A DORSAL, BUT NOT VENTRAL, HIPPOCAMPAL CIRCUIT IS REQUIRED FOR EXPRESSION OF HEROIN'S CONTEXTUALLY CONDITIONED IMMUNE EFFECTS

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ABSTRACT

Christina L. Lebonville: A dorsal, but not ventral, hippocampal circuit is required for expression of heroin's contextually conditioned immune effects.
(Under the direction of Donald T. Lysle)

Drugs of abuse, like opioids, cause a diverse array of physiological effects. These effects can become conditioned to occur to any stimulus, for example an environmental context, that becomes associated with drug use. In terms of drug reward conditioning, exposure to drug-paired contexts can elicit craving and re-engagement in drug seeking behaviors, promoting relapse to drug use. Similarly, the immune modulating effects of opioids can be conditioned to occur with exposure to an opioid-paired context. Therefore, exposure to drug-paired contexts can significantly exacerbate both the health consequences and risk of relapse with drugs of abuse. Understanding the neurological mechanisms that allow for the expression of contextually conditioned effects will allow us to better combat the problem of drug abuse. Brain regions governing opioid conditioned reward and immune responses have previously been investigated, but how these regions interact with each other is not fully understood. Previous studies show that the nucleus accumbens, basolateral amygdala, and hippocampus are required for expression of heroin contextually conditioned immune modulation, and this overlaps with what brain regions are required for contextually conditioned reward. The hippocampus is vital for encoding context, and we hypothesize that it instigates the motivational and immunological changes with exposure to a drug-paired context by engaging the other brain regions involved. The present studies are designed to further characterize the hippocampus' role in contextually conditioned drug

behaviors by manipulating this region's predominant outgoing projections prior to expression of heroin contextually conditioned immune modulation. The hippocampus is not a homogeneous structure, and the dorsal and ventral aspects of the hippocampus connect anatomically to distinct groups of brain regions. Therefore, understanding the relative importance of the dorsal and ventral outputs from the hippocampus will give a clearer understanding of how the hippocampus relays information about context to other brain regions. The chemogenetic technique, designer receptors exclusively activated by designer drugs (DREADDs), lends itself well to inhibiting particular neurons in hippocampal output regions and building toward an understanding of hippocampal circuits. An experiment in Chapter 2 tested whether output from the dorsal hippocampus is required for the expression of heroin contextually conditioned immune modulation. Chapter 3's experiment tested whether output from the ventral hippocampus is required for the expression of heroin contextually conditioned immune modulation. Chemogenetic inhibition of the dorsal hippocampus output, but not inhibition of the ventral hippocampus output, attenuated the expression of heroin conditioned immune modulation. Thus, Chapter 4's experiment tested whether a specific anatomical connection between the dorsal hippocampus and retrosplenial cortex is required for expression of conditioned immune modulation. Chemogenetic inhibition of the specific projection from the dorsal hippocampus to the retrosplenial cortex did not attenuate the expression of heroin conditioned immune modulation, leading to the conclusion that this specific projection is not required. The results from these experiments begin to build a picture of how the hippocampus, and its representation of context, can influence immune function and perhaps also the behaviors that lead to cycles of drug abuse.

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°C	degrees Celsius
ΔΔCt	delta delta threshold cycle (qPCR)
AAV	adeno-associated virus
AAV5	adeno-associated virus serotype 5
ANOVA	analysis of variance
AP	anterior-posterior
BLA	basolateral amygdala
CA1	Cornu Ammonis region 1 of the hippocampus
CA3	Cornu Ammonis region 3 of the hippocampus
CAMKIIα	calmodulin-dependent protein kinase II alpha
cAMP	cyclic adenosine monophosphate
CNO	clozapine-*N*-oxide
CS	conditioned stimulus
dCA1	dorsal Cornu Ammonis region 1 of the hippocampus
dCA3	dorsal Cornu Ammonis region 3 of the hippocampus
dDG	dorsal dentate gyrus of the hippocampus
DG	dentate gyrus of the hippocampus

dHpc	dorsal hippocampus
DMSO	dimethyl sulfoxide
DREADD	designer receptors exclusively activated by designer drugs
dSub	dorsal subiculum of the hippocampus
DV	dorsal-ventral
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FAD	flavine adenine dinucleotide
g	gram
g/rcf	gravitational force/relative centrifugal force
G-protein	guanine nucleotide-binding protein
GABA	gamma-aminobutyric acid
HC	home cage
HOV	homogeneity of variance
HPA	hypothalamic-pituitary-adrenal
HSD	Honestly Significant Difference
hSyn	human synapsin 1

IACUC	Institutional Animal Care and Use Committee
iNOS	inducible nitric oxide
kg	killogram
KORD	κ-opioid receptor derived DREADD (G_i-coupled)
LPS	lipopolysaccharide
LS	lateral septum
LTP	long-term potentiation
µL	microliter
µm	micrometer
mEC	medial entorhinal cortex
mg	milligram
min	minute
ML	medial-lateral
mL	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid

NAc	nucleus accumbens
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NIDA	National Institute on Drug Abuse
NO	nitric oxide
PAG	periaqueductal gray nucleus
PCR	polymerase chain reaction
RNA	ribonucleic acid
RSC	retrosplenial cortex
RSCgb	retrosplenial cortex granular area b/c
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
vCA1	ventral Cornu Ammonis region 1 of the hippocampus
vCA3	ventral Cornu Ammonis region 3 of the hippocampus
vDG	ventral dentate gyrus of the hippocampus
vHpc	ventral hippocampus
vSub	ventral subiculum of the hippocampus
VTA	ventral tegmental area

CHAPTER 1: GENERAL INTRODUCTION

The Role of Context in Drug Behaviors

Learning about events that occur in a particular environmental context allows us to use our past experiences to guide future behavior in that context. Generally, learning associations between contexts and important events confers an advantage, allowing for better prediction and adaptive responses to these events (Maren et al., 2013). However, some contextual associations contribute to maladaptive behavior, like drug addiction. Drug-associated contexts serve an important role in maintaining addiction by increasing the likelihood of relapse (Caprioli et al., 2007; Crombag et al., 2008; Janak and Chaudhri, 2010). For example, entering an environment where past drug experiences have occurred can increase motivation for seeking and taking a wide range of drugs including alcohol (Janak and Chaudhri, 2010), cocaine (Fuchs et al., 2008), nicotine (Diergaarde et al., 2008), and heroin (Bossert and Stern, 2014) in an animal model of relapse called "context-induced reinstatement of drug seeking". It is thought that being in a drug-associated context prompts the expression of learned behaviors and sensations surrounding drug use (ex. approach, seeking, craving). This might be due to the context becoming a Pavlovian conditioned stimulus (CS) for the rewarding/reinforcing effects of drugs or by the context acting as an occasion setter for drug seeking and taking behaviors that have led to obtaining drug reward previously (Crombag et al., 2008).

