LC cells may help to address this unexpected result. Thyrotropin-releasing factor (TRF) is a neuropeptide that can modulate activity of LC cells in a phospholipase-

C (PLC) dependent manner. Administration of TRF in slices results in elevated discharge from LC cells, but also decreases resting membrane K+ conductance, which facilitated burst activity following periods of inhibition (Ishibashi et al., 2009). Phasic activity in LC neurons is characterized by an overall suppression of baseline activity and task-related phasic bursts that are thought to facilitate a directed behavioral response. Therefore, it may be possible that CNO stimulation of Gq signaling pathways facilitates tonic and phasic activity and that switches between these modes of activity remain dependent on task contingencies. In this condition, it is uncertain that CNO administered in LC would exogenously and independently induce a tonic pattern of neuronal activity. In other words, CNO may only facilitate ongoing tonic or phasic activity in LC cells rather than unidirectionally biasing LC into a tonic mode. However, when CNO is administered in mPFC, tonic synaptic release of NA may be facilitated regardless of ongoing activity within the LC nucleus, which results in improved performance on the aSST. Although, this would still fail to explain the lack of effect on perseverative errors when CNO was administered in mPFC. But, as previously explained, an effect on perseveration may be difficult to attain within this particular behavioral framework by only manipulating LC inputs.

These regionally specific results raise the question of whether the lack of effect seen in the optogenetic experiments may have been due to similar circumstances. Recall, that these experiments only delivered light stimulation to the LC nucleus itself and not to efferent targets. It is possible then that exogenously stimulating LC optogenetically could produce the same conflicting behavioral outcomes depending on which neurons received

stimulation. A future experiment could reinforce these findings by placing light fibers in mPFC and stimulating ChR2 expressing LC terminals following the rule change on the EDS. If then no effect is seen, it may be reasonable to conclude that the lack of effects in ChR2 experiments might be the result of technical concerns already addressed. However, if the same regionally specific effect on TTC is witnessed, it would suggest that one or more of these proposed mechanisms may be at play. If tethering of the animal continued to be a concern, use of bistable opsins that can be persistently activated or inactivated with single pulses of light (Berndt et al., 2009) could be incorporated. These opsins could also potentially overcome concerns related to the induction of rhythmic activation of the cells in response to light input.

Further experiments will also be required to determine the target through which stimulated LC terminals are exerting the effect witnessed here. The most straightforward approach would be to co-infuse antagonists with CNO into mPFC. If this effect is simply due to facilitated release of NE, it should be blocked by co-infusion of the beta antagonist propranolol, the alpha-1 antagonist prazosin, the alpha-2 antagonist atipamezole, or any combination thereof. If none of these antagonists masked the improved performance, non-NA sources of this effect, such as DA, glutamate, or galanin would also have to be examined.

Although the mechanism responsible for these effects remains to be determined, these experiments do provide direct evidence that inputs from LC to mPFC are sufficient to improve set-shifting performance. However, these manipulations were not sufficient to independently interrupt an ongoing pattern of behavior as would be predicted by adaptive

gain theory. Adaptive gain theory posits that a pattern of tonic LC activity serves to actively disengage a respondent from a current pattern of behavior when the value of performing that behavior outweighs the cost. Perseveration then could be the result of LC remaining in a phasic pattern of activity until the point that enough evidence can be accumulated to update the declining value of continuing to perform that behavior. In these experiments, we attempted to force LC into a tonic mode independently of this lagging calculation. This claim comes with the caveat that an actual tonic stimulation was achieved from our manipulations, which as was previously addressed, remains to be confirmed. But, presuming this was the case, it is quite evident that our manipulations failed to induce a distractible or disengaged state. Two of the variables measured in the DREADD experiments speak directly to this question. First, following CNO microinjection, animals were still willing and able to accurately recall and perform the known behavior prior to the rule change. This was the case regardless of where the CNO microinjection was delivered. Second, these manipulations were not able to reduce perseverative behavior. This may indicate that LC alone is not able to force a distractible state independently of the ongoing calculation of behavioral utility. This would imply that LC is not solely responsible for initiating this transition. This is perhaps not surprising given the role of other systems such as VTA dopamine in mediating reward based motivated behavior. Further experiments may be able to verify that these manipulations are producing a physiologically relevant tonic mode of activity. However, if these hypothetical experiments still fail to capture a reduction of perseverative responding, any resulting explanation of the substrates of behavioral flexibility will not

be able proceed without fully integrating the influence of other neural systems. The necessary tools to conduct such a complex analyses are only now becoming available. Ideally, full control over multiple independent cell types could be achieved to completely dissect this behavior.

It may be the case that this task environment was not complex enough to witness the flexible phenotype these experiments were sought to capture. At its heart, any given trial was ultimately a 50/50 choice between the right or left lever. So, if the animal was using no particular strategy to perform the task, it will likely still receive the reward on 50% of trials. Given additional options, a subtle modification of strategy may become more obvious than what can be captured by this task. Future experiments to this end may need to incorporate an array of possible responses, additional stimulus dimensions, or variable reinforcement ratios in order to find an apt direct measure of flexibility beyond simple improvement in task performance.

Alternatively, it could be the case that perseveration, as modulated by NE, is mediated at a different site than PFC. Habitual type behaviors are commonly thought to be mediated by recurrent circuitry between PFC and dorsal striatum (Reading et al., 1991; Schmitzer-Torbert et al., 2014). However, in the rat, dorsal striatum does not receive innervation from LC (Sara, 2009) making this an unlikely target for NE in perseverative behavior. However, other studies have shown that tonic and phasic patterns of LC stimulation can selective strengthen or attenuate thalamic responses to incoming sensory stimuli(Berridge and Waterhouse, 2003; Devilbiss and Waterhouse, 2011). So, it may be possible that induction of tonic NE activity in thalamus could act to weaken

stimulus associations related to the old rule and mediate perseverative responding on this task.