In the study of addiction, a predominant focus on relapse behaviors makes sense. However, the rewarding and reinforcing effects are not the only physiological effect of drugs that become associated with a context through experience. Drugs of abuse, specifically opioids, can

potently modulate the immune system (Fecho et al., 1993; Fecho and Lysle, 2000; Fecho et al., 2000; Lysle et al., 1993; Lysle and How, 2000; Nelson et al., 2000). It has been shown that stimuli associated with immune altering substances can influence immune function by themselves through Pavlovian conditioning (Ader and Cohen, 1975). When the immune modulating effects of opioids become associated with a particular context, exposure to these contexts even in the absence of opioids can modulate the immune system to a similar degree (Coussons et al., 1992; Lysle and Ijames, 2002). Therefore, opioid contextually conditioned immune effects can add to the societal cost of addiction by contributing to the increased incidence of infection seen in opioid users (Govitrapong et al., 1998; Horsburgh et al., 1989; Louria et al., 1967; Risdahl et al., 1998). By understanding the neural mechanisms behind the recall or expression of contextually conditioned immune effects, we might be able to develop behavioral or pharmacological interventions to help ameliorate these costs.

The potent ability of a drug-associated context to not only increase the probability of relapse to abuse behavior but to also exert health consequences beyond those of drug use itself, makes it vital to understand the neural mechanisms of context-induced effects more broadly. If governed by a shared mechanism, both contextually conditioned immune and reward effects may be able to be blocked simultaneously with a single treatment. However, if each are governed by diverging mechanisms, it means that treatments blocking the abuse promoting effects of contexts will not address the immune compromising effects of contexts and vice versa. Ultimately, the study of contextually-elicited immune effects with drugs like opioids provides a unique perspective from which to study the general role of context in drug behaviors.

In order to investigate the mechanisms by which contexts can elicit both immune and reward behavior in greater detail, this thesis directly compares what is known about the neural

mechanisms of opioid contextually conditioned immune effects with the known mechanisms of the context-induced reinstatement of drug seeking model. The two animal models show complete overlap in required brain regions [for review see (Bossert et al., 2013; Szczytkowski et al., 2011)], and both share a dependency on the neural representation of a context. An additional benefit of comparing these two models is that they both use standard operant chambers as the behavior-producing context. Additional neural mechanisms engaged with larger or more complex environmental contexts could confound investigations of the neural mechanisms of context alone. For example, a frequently used animal model of contextual drug reward behaviors, conditioned place preference, involves movement between two or more connected chambers. As a result, in this and other models of drug reward, navigational and more complex spatial processing could be engaged. Furthermore, this processing might obscure mechanisms of contextual representation *per se* because both navigational and contextual representation occurs within the same brain region – the hippocampus [see (Riaz et al., 2017)].

The Hippocampus and Recall of Context-Drug Associations

The hippocampus has long been seen as the processor of context and context-relevant memories (Maren et al., 2013; Smith and Mizumori, 2006). The notorious case of Henry Molaison in the 1950's led to the hippocampus being accepted as a brain region important for retrieval of episodic memory (Scoville and Milner, 1957). In the 1970's, the hippocampus became considered a processor of physical space (spatial context) due to the discovery of location responsive "place cells" within this region (O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978). Researchers in the learning in memory field assert that the hippocampus' role in the formation of episodic memory is supported through the regions ability to encode location using place cells, since most events have a spatial context (Eichenbaum et al., 1999; Smith and

Mizumori, 2006). Recently, significant technological advances have led to the view that ensembles of cells (including place cells) are how the hippocampus represents context (Smith and Bulkin, 2014). Sparse cell ensembles that become active during exposure to a relevant context can be studied using specialized transgenic mice models with activity-dependent cell tagging techniques. Such studies have shown that a distinct set of cells form an ensemble with repeated exposure to a specific context, and this ensemble is preferentially active during re-exposure to that same context. It is proposed that activity of these ensembles allow for identification of the current context and retrieval of context-specific memories and therefore behaviors. This ability to represent unique contexts and their meaning with different ensembles of cells, may explain the hippocampus' role in controlling contextually motivated behaviors with drugs, leading to an increased likelihood of relapse.

Accordingly, addiction research has applied this foundational work from the learning and memory field in understanding the hippocampus' role in the ability of context to influence responses to drugs of abuse. A theoretical model has been proposed where the hippocampus encodes the context around the experiences with drugs and is involved in the recall of contextual memories that promote drug taking and seeking behavior (Everitt and Robbins, 2005; Robbins et al., 2008). A context-specific hippocampal ensemble has been shown to be sensitive to drug exposure that occurs in that context, to be particularly active around times when drug-seeking behavior occurs, and to be necessary for expression of context-specific drug behaviors (Trouche et al., 2016; Xia et al., 2017).

The hippocampus is also implicated in the ability of a drug-associated context to modulate the immune system. Inactivation of the hippocampus using a gamma-aminobutyric acid (GABA; an inhibitory neurotransmitter) agonist (activator), just before exposure to a heroin-

paired context prevented the context's ability to modulate the immune system (Szczytkowski et al., 2013). Since both contextually conditioned immune and reward drug responses rely on a functional hippocampus, this region may be of particular interest for pharmacological or behavioral interventions hoping to ameliorate both relapse behaviors and negative health outcomes associated with drug abuse. However, before such a treatment can be developed, we must understand how hippocampal signaling ultimately leads to these drug behaviors. Once the context is identified by increased activity in context-specific ensembles, the hippocampus is thought to promote drug behaviors by engaging reward and motivational brain areas. These connections between the hippocampus and the canonical "reward circuitry" are actually quite complex and frequently misunderstood. A close study of these connections reveals important unanswered questions about how the hippocampus processes context.