That is not to imply, however, that these data fail to support some tenets of adaptive gain theory. By its simplest interpretation, the manipulations employed by this set of experiments should be sufficient to improve performance on tasks measuring flexible behavior. That is exactly what has been achieved. That it does so by reducing regressive responding and strengthening the alternate behavioral response later in the EDS session rather than reducing perseveration does not disqualify a role for LC in producing a flexible behavioral phenotype. Rather, it could imply that the correct interpretation is more complex than can be captured by these analyses. An alternative explanation of how induction of tonic NE release in PFC could facilitate acquisition of the new reward contingency is that tonic LC activity may only disengage certain aspects of the behavior, such as arousal towards the task cues or overall attentiveness. Meanwhile, other relevant networks, remaining free of experimental influence, can maintain the ongoing behavior. However, once these systems relent to the new task demands, LC may have already sensitized to the new task contingencies and can subserve a more rapid utilization of the new behavior. However, these explanations are far beyond the scope of the current experiment.

These results could indicate an unforeseen issue with the unprecedented selectivity conveyed by these new experimental techniques, especially when they are used in such complex behaviors. The effect sizes witnessed in these results are fairly modest and the behaviors tested are quite complex. So, although we are able to control

select population with these new manipulations, it may only influence a small aspect of an otherwise very complex behavior. In other words, although our manipulations may reduce variability due to their specificity, that specificity may make it more difficult to witness drastic effects as a given behavior becomes more complex.

In its most straightforward interpretation, these data show that performance on a strategy set-shifting task can be improved by administering cell-specific stimulation to LC terminals in the mPFC. This improved performance results from a decrease in regressive responding, which is associated with an increase in win/stay behavior in the trials before the animal achieves a streak of 20 correct trials. So, stimulation of mPFC NA terminals this task is improving the animals' ability to consistently apply the new behavior sooner in the EDS. Although the physiological mechanism behind this effect remains to be determined, these results strengthen understanding of the role of LC in mediating flexible behavior and managing trade-offs in light of dynamic environmental contingencies.

6. REFERENCES

Abbott SBG, Stornetta RL, Fortuna MG, Depuy SD, West GH, Harris TE, Guyenet PG (2009) Photostimulation of Retrotrapezoid Nucleus Phox2b-Expressing Neurons In Vivo Produces Long-Lasting Activation of Breathing in Rats. J Neurosci 29:5806-5819.

Agster KL, Mejias-Aponte CA, Clark BD, Waterhouse BD (2013) Evidence for a regional specificity in the density and distribution of noradrenergic varicosities in rat cortex. J Comp Neurol 521:2195-2207.

Alexander GM, Rogan SC, Abbas AI, Armbruster BN, Pei Y, Allen JA, Nonneman RJ, Hartmann J, Moy SS, Nicolelis MA, McNamara JO, Roth BL (2009) Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. Neuron 63:27-39.

American Psychiatric Association., American Psychiatric Association. DSM-5 Task Force. (2013) Diagnostic and statistical manual of mental disorders : DSM-5, 5th Edition. Washington, D.C.: American Psychiatric Association.

Aponte Y, Atasoy D, Sternson SM (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat Neurosci 14:351-355.

Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A 104:5163-5168.

Aston-Jones G, Bloom FE (1981) Norepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. J Neurosci 1:887-900.

Aston-Jones G, Cohen JD (2005a) Adaptive gain and the role of the locus coeruleus-norepinephrine system in optimal performance. J Comp Neurol 493:99-110.

Aston-Jones G, Cohen JD (2005b) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. Annu Rev Neurosci 28:403-450.

Aston-Jones G, Rajkowski J, Cohen J (2000) Locus coeruleus and regulation of behavioral flexibility and attention. Prog Brain Res 126:165-182.

Aston-Jones G, Ennis M, Pieribone VA, Nickell WT, Shipley MT (1986) The brain nucleus locus coeruleus: restricted afferent control of a broad efferent network. Science 234:734-737.

Aston-Jones G, Rajkowski J, Kubiak P, Valentino RJ, Shipley MT (1996) Role of the locus coeruleus in emotional activation. Prog Brain Res 107:379-402.

Barch DM, Braver TS, Sabb FW, Noll DC (2000) Anterior cingulate and the monitoriing of response conflict: evidence from an fMRI study of overt verb generation. J Cogn Neurosci 12:298-309.

Barch DM, Braver TS, Nystrom LE, Forman SD, Noll DC, Cohen JD (1997) Dissociating working memory from task difficulty in human prefrontal cortex. Neuropsychologia 35:1373-1380.
Barch DM, Braver TS, Akbudak E, Conturo T, Ollinger J, Snyder A (2001) Anterior cingulate cortex and response conflict: effects of response modality and processing domain. Cereb Cortex 11:837-848.
Bari A, Eagle DM, Mar AC, Robinson ESJ, Robbins TW (2009) Dissociable effects of noradrenaline, dopamine, and serotonin uptake blockade on stop task performance in rats. Psychopharmacology (Berl) 205:273-283.
Berridge C, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res Brain Res Rev 42:33-84.
Birrell JM, Brown VJ (2000) Medial frontal cortex mediates perceptual attentional set shifting in the rat. J Neurosci 20:4320-4324.
Bouret S, Sara S (2004) Reward expectation, orientation of attention and locus coeruleus-medial frontal cortex interplay during learning. Eur J Neurosci 20:791-802.
Bouret S, Sara SJ (2005) Network reset: a simplified overarching theory of locus coeruleus noradrenaline function. TRENDS in Neurosciences 28:574-582.
Boyden ES (2011) A history of optogenetics: the development of tools for controlling brain circuits with light. F1000 Biol Rep 3:11.
Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci 8:1263-1268.
Braff DL, Geyer MA, Swerdlow NR (2001) Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology 156:234-258.
Brázdil M, Roman R, Falkenstein M, Daniel P, Jurák P, Rektor I (2002) Error processing--evidence from intracerebral ERP recordings. Experimental brain research Experimentelle Hirnforschung Expérimentation cérébrale 146:460-466.
Bullitt E (1990) Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol 296:517-530.
Carter ME, De Lecea L, Adamantidis A (2013) Functional wiring of hypocretin and LC-NE neurons: implications for arousal. Front Behav Neurosci 7:43.
Carter ME, Yizhar O, Chikahisa S, Nguyen H, Adamantidis A, Nishino S, Deisseroth K, De Lecea L (2010) Tuning arousal with optogenetic modulation of locus coeruleus neurons. Nat Neurosci.
Chandler D, Waterhouse BD (2012) Evidence for Broad Versus Segregated Projections from Cholinergic and Noradrenergic Nuclei to Functionally and Anatomically Discrete Subregions of Prefrontal Cortex. Front Behav Neurosci 6:1-9.
Choi J, Kurtz MM (2009) A comparison of remediation techniques on the Wisconsin Card Sorting Test in schizophrenia. Schizophr Res 107:76-82.
Clayton EC, Rajkowski J, Cohen JD, Aston-Jones G (2004) Phasic activation of monkey locus ceruleus neurons by simple decisions in a forced-choice task. J Neurosci 24:9914-9920.

Corbetta M, Patel G, Shulman GL (2008) The reorienting system of the human brain: from environment to theory of mind. Neuron 58:306-324.
Dalton GL, Ma LM, Phillips AG, Floresco SB (2011) Blockade of NMDA GluN2B receptors selectively impairs behavioral flexibility but not initial discrimination learning. Psychopharmacology (Berl).
Darrah JM, Stefani MR, Moghaddam B (2008) Interaction of N-methyl-D-aspartate and group 5 metabotropic glutamate receptors on behavioral flexibility using a novel operant set-shift paradigm. Behavioural pharmacology 19:225-234.
Delfs JM, Zhu Y, Druhan JP, Aston-Jones GS (1998) Origin of noradrenergic afferents to the shell subregion of the nucleus accumbens: anterograde and retrograde tract-tracing studies in the rat. Brain Research 806:127-140.
Devilbiss DM, Berridge CW (2006) Low-dose methylphenidate actions on tonic and phasic locus coeruleus discharge. J Pharmacol Exp Ther 319:1327-1335.
Devoto P, Flore G, Pani L, Gessa GL (2001) Evidence for co-release of noradrenaline and dopamine from noradrenergic neurons in the cerebral cortex. Mol Psychiatry 6:657-664.
Devoto P, Flore G, Saba P, Fa M, Gessa GL (2005) Stimulation of the locus coeruleus elicits noradrenaline and dopamine release in the medial prefrontal and parietal cortex. J Neurochem 92:368-374.
Dragunow M, Faull R (1989) The use of c-fos as a metabolic marker in neuronal pathway tracing. J Neurosci Methods 29:261-265.
Durstewitz D, Seamans JK (2008) The dual-state theory of prefrontal cortex dopamine function with relevance to catechol-o-methyltransferase genotypes and schizophrenia. Biological psychiatry 64:739-749.
Durstewitz D, Vittoz NM, Floresco SB, Seamans JK (2010) Abrupt transitions between prefrontal neural ensemble states accompany behavioral transitions during rule learning. Neuron 66:438-448.
Eisenberger NI, Lieberman MD, Williams KD (2003) Does rejection hurt? An FMRI study of social exclusion. Science 302:290-292.
Emeric EE, Brown JW, Leslie M, Pouget P, Stuphorn V, Schall JD (2008) Performance monitoring local field potentials in the medial frontal cortex of primates: anterior cingulate cortex. J Neurophysiol 99:759-772.
Enomoto T, Tse MT, Floresco SB (2010) Reducing Prefrontal Gamma-Aminobutyric Acid Activity Induces Cognitive, Behavioral, and Dopaminergic Abnormalities That Resemble Schizophrenia. Biol Psychiatry.
Fenno L, Yizhar O, Deisseroth K (2011) The development and application of optogenetics. Annual review of neuroscience 34:389-412.
Ferguson SM, Neumaier JF (2012) Grateful DREADDs: engineered receptors reveal how neural circuits regulate behavior. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37:296-297.
Ferguson SM, Phillips PEM, Roth BL, Wess J, Neumaier JF (2013) Direct-pathway striatal neurons regulate the retention of decision-making strategies. J Neurosci 33:11668-11676.

Ferguson SM, Eskenazi D, Ishikawa M, Wanat MJ, Phillips PE, Dong Y, Roth BL, Neumaier JF (2011) Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. Nat Neurosci 14:22-24.
Fitzgerald KD, Welsh RC, Gehring WJ, Abelson JL, Himle JA, Liberzon I, Taylor SF (2005) Error-related hyperactivity of the anterior cingulate cortex in obsessive-compulsive disorder. Biol Psychiatry 57:287-294.
Floresco SB, Jentsch JD (2011) Pharmacological enhancement of memory and executive functioning in laboratory animals. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36:227-250.
Floresco SB, Block AE, Tse MTL (2008) Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure. Behav Brain Res 190:85-96.
Fung SJ, Reddy VK, Liu RH, Wang Z, Barnes CD (1994) Existence of glutamate in noradrenergic locus coeruleus neurons of rodents. Brain Res Bull 35:505-512.
Garner AR, Rowland DC, Hwang SY, Baumgaertel K, Roth BL, Kentros C, Mayford M (2012) Generation of a synthetic memory trace. Science 335:1513-1516.
George SA, Knox D, Curtis AL, Aldridge JW, Valentino RJ, Liberzon I (2012) Altered locus coeruleus-norepinephrine function following single prolonged stress. Eur J Neurosci.
Haluk DM, Floresco SB (2009) Ventral Striatal Dopamine Modulation of Different Forms of Behavioral Flexibility. Neuropsychopharmacology.
Harris GC, Wimmer M, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. Nature 437:556-559.
Harris GC, Wimmer M, Randall-Thompson JF, Aston-Jones G (2007) Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. Behavioural Brain Research 183:43-51.
Hickey L, Li Y, Fyson SJ, Watson TC, Perrins R, Hewinson J, Teschemacher AG, Furue H, Lumb BM, Pickering AE (2014) Optoactivation of locus ceruleus neurons evokes bidirectional changes in thermal nociception in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 34:4148-4160.
Holets VR, Hokfelt T, Rokaeus A, Terenius L, Goldstein M (1988) Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. Neuroscience 24:893-906.
Hwang DY, Carlezon WA, Jr., Isacson O, Kim KS (2001) A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. Hum Gene Ther 12:1731-1740.
Ishibashi H, Nakahata Y, Eto K, Nabekura J (2009) Excitation of locus coeruleus noradrenergic neurons by thyrotropin-releasing hormone. The Journal of Physiology 587:5709-5722.
Ito S, Stuphorn V, Brown JW, Schall JD (2003) Performance monitoring by the anterior cingulate cortex during saccade countermanding. Science 302:120-122.
Jacobs GH, Fenwick JA, Williams GA (2001) Cone-based vision of rats for ultraviolet and visible lights. J Exp Biol 204:2439-2446.