Hippocampal Function and Connectivity

In order to accurately characterize relevant hippocampal connections (to other areas) for contextually-elicited drug behaviors, it is vital to understand that the hippocampus is not a homogenous structure. The hippocampus has both distinct subregions and variation in connectivity along the septo-temporal (dorsal-ventral) axis. A systematic analysis of gene expression using the Allen Brain Atlas demonstrated that gene expression segregates into spatial domains across subregions and along the axis (Bienkowski et al., 2018; Dong et al., 2009; Thompson et al., 2008). In further support of this distinction, the dorsalmost and ventralmost aspects of the hippocampus (dHpc and vHpc, respectively) send neuronal projections to mostly different, non-overlapping regions – with dHpc projecting to areas such as the retrosplenial cortex, anterior thalamus, and medial mammillary nucleus; and the vHpc projecting to the

basolateral amygdala, hypothalamus, and infralimbic cortex (Amaral and Witter, 1989; Bienkowski et al., 2018; Naber and Witter, 1998; Witter, 2006).

Since dHpc and vHpc connections are incredibly distinct from one another and non-overlapping, it may be that by virtue of engaging one set of targets versus the other, different functions of the dHpc and vHpc arise. In fact, experiments have shown that the dHpc and vHpc have dissociable roles in different types of behavior [see (Fanselow and Dong, 2010)]. The dHpc is thought to primarily support spatial, navigational, and episodic memory, while the vHpc is thought to be involved in contextually motivated fear and reward memory ("emotional memory"), although not all evidence supports this clear distinction (see Chapter 5: General Discussion). Despite these functional distinctions, inactivation of neural signaling in either the dHpc (Fuchs et al., 2005; Ge et al., 2017; Xie et al., 2010) or vHpc (Lasseter et al., 2010) attenuates the expression of context-induced reinstatement with cocaine or heroin. Likewise, inactivation of neural signaling in the dHpc attenuates expression of heroin contextually conditioned immune modulation (Szczytkowski et al., 2013), but the role of the vHpc has yet to be tested. There is reason to believe that the dHpc, and perhaps also the vHpc, is involved in the expression of contextually conditioned drug behaviors, generally. What follows is a summary of which connections are theoretically important to contextually driven drug behaviors and which regions of the hippocampus, the dHpc or vHpc, might make these connections.

Specific Hippocampal Connections and Contextually Conditioned Drug Behaviors

Connections between the hippocampus and ventral striatum have been proposed to support the expression of contextually conditioned reward behaviors (van der Meer and Redish, 2011). Regions in the ventral striatum are well-known to modulate motivational states, specifically the nucleus accumbens (NAc). During re-exposure to a reward paired context, the

hippocampus might represent the context and then signal to the NAc which would be able to promote the necessary reward-motivated behaviors (Lansink et al., 2009; van der Meer and Redish, 2011).The hypothesis that the hippocampus initiates retrieval of contextually conditioned drug behaviors via the NAc is supported by the fact that in recall (replay) of contextual drug reward associations, activity the hippocampus precedes activity in the NAc (Lansink et al., 2009). Thus, the activation of a contextual ensemble for a drug-paired context is theoretically able to contribute to expression of drug-related behavior in that context by reactivating relevant experiential and motivational states mediated by the NAc (Destexhe et al., 2015).

There is support for the importance of this connection between the hippocampus and NAc to contextual initiation of drug behaviors (Sjulson et al., 2018), but it is unclear whether this connection arises from the dHpc or vHpc. Their respective roles likely depend on which region(s) of the NAc is/are important. Even in cases where dHpc and vHpc projections reach the same brain region, like they do with the NAc, they are topographically segregated to different aspects of that region. For example, the dHpc projects to NAc core and rostrolateral shell, while the vHpc projects to the NAc caudomedial shell (Brog et al., 1993; Groenewegen et al., 1987; Kelley and Domesick, 1982; Witter, 2006). In paradigms where a context causes expression of either conditioned reward or immune behaviors with drugs of abuse, activity in the medial NAc shell has been shown to be involved while not activity in the NAc core (Bossert et al., 2007; Chaudhri et al., 2010; Saurer et al., 2008). This evidence would seem to indicate that perhaps the connection between the vHpc and medial NAc shell would be most important for the conditioned effects caused by contexts associated with drugs of abuse. Recent experiments support this hypothesis. Functional disruption of connections between the vHpc and the medial NAc shell has been shown to inhibit context-induced reinstatement of heroin seeking (Bossert et al., 2016). In

one study, lateral NAc shell also mediated context-induced reinstatement of heroin seeking (Bossert et al., 2007), which could suggest a role for dHpc communication to the NAc shell. As a result, either the dHpc, vHpc, or both regions of the hippocampus could mediate context-induced drug behaviors by engaging with different regions of the NAc shell.

Connections between the hippocampus and other reward/motivational regions have also been implicated in the expression of contextually conditioned behaviors with drugs of abuse. Specifically, serial information processing between the dHpc and basolateral amygdala (BLA) has been shown to be required for expression of context-induced reinstatement of cocaine seeking (Fuchs et al., 2007b). Similarly, the BLA has also been implicated in contextually conditioned immune modulation with opioids (Szczytkowski and Lysle, 2008, 2010). A hippocampal projection to the BLA arises only from the vHpc, which has direct, reciprocal connections with the BLA (Pikkarainen et al., 1999; Pitkänen et al., 2000). This means that despite the fact that functional disconnection of the dHpc and BLA interferes with expression of contextually conditioned reward behaviors, there is no direct connection between these regions. Thus, the dHpc must interact with the BLA indirectly through some area(s) with connectivity to both regions, whereas the vHpc is well-positioned to mediate contextually conditioned drug behaviors along with the BLA. To our knowledge, no one has determined how the dHpc and BLA can interact even though this interaction is implicated in not only expression of contextually conditioned drug reward but also with contextually conditioned fear behavior (Maren and Hobin, 2007). The medial NAc shell, prelimbic cortex, and infralimbic cortex all receive converging input from both the hippocampus and BLA and could potentially be sites of this interaction, but the hippocampal projections to these regions arise only from vHpc (Britt et al., 2012; Ishikawa and Nakamura, 2003).