Jodo E, Chiang C, Aston-Jones G (1998) Potent excitatory influence of prefrontal cortex activity on noradrenergic locus coeruleus neurons. Neuroscience 83:63-79.
Joel D, Tarrasch R, Feldon J, Weiner I (1997) Effects of electrolytic lesions of the medial prefrontal cortex or its subfields on 4-arm baited, 8-arm radial maze, two-way active avoidance and conditioned fear tasks in the rat. Brain Res 765:37-50.
Kaland N, Smith L, Mortensen EL (2008) Brief report: cognitive flexibility and focused attention in children and adolescents with Asperger syndrome or high-functioning autism as measured on the computerized version of the Wisconsin Card Sorting Test. Journal of autism and developmental disorders 38:1161-1165.
Kanbar R, Stornetta RL, Cash DR, Lewis SJ, Guyenet PG (2010) Photostimulation of Phox2b medullary neurons activates cardiorespiratory function in conscious rats. Am J Respir Crit Care Med 182:1184-1194.
Kim T-i et al. (2013) Injectable, cellular-scale optoelectronics with applications for wireless optogenetics. Science 340:211-216.
Kozorovitskiy Y, Saunders A, Johnson CA, Lowell BB, Sabatini BL (2012) Recurrent network activity drives striatal synaptogenesis. Nature 485:646-650.
Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J Clin Invest 121:1424-1428.
Lacerda AL, Dalgalarrondo P, Caetano D, Haas GL, Camargo EE, Keshavan MS (2003) Neuropsychological performance and regional cerebral blood flow in obsessive-compulsive disorder. Prog Neuropsychopharmacol Biol Psychiatry 27:657-665.
Lyvers M, Yakimoff M (2003) Neuropsychological correlates of opioid dependence and withdrawal. Addictive behaviors 28:605-611.
Mahler SV, Aston-Jones GS (2012) Fos activation of selective afferents to ventral tegmental area during cue-induced reinstatement of cocaine seeking in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:13309-13326.
Mahler SV, Vazey EM, Beckley JT, Keistler CR, McGlinchey EM, Kaufling J, Wilson SP, Deisseroth K, Woodward JJ, Aston-Jones G (2014) Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. Nat Neurosci 17:577-585.
McGaughy J, Ross RS, Eichenbaum H (2008) Noradrenergic, but not cholinergic, deafferentation of prefrontal cortex impairs attentional set-shifting. Neuroscience 153:63-71.
Moore RY, Bloom FE (1979) Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. Annual review of neuroscience 2:113-168.
Mueller D, Cahill SP (2010) Noradrenergic modulation of extinction learning and exposure therapy. Behavioural Brain Research 208:1-11.
Murchison CF, Zhang X-Y, Zhang W-P, Ouyang M, Lee A, Thomas SA (2004) A distinct role for norepinephrine in memory retrieval. Cell 117:131-143.

Nair SG, Strand NS, Neumaier JF (2013) DREADDing the lateral habenula: a review of methodological approaches for studying lateral habenula function. Brain research 1511:93-101.
Nakamura K, Roesch MR, Olson CR (2005) Neuronal activity in macaque SEF and ACC during performance of tasks involving conflict. J Neurophysiol 93:884-908.
Newman LA, Darling J, McGaughy J (2008) Atomoxetine reverses attentional deficits produced by noradrenergic deafferentation of medial prefrontal cortex. Psychopharmacology (Berl) 200:39-50.
Paxinos G, Watson C (2008) The Rat Brain in Stereotaxic Coordinates 6th Edition.1-166.
Pirastu R, Fais R, Messina M, Bini V, Spiga S, Falconieri D, Diana M (2006) Impaired decision-making in opiate-dependent subjects: effect of pharmacological therapies. Drug and alcohol dependence 83:163-168.
Ragozzino ME, Detrick S, Kesner RP (1999) Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. J Neurosci 19:4585-4594.
Rajkowski J, Majczynski H, Clayton E, Aston-Jones G (2004) Activation of monkey locus coeruleus neurons varies with difficulty and performance in a target detection task. Journal of Neurophysiology 92:361-371.
Reeve WV, Schandler SL (2001) Frontal lobe functioning in adolescents with attention deficit hyperactivity disorder. Adolescence 36:749-765.
Robertson SD, Plummer NW, de Marchena J, Jensen P (2013) Developmental origins of central norepinephrine neuron diversity. Nat Neurosci 16:1016-1023.
Romine CB, Lee D, Wolfe ME, Homack S, George C, Riccio CA (2004) Wisconsin Card Sorting Test with children: a meta-analytic study of sensitivity and specificity. Arch Clin Neuropsychol 19:1027-1041.
Sara S (2009) The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci 10:211-223.
Sara SJ (2010) Reactivation, retrieval, replay and reconsolidation in and out of sleep: connecting the dots. Front Behav Neurosci 4:185.
Sartor GC, Aston-Jones GS (2012) A septal-hypothalamic pathway drives orexin neurons, which is necessary for conditioned cocaine preference. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:4623-4631.
Schneider SG, Asarnow RF (1987) A comparison of cognitive/neuropsychological impairments of nonretarded autistic and schizophrenic children. Journal of abnormal child psychology 15:29-45.
Seidman LJ, Biederman J, Faraone SV, Weber W, Ouellette C (1997) Toward defining a neuropsychology of attention deficit-hyperactivity disorder: performance of children and adolescents from a large clinically referred sample. J Consult Clin Psychol 65:150-160.
Servan-Schreiber D, Printz H, Cohen JD (1990) A network model of catecholamine effects: gain, signal-to-noise ratio, and behavior. Science 249:892-895.
Shin MS, Choi H, Kim H, Hwang JW, Kim BN, Cho SC (2008) A study of neuropsychological deficit in children with obsessive-compulsive disorder. Eur Psychiatry 23:512-520.

Smith KS, Virkud A, Deisseroth K, Graybiel AM (2012) Reversible online control of habitual behavior by optogenetic perturbation of medial prefrontal cortex. Proc Natl Acad Sci USA.
Stachniak TJ, Ghosh A, Sternson SM (2014) Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus→midbrain pathway for feeding behavior. Neuron 82:797-808.
Sternson SM, Roth BL (2014) Chemogenetic tools to interrogate brain functions. Annual review of neuroscience 37:387-407.
Sterpenich V, D'Argembeau A, Desseilles M, Balteau E, Albouy G, Vandewalle G, Degueldre C, Luxen A, Collette F, Maquet P (2006) The locus ceruleus is involved in the successful retrieval of emotional memories in humans. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:7416-7423.
Stuber GD (2010) Dissecting the neural circuitry of addiction and psychiatric disease with optogenetics. Neuropsychopharmacology 35:341-342.
Stuber GD, Hnasko TS, Britt JP, Edwards RH, Bonci A (2010) Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. J Neurosci 30:8229-8233.
Tait D, Brown V, Farovik A, Theobald D, Dalley J, Robbins T (2007) Lesions of the dorsal noradrenergic bundle impair attentional set-shifting in the rat. Eur J Neurosci 25:3719-3724.
Tye KM, Prakash R, Kim S-Y, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature:1-5.
Usher M, Cohen JD, Servan-Schreiber D, Rajkowski J, Aston-Jones G (1999) The role of locus coeruleus in the regulation of cognitive performance. Science 283:549-554.
Vazey E, Cope ZA, Keren NI, Lavin A, Hinson VK, Hinson VK, Eckert MA, Granholm AC, Aston-Jones G (2011a) Optogenetic methods for modulating locus coeruleus noradrenergic function in vivo. In: 11th Annual International Neural Transplantation and Repair Conference. Clearwater, FL.
Vazey E, Cope ZA, Keren NI, Moorman D, Lavin A, Eckert MA, Granholm AC, Hinson VK, Aston-Jones G (2011b) Optogenetic methods for modulating locus coeruleus noradrenergic neurons in vivo. In: 15th International Congress of Parkinson's Disease and Movement Disorders. Toronto, Canada.
Vazey EM, Aston-Jones G (2014) Designer receptor manipulations reveal a role of the locus coeruleus noradrenergic system in isoflurane general anesthesia. Proc Natl Acad Sci U S A 111:3859-3864.
Williams ZM, Bush G, Rauch SL, Cosgrove GR, Eskandar EN (2004) Human anterior cingulate neurons and the integration of monetary reward with motor responses. Nat Neurosci 7:1370-1375.
Yeung N, Cohen JD (2006) The impact of cognitive deficits on conflict monitoring. Predictable dissociations between the error-related negativity and N2. Psychol Sci 17:164-171

Abbott SBG, Stornetta RL, Fortuna MG, Depuy SD, West GH, Harris TE, Guyenet PG (2009) Photostimulation of Retrotrapezoid Nucleus Phox2b-Expressing Neurons In Vivo Produces Long-Lasting Activation of Breathing in Rats. J Neurosci 29:5806-5819.

Agster KL, Mejias-Aponte CA, Clark BD, Waterhouse BD (2013) Evidence for a regional specificity in the density and distribution of noradrenergic varicosities in rat cortex. J Comp Neurol 521:2195-2207.

Alexander GM, Rogan SC, Abbas AI, Armbruster BN, Pei Y, Allen JA, Nonneman RJ, Hartmann J, Moy SS, Nicolelis MA, McNamara JO, Roth BL (2009) Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. Neuron 63:27-39.

American Psychiatric Association., American Psychiatric Association. DSM-5 Task Force. (2013) Diagnostic and statistical manual of mental disorders : DSM-5, 5th Edition. Washington, D.C.: American Psychiatric Association.

Aponte Y, Atasoy D, Sternson SM (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat Neurosci 14:351-355.

Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A 104:5163-5168.

Aston-Jones G, Bloom FE (1981) Norepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. J Neurosci 1:887-900.

Aston-Jones G, Cohen JD (2005a) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. Annu Rev Neurosci 28:403-450.

Aston-Jones G, Cohen JD (2005b) Adaptive gain and the role of the locus coeruleus-norepinephrine system in optimal performance. J Comp Neurol 493:99-110.

Aston-Jones G, Rajkowski J, Cohen J (2000) Locus coeruleus and regulation of behavioral flexibility and attention. Prog Brain Res 126:165-182.

Aston-Jones G, Ennis M, Pieribone VA, Nickell WT, Shipley MT (1986) The brain nucleus locus coeruleus: restricted afferent control of a broad efferent network. Science 234:734-737.

Aston-Jones G, Rajkowski J, Kubiak P, Valentino RJ, Shipley MT (1996) Role of the locus coeruleus in emotional activation. Prog Brain Res 107:379-402.

Barch DM, Braver TS, Sabb FW, Noll DC (2000) Anterior cingulate and the monitoriing of response conflict: evidence from an fMRI study of overt verb generation. J Cogn Neurosci 12:298-309.

Barch DM, Braver TS, Nystrom LE, Forman SD, Noll DC, Cohen JD (1997) Dissociating working memory from task difficulty in human prefrontal cortex. Neuropsychologia 35:1373-1380.

Barch DM, Braver TS, Akbudak E, Conturo T, Ollinger J, Snyder A (2001) Anterior cingulate cortex and response conflict: effects of response modality and processing domain. Cereb Cortex 11:837-848.