In summary, there are important gaps in understanding the roles of connections originating from the dHpc and vHpc in contextually conditioned behaviors with drugs, especially with conditioned immune modulation. The evidence indicates that both the dHpc and vHpc may be necessary, but approaching their role at the circuit level highlights outstanding questions that need to be answered. First, how is the dHpc initiating immune modulating or reward behavior to a context, especially if it needs to interact with the BLA to do so? Second, is the vHpc important to contextually conditioned immune modulation as it is in conditioned reward? A powerful chemogenetic tool allows us to begin to answer these important questions.

DREADDs Have the Power to Reveal Hippocampal Neurocircuitry

Looking only at hippocampal ensembles does not reveal any information about how the hippocampus is engaging behavior through communication with other brain regions. Hippocampal outputs to other areas might convey the net processing from such activated ensembles and may be a simpler way to investigate hippocampal contextual initiation of behavior. If we could inhibit these outputs specifically, then we would be able to investigate the respective roles of the dHpc and vHpc. This is where designer receptors exclusively activated by designer drugs (DREADDs) are particularly useful.

DREADDs are mutated human muscarinic receptors that no longer show an affinity for their native agonist, acetylcholine (Armbruster et al., 2007). Instead, these mutant receptors can only be activated by a synthetic drug called clozapine-*N*-oxide (CNO), which is otherwise biologically inert (but see Chapter 5: General Discussion). A viral vector containing the genetic sequence that encodes for these receptors can be intracranially administered to specific neural cell populations. After cells have had the viral genetic code delivered to them, their native cellular machinery will then produce and express the DREADD in the cell's plasma membrane

after a 3-4 week-long incubation period. As modified guanine nucleotide-binding protein (G-protein) coupled receptors, several different types of DREADDs have been produced that couple to different types of G-proteins. The family of G-protein coupled receptors produces different intracellular signaling cascades and effects upon activation. A DREADD coupled with a G_i protein and expressed in neurons, when activated by systemically delivered CNO, will inhibit adenylyl cyclase, decreasing levels of cyclic adenosine monophosphate (cAMP) and preventing numerous cellular functions. The activated G_i-protein also activates inwardly-rectifying potassium channels which hyperpolarize the neuron and prevent action potentials. Thus, a G_i-coupled DREADD inhibits neuronal function upon activation with CNO (Armbruster et al., 2007; Roth, 2016).

Whereas GABA agonists can be used for localized cellular inhibition of hippocampal output regions, DREADD technology allows for specific cells and their projections to be inhibited. For example, by including promotor elements in the genetic construct, you can limit expression of DREADDs to cells expressing particular genes (e.g. calmodulin-dependent kinase II alpha, CAMKIIα). Furthermore, once you determine that a population of cells is important for an effect, you can then take advantage of the fact that DREADDs are expressed throughout the cell, including efferent projections to other brain regions. By delivering CNO specifically to where the projections terminate in these regions, specific projections arising from your DREADD-expressing region can be inhibited to determine function. DREADD technology, as opposed to the classic GABA agonist neural inactivation strategy, was selected to investigate the roles of the dHpc and vHpc because of its usefulness not only as a regional inactivator, but as a way to selectively inhibit projections from these hippocampal regions to other brain regions during the expression of heroin contextually conditioned immune modulation. To look at the role

of hippocampal regions/projections in contextually conditioned immune effects, it is important to have a model that captures what impact opioid-associated contexts might have on the health consequences of opioid use.

Examining the Diverse Hippocampus in Opioid Contextually Conditioned Immune Effects

Exposure to opioid-associated contexts in animals suppresses several peripheral measures of immunity including lymphocyte proliferation, natural killer cell activity, the production of proinflammatory cytokines, and the production of nitric oxide [NO, (Coussons et al., 1992; Lysle and Ijames, 2002; Saurer et al., 2008; Szczytkowski and Lysle, 2008)]. NO production has been particularly useful as an *in vivo* measure of immune function after exposure to opioids. NO is released by multiple immune cells, greatly aids resistance to infections, and serves as a regulator of immune function (Bogdan, 2001; Lewis et al., 2010; MacMicking et al., 1995; Nathan and Shiloh, 2000; Uehara et al., 2015). By strongly inducing NO production with lipopolysaccharide (LPS), an immunogenic component of gram-negative bacterial walls, you can measure NO by looking at levels of splenic inducible nitric oxide (iNOS), the enzyme responsible for producing NO, and plasma nitrate/nitrite, byproducts of NO degradation. Using this model of immune challenge, numerous studies have sought to characterize the neural mechanisms of heroin contextually conditioned suppression of NO (Lysle and Ijames, 2002; Paniccia et al., 2018; Szczytkowski et al., 2011; Szczytkowski et al., 2013; Szczytkowski and Lysle, 2007, 2008, 2010). It is through these experiments that the hippocampus was found to be important for the expression of this contextually conditioned immune effect.

There are important gaps in knowledge about the function of the hippocampus in mediating context-driven behaviors with drugs of abuse. We are able to leverage the heroin contextually conditioned NO suppression paradigm to study contextual associations with drugs

more broadly and to complement the work that has been done so far with drug reward. The goals of following experiments are to 1) delineate the specific roles of dHpc and vHpc outputs in heroin contextually conditioned suppression of NO and 2) to build on this information to probe functionally relevant connections between the hippocampus and other structures.

It is unclear how the dHpc contributes to heroin conditioned immune modulation. The dHpc has also been implicated in conditioned drug-reward behavior to a context (Fuchs et al., 2005) and disconnection of the dHpc and the BLA attenuates context-induced reinstatement of cocaine seeking, despite the two areas not having direct connectivity (Fuchs et al., 2007b). Therefore, understanding how the dHpc communicates with these reward structures would be valuable for understanding both context-immune and context-reward associations with drugs of abuse. Chapter 2 of this dissertation describes an experiment employing DREADD-mediated inhibition of dHpc output regions during expression of heroin contextually conditioned suppression of NO. This experiment validates the involvement of dHpc outputs and identifies specific dHpc projection targets that could support hippocampal initiation of contextually conditioned drug behaviors. In Chapter 4, the dHpc output to one of these targets, the retrosplenial cortex, is specifically inhibited using a combination of dHpc DREADD expression and intra-retrosplenial CNO administration during expression of heroin contextually conditioned suppression of NO.