Bari A, Eagle DM, Mar AC, Robinson ESJ, Robbins TW (2009) Dissociable effects of noradrenaline, dopamine, and serotonin uptake blockade on stop task performance in rats. Psychopharmacology (Berl) 205:273-283.
Berndt A, Yizhar O, Gunaydin LA, Hegemann P, Deisseroth K (2009) Bi-stable neural state switches. Nat Neurosci 12:229-234.
Berridge C, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res Brain Res Rev 42:33-84.
Birrell JM, Brown VJ (2000) Medial frontal cortex mediates perceptual attentional set shifting in the rat. J Neurosci 20:4320-4324.
Bouret S, Sara S (2004) Reward expectation, orientation of attention and locus coeruleus-medial frontal cortex interplay during learning. Eur J Neurosci 20:791-802.
Bouret S, Sara SJ (2005) Network reset: a simplified overarching theory of locus coeruleus noradrenaline function. TRENDS in Neurosciences 28:574-582.
Boyden ES (2011) A history of optogenetics: the development of tools for controlling brain circuits with light. F1000 Biol Rep 3:11.
Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci 8:1263-1268.
Braff DL, Geyer MA, Swerdlow NR (2001) Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology 156:234-258.
Brázdil M, Roman R, Falkenstein M, Daniel P, Jurák P, Rektor I (2002) Error processing--evidence from intracerebral ERP recordings. Experimental brain research Experimentelle Hirnforschung Expérimentation cérébrale 146:460-466.
Bullitt E (1990) Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol 296:517-530.
Carter ME, De Lecea L, Adamantidis A (2013) Functional wiring of hypocretin and LC-NE neurons: implications for arousal. Front Behav Neurosci 7:43.
Carter ME, Yizhar O, Chikahisa S, Nguyen H, Adamantidis A, Nishino S, Deisseroth K, De Lecea L (2010) Tuning arousal with optogenetic modulation of locus coeruleus neurons. Nat Neurosci.
Chandler D, Waterhouse BD (2012) Evidence for Broad Versus Segregated Projections from Cholinergic and Noradrenergic Nuclei to Functionally and Anatomically Discrete Subregions of Prefrontal Cortex. Front Behav Neurosci 6:1-9.
Choi J, Kurtz MM (2009) A comparison of remediation techniques on the Wisconsin Card Sorting Test in schizophrenia. Schizophr Res 107:76-82.
Clayton EC, Rajkowski J, Cohen JD, Aston-Jones G (2004) Phasic activation of monkey locus ceruleus neurons by simple decisions in a forced-choice task. J Neurosci 24:9914-9920.
Corbetta M, Patel G, Shulman GL (2008) The reorienting system of the human brain: from environment to theory of mind. Neuron 58:306-324.
Dalton GL, Ma LM, Phillips AG, Floresco SB (2011) Blockade of NMDA GluN2B receptors selectively impairs behavioral flexibility but not initial discrimination learning. Psychopharmacology (Berl).

Darrah JM, Stefani MR, Moghaddam B (2008) Interaction of N-methyl-D-aspartate and group 5 metabotropic glutamate receptors on behavioral flexibility using a novel operant set-shift paradigm. Behavioural pharmacology 19:225-234.
Delfs JM, Zhu Y, Druhan JP, Aston-Jones GS (1998) Origin of noradrenergic afferents to the shell subregion of the nucleus accumbens: anterograde and retrograde tract-tracing studies in the rat. Brain Research 806:127-140.
Devilbiss DM, Berridge CW (2006) Low-dose methylphenidate actions on tonic and phasic locus coeruleus discharge. J Pharmacol Exp Ther 319:1327-1335.
Devilbiss DM, Waterhouse BD (2011) Phasic and tonic patterns of locus coeruleus output differentially modulate sensory network function in the awake rat. J Neurophysiol 105:69-87.
Devoto P, Flore G, Pani L, Gessa GL (2001) Evidence for co-release of noradrenaline and dopamine from noradrenergic neurons in the cerebral cortex. Mol Psychiatry 6:657-664.
Devoto P, Flore G, Saba P, Fa M, Gessa GL (2005) Stimulation of the locus coeruleus elicits noradrenaline and dopamine release in the medial prefrontal and parietal cortex. J Neurochem 92:368-374.
Dragunow M, Faull R (1989) The use of c-fos as a metabolic marker in neuronal pathway tracing. J Neurosci Methods 29:261-265.
Durstewitz D, Seamans JK (2008) The dual-state theory of prefrontal cortex dopamine function with relevance to catechol-o-methyltransferase genotypes and schizophrenia. Biological psychiatry 64:739-749.
Durstewitz D, Vittoz NM, Floresco SB, Seamans JK (2010) Abrupt transitions between prefrontal neural ensemble states accompany behavioral transitions during rule learning. Neuron 66:438-448.
Eisenberger NI, Lieberman MD, Williams KD (2003) Does rejection hurt? An FMRI study of social exclusion. Science 302:290-292.
Emeric EE, Brown JW, Leslie M, Pouget P, Stuphorn V, Schall JD (2008) Performance monitoring local field potentials in the medial frontal cortex of primates: anterior cingulate cortex. J Neurophysiol 99:759-772.
Enomoto T, Tse MT, Floresco SB (2010) Reducing Prefrontal Gamma-Aminobutyric Acid Activity Induces Cognitive, Behavioral, and Dopaminergic Abnormalities That Resemble Schizophrenia. Biol Psychiatry.
Fenno L, Yizhar O, Deisseroth K (2011) The development and application of optogenetics. Annual review of neuroscience 34:389-412.
Ferguson SM, Neumaier JF (2012) Grateful DREADDs: engineered receptors reveal how neural circuits regulate behavior. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37:296-297.
Ferguson SM, Phillips PEM, Roth BL, Wess J, Neumaier JF (2013) Direct-pathway striatal neurons regulate the retention of decision-making strategies. J Neurosci 33:11668-11676.
Ferguson SM, Eskenazi D, Ishikawa M, Wanat MJ, Phillips PE, Dong Y, Roth BL, Neumaier JF (2011) Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. Nat Neurosci 14:22-24.

Fitzgerald KD, Welsh RC, Gehring WJ, Abelson JL, Himle JA, Liberzon I, Taylor SF (2005) Error-related hyperactivity of the anterior cingulate cortex in obsessive-compulsive disorder. Biol Psychiatry 57:287-294.
Floresco SB, Jentsch JD (2011) Pharmacological enhancement of memory and executive functioning in laboratory animals. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36:227-250.
Floresco SB, Block AE, Tse MTL (2008) Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure. Behav Brain Res 190:85-96.
Fung SJ, Reddy VK, Liu RH, Wang Z, Barnes CD (1994) Existence of glutamate in noradrenergic locus coeruleus neurons of rodents. Brain Res Bull 35:505-512.
Garner AR, Rowland DC, Hwang SY, Baumgaertel K, Roth BL, Kentros C, Mayford M (2012) Generation of a synthetic memory trace. Science 335:1513-1516.
George SA, Knox D, Curtis AL, Aldridge JW, Valentino RJ, Liberzon I (2012) Altered locus coeruleus-norepinephrine function following single prolonged stress. Eur J Neurosci.
Haluk DM, Floresco SB (2009) Ventral Striatal Dopamine Modulation of Different Forms of Behavioral Flexibility. Neuropsychopharmacology.
Harris GC, Wimmer M, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. Nature 437:556-559.
Harris GC, Wimmer M, Randall-Thompson JF, Aston-Jones G (2007) Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. Behavioural Brain Research 183:43-51.
Hickey L, Li Y, Fyson SJ, Watson TC, Perrins R, Hewinson J, Teschemacher AG, Furue H, Lumb BM, Pickering AE (2014) Optoactivation of locus ceruleus neurons evokes bidirectional changes in thermal nociception in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 34:4148-4160.
Holets VR, Hokfelt T, Rokaeus A, Terenius L, Goldstein M (1988) Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. Neuroscience 24:893-906.
Hwang DY, Carlezon WA, Jr., Isacson O, Kim KS (2001) A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. Hum Gene Ther 12:1731-1740.
Ishibashi H, Nakahata Y, Eto K, Nabekura J (2009) Excitation of locus coeruleus noradrenergic neurons by thyrotropin-releasing hormone. The Journal of Physiology 587:5709-5722.
Ito S, Stuphorn V, Brown JW, Schall JD (2003) Performance monitoring by the anterior cingulate cortex during saccade countermanding. Science 302:120-122.
Jacobs GH, Fenwick JA, Williams GA (2001) Cone-based vision of rats for ultraviolet and visible lights. J Exp Biol 204:2439-2446.
Jodo E, Chiang C, Aston-Jones G (1998) Potent excitatory influence of prefrontal cortex activity on noradrenergic locus coeruleus neurons. Neuroscience 83:63-79.
Joel D, Tarrasch R, Feldon J, Weiner I (1997) Effects of electrolytic lesions of the medial prefrontal cortex or its subfields on 4-arm baited, 8-arm radial maze, two-way active avoidance and conditioned fear tasks in the rat. Brain Res 765:37-50.

Kaland N, Smith L, Mortensen EL (2008) Brief report: cognitive flexibility and focused attention in children and adolescents with Asperger syndrome or high-functioning autism as measured on the computerized version of the Wisconsin Card Sorting Test. Journal of autism and developmental disorders 38:1161-1165.
Kanbar R, Stornetta RL, Cash DR, Lewis SJ, Guyenet PG (2010) Photostimulation of Phox2b medullary neurons activates cardiorespiratory function in conscious rats. Am J Respir Crit Care Med 182:1184-1194.
Kim T-i et al. (2013) Injectable, cellular-scale optoelectronics with applications for wireless optogenetics. Science 340:211-216.
Kozorovitskiy Y, Saunders A, Johnson CA, Lowell BB, Sabatini BL (2012) Recurrent network activity drives striatal synaptogenesis. Nature 485:646-650.
Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J Clin Invest 121:1424-1428.
Lacerda AL, Dalgalarrondo P, Caetano D, Haas GL, Camargo EE, Keshavan MS (2003) Neuropsychological performance and regional cerebral blood flow in obsessive-compulsive disorder. Prog Neuropsychopharmacol Biol Psychiatry 27:657-665.
Lyvers M, Yakimoff M (2003) Neuropsychological correlates of opioid dependence and withdrawal. Addictive behaviors 28:605-611.
Mahler SV, Aston-Jones GS (2012) Fos activation of selective afferents to ventral tegmental area during cue-induced reinstatement of cocaine seeking in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:13309-13326.
Mahler SV, Vazey EM, Beckley JT, Keistler CR, McGlinchey EM, Kaufling J, Wilson SP, Deisseroth K, Woodward JJ, Aston-Jones G (2014) Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. Nat Neurosci 17:577-585.
McGaughy J, Ross RS, Eichenbaum H (2008) Noradrenergic, but not cholinergic, deafferentation of prefrontal cortex impairs attentional set-shifting. Neuroscience 153:63-71.
Moore RY, Bloom FE (1979) Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. Annual review of neuroscience 2:113-168.
Mueller D, Cahill SP (2010) Noradrenergic modulation of extinction learning and exposure therapy. Behavioural Brain Research 208:1-11.
Murchison CF, Zhang X-Y, Zhang W-P, Ouyang M, Lee A, Thomas SA (2004) A distinct role for norepinephrine in memory retrieval. Cell 117:131-143.
Nair SG, Strand NS, Neumaier JF (2013) DREADDing the lateral habenula: a review of methodological approaches for studying lateral habenula function. Brain research 1511:93-101.
Nakamura K, Roesch MR, Olson CR (2005) Neuronal activity in macaque SEF and ACC during performance of tasks involving conflict. J Neurophysiol 93:884-908.
Newman LA, Darling J, McGaughy J (2008) Atomoxetine reverses attentional deficits produced by noradrenergic deafferentation of medial prefrontal cortex. Psychopharmacology (Berl) 200:39-50.
Paxinos G, Watson C (2008) The Rat Brain in Stereotaxic Coordinates 6th Edition.1-166.