Given that the BLA and medial NAc shell have both been shown to play a role in heroin conditioned immune modulation to a context, it is important to determine whether the vHpc outputs to these areas serve as a conduit for contextual-cueing of this Pavlovian response. Thus, an experiment described in Chapter 3 tests the hypothesis that inactivating the vHpc output

regions using an inhibitory DREADD will attenuate heroin contextually conditioned suppression of NO.

CHAPTER 2: DORSAL HIPPOCAMPAL OUTPUT REGIONS ARE REQUIRED FOR THE EXPRESSION OF HEROIN'S CONTEXTUALLY CONDITIONED IMMUNE EFFECTS

Introduction

The dorsal hippocampus (dHpc), like the rest of the hippocampus, is thought to process information mostly in a serial, unidirectional fashion. Sensory information from cortex arrives at the dorsal dentate gyrus (dDG) via the perforant path, is then transmitted from the dDG to the dorsal CA3 (dCA3) subregion via the mossy fiber path, and is conveyed to the dorsal CA1 subregion (dCA1) via Schaffer Collaterals. Information from this tri-synaptic circuit is then finally routed to the dorsal subiculum (dSub) by dCA1 (Knierim, 2015; O'Mara, 2005). Information processed by the dHpc in this way is then relayed to other brain regions predominantly through projections directly from dCA1 and dSub, which are considered the main output regions of the dHpc (Witter, 2006). Fitting with the vital role of the dHpc in processing contextual information, these regions have been shown to be important in contextually-influenced behaviors with drugs of abuse. Xia et al showed that dCA1 neurons encode associations between nicotine reward and context in a conditioned place preference paradigm (Xia et al., 2017). Trouche et al similarly showed that more dCA1 neurons active during context-cocaine association, were re-activated during re-exposure to the cocaine-paired context than to a saline-paired context. Repeated inhibited of these cocaine-context representing neurons abolished conditioned drug behavior [conditioned place preference, (Trouche et al., 2016)]. These studies convincingly demonstrate that drug-associated contexts are meaningfully encoded by dCA1 neurons.

Much less direct evidence exists to evaluate the likely importance of dSub neurons to recall of a drug-associated context. One study showed that exposure to a cocaine-associated context increased activity in dSub neurons, although this did not differ significantly from unpaired controls where the context was not associated with cocaine (Franklin and Druhan, 2000). Another study showed that inactivation of the dSub disrupted forming a context-cocaine reward association as measured by cocaine-seeking behavior in a subsequent re-exposure to the associated context (Martin-Fardon et al., 2007), but this study manipulated the learning, not the expression of the conditioned behavior. Despite the scarcity of supporting evidence, if we accept the dSub as a predominant output of dHpc information, the evidence implicating dCA1 in context-drug associations and behavior would predict that dSub would be equally important to such processing.

Furthermore, outgoing projections from these output regions of the dHpc have been indirectly implicated in contextual models of drug behaviors from circuit-level analyses. For example, Fuchs et al have shown that inhibition of the dHpc prevents context-induced reinstatement of cocaine seeking (Fuchs et al., 2005) and that functional disconnection of the dHpc and the BLA attenuates context-induced reinstatement of cocaine seeking through interfering with context-cocaine memory reconsolidation (Fuchs et al., 2007b; Wells et al., 2011).

It would seem to follow that hippocampal output activity would be required in any paradigm requiring contextual processing. Our published experiments demonstrate that the dHpc is required for heroin contextually conditioned immunomodulation (Szczytkowski et al., 2013). What is not clear from this experiment is whether activity in these output regions is specifically required in this paradigm, since the dHpc was globally inactivated. The mainly serial processing

of information within the hippocampus would lead to the prediction that dCA1 and dSub would be critically important for the expression of heroin contextually conditioned immune modulation, but there is a possibility that this information might be relayed in some manner other than through these classical output regions (see Chapter 5: General Discussion). Additionally, it is unclear which projection neurons in these heterogeneous regions would mediate the relay of this information. Knowing which neuronal activity in the dHpc is required for the expression of heroin conditioned immune modulation will be an important step toward understanding how the dHpc might relay contextual information in this phenomenon.

The current experiment aimed to chemogenetically inhibit neurons in dCA1 and dSub just prior to re-exposure to a heroin-paired context in order to test their hypothesized role in mediating the expression of heroin contextually conditioned immune modulation.

Materials & Methods

Animals

Adult, male Lewis rats weighing initially 225-250 g (N = 36) were purchased from Charles River Laboratories (Kingston, NY, USA). Rats were housed individually on a reversed, 12-h light-dark cycle and all experimental procedures took place during the animals' active dark period (7 am – 7 pm). Food and water were provided *ad libitum* in home cages and animals were handled regularly. All experimental procedures were conducted in accordance with federal guidelines and with approval from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC).

Drugs and Delivery

Heroin (diacetylmorphine hydrochloride) was procured from the National Institute on Drug Abuse (NIDA) drug supply program, dissolved in sterile 0.9% saline to produce a 1.0

mg/mL solution, and stored at 4°C. During each conditioning session, rats were administered 1.0 mg/kg heroin subcutaneously. This dosage was based on our experiments showing that it reliably alters measures of nitric oxide (NO) in spleen and blood plasma following endotoxin immune challenge (Lysle and How, 2000; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Lipopolysaccharide (LPS, derived from *E. coli*, serotype O55:B5, Cat# L2880, MilliporeSigma, St. Louis, MO, USA) was dissolved in sterile 0.9% saline the day before use to produce a 1 mg/mL solution, which was then stored at 4°C. Following the test session, LPS was injected subcutaneously at a dose of 1.0 mg/kg which produces sickness behavior and production of NO measures. We have used this particular LPS serotype to previously investigate heroin-and conditioning-induced changes in immune response. Replications of these experiments should employ the same serotype, if possible, as activity between serotypes can vary (Caroff et al., 2002). The synthetic DREADD agonist clozapine-*N*-oxide (CNO, Cat# C0832, MilliporeSigma) was prepared as a solution on the same day as it was used while also protecting this light-sensitive reagent from light. CNO first dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with sterile 0.9% saline to a final concentration of 3.0 mg/mL CNO and 0.5% DMSO. CNO was injected subcutaneously at a dose of 3.0 mg/kg. All injected reagents stored at 4°C were allowed to come to room temperature just before use.