Pirastu R, Fais R, Messina M, Bini V, Spiga S, Falconieri D, Diana M (2006) Impaired decision-making in opiate-dependent subjects: effect of pharmacological therapies. Drug and alcohol dependence 83:163-168.
Ragozzino ME, Detrick S, Kesner RP (1999) Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. J Neurosci 19:4585-4594.
Rajkowski J, Majczynski H, Clayton E, Aston-Jones G (2004) Activation of monkey locus coeruleus neurons varies with difficulty and performance in a target detection task. Journal of Neurophysiology 92:361-371.
Reading PJ, Dunnett SB, Robbins TW (1991) Dissociable roles of the ventral, medial and lateral striatum on the acquisition and performance of a complex visual stimulus-response habit. Behav Brain Res 45:147-161.
Reeve WV, Schandler SL (2001) Frontal lobe functioning in adolescents with attention deficit hyperactivity disorder. Adolescence 36:749-765.
Robertson SD, Plummer NW, de Marchena J, Jensen P (2013) Developmental origins of central norepinephrine neuron diversity. Nat Neurosci 16:1016-1023.
Romine CB, Lee D, Wolfe ME, Homack S, George C, Riccio CA (2004) Wisconsin Card Sorting Test with children: a meta-analytic study of sensitivity and specificity. Arch Clin Neuropsychol 19:1027-1041.
Sara S (2009) The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci 10:211-223.
Sara SJ (2010) Reactivation, retrieval, replay and reconsolidation in and out of sleep: connecting the dots. Front Behav Neurosci 4:185.
Sartor GC, Aston-Jones GS (2012) A septal-hypothalamic pathway drives orexin neurons, which is necessary for conditioned cocaine preference. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:4623-4631.
Schmitzer-Torbert N, Apostolidis S, Amoa R, O'Rear C, Kaster M, Stowers J, Ritz R (2014) Post-training cocaine administration facilitates habit learning and requires the infralimbic cortex and dorsolateral striatum. Neurobiol Learn Mem.
Schneider SG, Asarnow RF (1987) A comparison of cognitive/neuropsychological impairments of nonretarded autistic and schizophrenic children. Journal of abnormal child psychology 15:29-45.
Seidman LJ, Biederman J, Faraone SV, Weber W, Ouellette C (1997) Toward defining a neuropsychology of attention deficit-hyperactivity disorder: performance of children and adolescents from a large clinically referred sample. J Consult Clin Psychol 65:150-160.
Servan-Schreiber D, Printz H, Cohen JD (1990) A network model of catecholamine effects: gain, signal-to-noise ratio, and behavior. Science 249:892-895.
Shin MS, Choi H, Kim H, Hwang JW, Kim BN, Cho SC (2008) A study of neuropsychological deficit in children with obsessive-compulsive disorder. Eur Psychiatry 23:512-520.
Smith KS, Virkud A, Deisseroth K, Graybiel AM (2012) Reversible online control of habitual behavior by optogenetic perturbation of medial prefrontal cortex. Proc Natl Acad Sci USA.

Stachniak TJ, Ghosh A, Sternson SM (2014) Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus→midbrain pathway for feeding behavior. Neuron 82:797-808.
Sternson SM, Roth BL (2014) Chemogenetic tools to interrogate brain functions. Annual review of neuroscience 37:387-407.
Sterpenich V, D'Argembeau A, Desseilles M, Balteau E, Albouy G, Vandewalle G, Degueldre C, Luxen A, Collette F, Maquet P (2006) The locus ceruleus is involved in the successful retrieval of emotional memories in humans. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:7416-7423.
Stuber GD (2010) Dissecting the neural circuitry of addiction and psychiatric disease with optogenetics. Neuropsychopharmacology 35:341-342.
Stuber GD, Hnasko TS, Britt JP, Edwards RH, Bonci A (2010) Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. J Neurosci 30:8229-8233.
Tait D, Brown V, Farovik A, Theobald D, Dalley J, Robbins T (2007) Lesions of the dorsal noradrenergic bundle impair attentional set-shifting in the rat. Eur J Neurosci 25:3719-3724.
Tye KM, Prakash R, Kim S-Y, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature:1-5.
Usher M, Cohen JD, Servan-Schreiber D, Rajkowski J, Aston-Jones G (1999) The role of locus coeruleus in the regulation of cognitive performance. Science 283:549-554.
Van Bockstaele EJ, Bajic D, Proudfit H, Valentino RJ (2001) Topographic architecture of stress-related pathways targeting the noradrenergic locus coeruleus. Physiol Behav 73:273-283.
Vazey E, Cope ZA, Keren NI, Lavin A, Hinson VK, Hinson VK, Eckert MA, Granholm AC, Aston-Jones G (2011a) Optogenetic methods for modulating locus coeruleus noradrenergic function in vivo. In: 11th Annual International Neural Transplantation and Repair Conference. Clearwater, FL.
Vazey E, Cope ZA, Keren NI, Moorman D, Lavin A, Eckert MA, Granholm AC, Hinson VK, Aston-Jones G (2011b) Optogenetic methods for modulating locus coeruleus noradrenergic neurons in vivo. In: 15th International Congress of Parkinson's Disease and Movement Disorders. Toronto, Canada.
Vazey EM, Aston-Jones G (2014) Designer receptor manipulations reveal a role of the locus coeruleus noradrenergic system in isoflurane general anesthesia. Proc Natl Acad Sci U S A 111:3859-3864.
Williams ZM, Bush G, Rauch SL, Cosgrove GR, Eskandar EN (2004) Human anterior cingulate neurons and the integration of monetary reward with motor responses. Nat Neurosci 7:1370-1375.
Yeung N, Cohen JD (2006) The impact of cognitive deficits on conflict monitoring. Predictable dissociations between the error-related negativity and N2. Psychol Sci 17:164-171.

BIOGRAPHY

Zackary (Zack) A. Cope received his B.S. degree in Psychology and Biology the East Tennessee State University in Johnson City, TN. During his time as an undergraduate, Zack performed undergraduate research in the laboratory of Russell W. Brown while also working as a psychiatric tech at Woodridge Psychiatric Hospital. Following graduation, Zack worked as an in-home mental health case manager with Holston Counseling Center in Kingsport, TN.

In 2008, Zack returned to the lab of Russell W. Brown to pursue a Master's degree in Experimental Psychology. During this time, Zack led a research project examining dopamine overflow in the nucleus accumbens in rodent model of schizophrenia and d-amphetamine addiction comorbidity. This project revealed a synergistic action of neonatal treatment with quinpirole and sensitization to amphetamine in increasing DA release in the nucleus accumbens, as measured with *in-vivo* microdialysis.

After receiving his Master's degree, Zack attended the Medical University of South Carolina in Charleston, SC to pursue his PhD in Neurosciences under the advisement of Dr. Gary S. Aston-Jones. The research presented in this dissertation comprises the body of work conducted during Zack's time at MUSC. Zack has continuing research interests in the neural substrates of normal and maladaptive behavior such as that seen in addiction, schizophrenia, and PTSD.