Surgical Procedure

To deliver a designer receptors exclusively activated by designer drugs (DREADD) containing virus *in vivo* to the dorsal hippocampus (dHpc) output regions, rats underwent intracranial surgery. Rats were anesthetized with 1.0 mL/kg of a 9:1 (volume:volume) mixture of ketamine hydrochloride (100 mg/mL) and xylazine (100 mg/mL) injected intraperitoneally. Head-shaven animals were placed in a stereotaxic apparatus, the surgical site sterilized, and an

incision was made in the skin covering the skull. Holes were drilled into the exposed skull at bilateral coordinates for dorsal subiculum (dSub). Coordinates relative to bregma were AP -6.0 mm, ML ±2.8 mm, DV -3.5 mm (Paxinos and Watson, 2007). Injectors (33 gauge, Plastics One, Roanoke, VA, USA) containing virus and connected with tubing to syringes and a microinfusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) were lowered at a 0° lateral angle to the appropriate DV coordinate but were raised 0.1 mm DV to create a pocket before virus infusion. The virus was infused at the received titer of $4.4x10^{12}$ GC/mL, volume of 0.7 µL per hemisphere, and a rate of 0.05 µL/min. Injectors were then left in place for 10 min to allow for diffusion away from the injection site. Injectors were raised slowly (over 1-2 min) to limit spread of the virus up the injection tract. All animals received DREADD virus. The incision was closed using 4-0 nylon monofilament non-absorbable suture. Animals were given at least two weeks to recover prior to undergoing conditioning.

DREADD Virus and Incubation

DREADDs are delivered *in vivo* using viruses. In these experiments, the DREADD plasmid CAMKIIα-hM4D(G_i)-mCherry, a gift from Bryan Roth, was purchased pre-packaged in an adeno-associated virus serotype 5 (AAV5) from Addgene (Viral prep 50477-AAV5; http://n2t.net/addgene:50477; RRID:Addgene_50477, Cambridge, MA, USA). The Ca^{2+}/calmodulin-dependent protein kinase II alpha (CAMKIIα) promoter element was chosen based on pilot studies showing that virus with this promoter exhibited stronger expression and less dorsal-ventral spread relative to virus with the human synapsin 1 (hSyn) promoter (unpublished data). Furthermore, the CAMKIIα promoter element may allow for the DREADD to be expressed preferentially in excitatory neurons, especially CA1 pyramidal neurons (Achterberg et al., 2014; Guo et al., 2010; Johansen et al., 2010; Liu and Jones, 1996; Tsien et

al., 1996), which is one of our main target populations. The mCherry reporter element produced fluorescent signal in transduced cells that allowed for localization of DREADD-expressing cells postmortem. Following virus delivery, the virus was allowed to incubate to promote DREADD expression for at least two weeks prior to the initiation of any training. By the time animals were treated with CNO, total incubation time was at least four weeks.

Conditioning & Testing Procedure

All rats in these studies were Pavlovian conditioned using five, 60-min sessions every 48 hours where they received an injection of heroin, the unconditioned stimulus (US), and were immediately placed in a distinct context, the conditioned stimulus (CS). This training regimen has repeatedly produced a conditioned immunomodulatory response to the heroin-paired context alone in our laboratory (Lebonville et al., 2016; Paniccia et al., 2018; Szczytkowski et al., 2011; Szczytkowski et al., 2013; Szczytkowski and Lysle, 2010). The CS was a standard operant chamber (BRS/LVE, Laurel, MD, USA; W 30.5 cm x H 26.7 cm x D 24.1 cm) that was enclosed by a sound and light attenuating outer chamber (W 50.8 cm x H 36.8 cm x D 34.3 cm). To distinguish these chambers from any home cage stimuli, the conditioning chambers were housed in a separate room from the vivarium and contained distinct auditory (noise-masking house fan), tactile (metal footshock bar floor), visual (metal side walls), and olfactory (cedar bedding) cues. Between animals, the chambers were thoroughly cleaned with Roccal-D Plus (Zoetis, Kalamazoo, MI).

Six days after the final conditioning session (day 15), animals were tested for the expression of a conditioned immune response by being re-exposed to the CS (conditioning chamber) for 60 min. Thirty-minutes before CS re-exposure, animals received either an injection of CNO (experimental) or vehicle (control). As a behavioral control representing a typical

immune response to LPS, another group of animals remained in home cage instead of being re-exposed to the CS before LPS challenge. Prior work in our laboratory has demonstrated that the LPS response of these heroin-conditioned home-cage control animals are not different than unmanipulated animals, saline conditioned controls, or animals that received heroin and CS-exposure in an unpaired manner (Lysle and Ijames, 2002). Collectively, these results indicate not only that immunomodulation to a heroin-paired context is a conditioned response to the CS and not ancillary effects of conditioning procedures or heroin dosing, but also that the use of only one of these equivalent control groups in future experiments is valid. We believe the heroin-conditioned home-cage control to be the most important and thus it is used here.

Blood and Spleen Tissue Collection

Immediately after CS (or home cage control) exposure, all animals received an LPS immune challenge and were sacrificed by cervical dislocation without anesthesia 6 h later for brain, blood, and spleen collection. This time point is optimized to detect measures of NO production in spleen and blood plasma. Blood was collected in heparinized syringes, transferred to tubes, and spun at 2000 g/rcf and 4°C for 20 min. Plasma was collected and stored at -80°C. Spleen tissue has shown robust expression of iNOS in response to LPS in multiple immune cell types (Bandaletova et al., 1993) and has reliably demonstrated opioid-conditioned immunomodulation in our studies (Lebonville et al., 2016; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Spleen tissue was dissected out postmortem and cut into approximately 100 mg pieces for RT-qPCR and ELISA. For RT-qPCR analysis, tissue was stored in RNA*later* (ThermoFisher Scientific, Waltham, MA, USA) for two days at 4°C and then -80°C. For ELISA analysis, spleen tissue was stored in protease inhibitor buffer (Pierce™, ThermoFisher Scientific) at -80°C.

Brain Histology and DREADD Expression Analysis

Whole brains were extracted and post-fixed in 4% paraformaldehyde at 4°C for 48 hours with agitation. Then brains were cryoprotected at 4°C in 30% sucrose containing 0.1% sodium azide until sunk (from 6-8 days). Brains were next embedded in frozen section compound (VWR, Radnor, PA, USA), frozen in a -23 to -25°C freezing microtome, covered with aluminum foil, and stored at -80°C. Brains were allowed to warm to -20 to -21°C before being sliced into 40 µm coronal sections on a cryostat (Leica CM 3050 S, Leica Microsystems, Buffalo Grove, IL, USA). Free-floating slices were stored in a cold solution of ethylene glycol and polyvinylpyrrolidone at -20°C. Desired sections were slide mounted onto charged glass slides (FisherBrand Superfrost, ThermoFisher Scientific, Waltham, MA, USA), allowed to air dry under dark conditions, and then coverslip mounted using HardSet VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescent microscopy (Leica DM6000 B widefield light microscope, Leica Microsystems, Buffalo Grove, IL, USA) was used to verify positive bilateral DREADD expression in the hippocampal region of interest though localization of the mCherry fluorescent tag. Any animals that did not show bilateral mCherry expression in dSub were removed from all subsequent analyses.

Nitrate/nitrite Assay

The byproducts of NO degradation in plasma, nitrate and nitrite, were measured using a Greiss reagent assay as previously described (Szczytkowski and Lysle, 2007). Recovery of nitrate is greater than 95% using this assay. Briefly, in a 96-well plate in triplicate, 12 µL plasma, 38 µL dH_2O, 10 µL nitrate reductase (1.0 U/mL), 20 µL of 0.31 M phosphate buffer (pH 7.5), 10 µL 0.86 mM NADPH, and 10 µL 0.11 mM FAD were incubated in the dark at room temperature for 90 min. Next, 200 µL of Greiss reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric

acid and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride in dH_2O) was added and color allowed to develop for 10 min at room temperature. Bubbles were popped using a syringe needle and the bottom of the plate was wiped clean before measuring absorbance at 550 nm in a spectrophotometer. Total nitrate/nitrite concentration was determined from a concurrently run known standard dilution series with a 4-parameter logistic curve fit.

RNA Extraction & RT-qPCR

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was performed on spleen samples to measure iNOS messenger ribonucleic acid (mRNA) expression. First, spleen tissue free of residual RNA*later* was homogenized in 1 mL TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) using a bead mill homogenizer (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France) and the following parameters: 7500 rpm, 30 s, 30 s pause, 6 cycles, cooling samples on ice between every three cycles. RNA was purified using TRI-Reagent's manufacturer protocols with the following modifications: performed optional homogenization step to remove debris, added 100 µL RNAse Free H_2O in conjunction with 100 µL of BCP to reduce the density of the homogenate and aid phase separation in PhaseLock Gel Tubes (Heavy formulation, 5Prime/Quantabio, Beverly, MA, USA), and conducted three RNA pellet washes with 75% ethanol. The purified RNA pellet was dissolved by 55-60°C incubation in 150 µL of RNase Free H_2O.

RNA purity and concentration were assessed by spectrophotometry (Take3 microdot plate and the Epoch™ spectrophotometer, BioTek Instruments Inc., Winooski, VT). RNA was diluted 1:20 in 1xTE (pH = 7.5) for spectrophotometric readings. A260 nm/280 nm ratios were used to assess RNA purity. A260 nm values were used to determine RNA concentration. RNA quality was assessed using the RNA 6000 Nano kit on a 2100 Bioanalyzer instrument (Agilent

Technologies, Inc., Santa Clara, CA, USA). A minimum RNA integrity number (RIN) of 8.0 was considered indicative of high quality, intact RNA. All samples demonstrated A260 nm/280 nm values close to 2.1, indicating high purity.

cDNA synthesis was performed on a Veriti 96 Well Fast Thermal Cycler (Applied Biosystems, ThermoFisher Scientific) using the Advantage RT-for-PCR Kit, according to the manufacturer protocol (Clontech/Takara Bio, Mountain View, CA, USA). Priming for the RT reaction was carried out using Oligo(dT) primers. Input RNA concentration was equalized across samples (1 µg). Undiluted cDNA from each sample was pooled into a single sample of which five serial 1:10 dilutions were made to evaluate qPCR efficiency. Remaining cDNA from each sample was then diluted 1:5 in PCR-grade H_2O.

qPCR was run on a QuantStudio™ 6 Flex system (AP Biosystems, ThermoFisher Scientific) using TaqMan™ Fast Advanced Master Mix (AP Biosystems) and predesigned fluorescein (FAM) assays for iNOS (NOS2, gene of interest, Assay ID: Rn00561646_m1, ThermoFisher Scientific) and L13A (Rpl13a, reference gene, Assay ID: Rn01475911_g1, ThermoFisher Scientific). Individual reactions containing 1.5 µL cDNA template, 3.0 µL of PCR-grade water, 5.0 µL master mix, and 0.5 µL assay were run in triplicate on a 384-well plate. Cycling parameters were 50°C for 2 min for degradation of any qPCR product contamination, 90°C for 20 s for polymerase activation, and 45 cycles of 95°C for 1 s and 60°C for 20 s for target amplification. Fluorescent data was collected at the end of each of each cycle. Efficiency was estimated by plotting the dilution number (5, 4, 3…with 5 being the most concentrated) by the Ct and fitting a linear regression to the data. The slope of the line was then used to estimate the efficiency using an online calculator (Agilent Genomics: Tools – Bio Calculators) which used the equation: Efficiency = $-1+10^{(-1/\text{slope})}$. For all qPCR assays, iNOS and L13A amplification

efficiency of cDNA pools were at least 92% and roughly equivalent between the two genes, two important prerequisites for relative qPCR analysis. The comparative delta delta Ct method (ΔΔCt) was used for data analysis (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Any Ct value in a triplicate that differed by 0.5 or more from the other two was removed from analysis. The validity of L13A as a reference gene was verified using a 2 x 2 ANOVA to show that L13A expression did not differ in any way by group ($F_{(3,28)} = .178$, $p = .910$). The first normalization (ΔCt) was to the reference gene, L13A, while the second normalization was to an average of iNOS ΔCt for the experiment, since the experimental design did not have a single control group. ΔΔCt values were linearly transformed into $2^{-\Delta\Delta Ct}$ values for graphical representation.

Protein Extraction & iNOS ELISA

Spleen tissue was thawed and homogenized on ice in sterile, glass, Dounce grinders. Cells were lysed using two freeze-thaw cycles, where the protein was released into supernatant (protease inhibitor buffer). The homogenate was then centrifuged and the supernatant containing isolated protein collected. Total protein was quantified by Bradford Assay as previously described (Lebonville et al., 2016). To quantify iNOS protein, 38 µg of total protein from each sample was run in triplicate in a rat iNOS sandwich ELISA (Cat #: abx256135, Abbexa Ltd., Cambridge, UK) following the manufacturer's protocol. An automatic plate washer (EL 403, BioTek Instruments Inc., Winooski, VT, USA) was used to uniformly wash the plate. Absorbance of the finished ELISA was read at 450 nm. Technical replicates greater than 2 standard deviations from the triplicate mean were removed from analysis. The amount of iNOS protein per sample was determined from the 4-parameter logistic curve fit to a dilution series of the included standard. Quantity of iNOS protein is reported in pg per 38 µg total protein.

Statistical Analysis

A 2 x 2 analysis of variance (ANOVA) was performed on all data sets using statistical software (SPSS Statistics 24 and 25, IBM, Armonk, NY, USA). For all tests, the level of significance was set to $p \leq .05$. The validity of using an ANOVA with the current data was tested using Shapiro-Wilk Test for normality and Levene's Test for homogeneity of variance (HOV). All assumptions were supported for this experimental data. Planned contrasts were performed with the CS and HC groups within each drug treatment (vehicle or CNO) to test for a conditioned effect with exposure to the CS. Any ancillary effects were probed using Tukey's Honestly Significant Difference (HSD) post-hoc test. The presence of statistical outliers was probed using Grubb's test. Statistical outliers were removed from final analysis. For RT-qPCR data, statistical analysis was performed with ΔΔCt values (without linear transformation) because these values tend to better meet the assumptions of an ANOVA in our experience.

Results

These studies tested the effect of DREADD-mediated inhibition of dorsal hippocampal (dHpc) outputs on the expression of heroin contextually conditioned suppression of NO. The experimental design is displayed in **Figure 1A**. Virally-transduced cells, as determined by mCherry staining, were present in multiple areas of the dorsal hippocampus (dCA1, dSub, dDG, and dCA2), post-subiculum, overlying proximal retrosplenial granular and dysgranular cortex, and deep layers of visual cortex (**Figure 1B**).

The most consistent and thorough staining across subjects (most opaque regions) was present in dCA1 and dSub subregions of the dHpc. No subjects needed to be dropped due to not having bilateral virus expression in dSub. One subject was dropped from nitrate/nitrite analysis

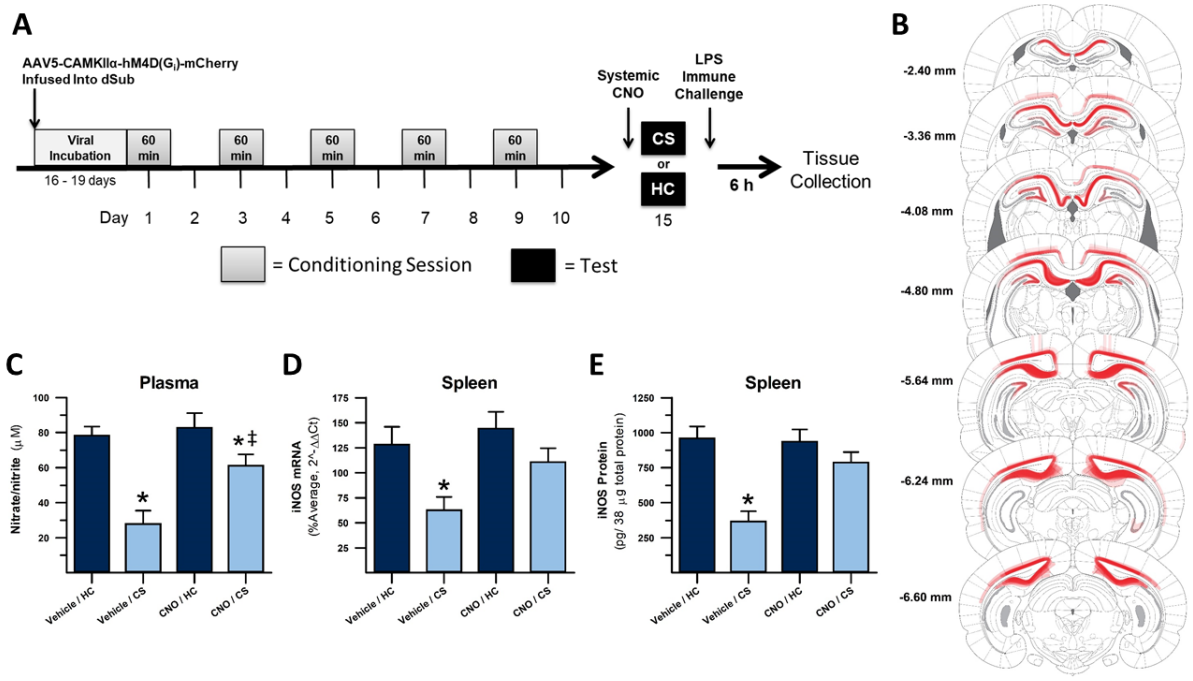

Figure 1. Inactivation of dHpc output regions, including dSub and dCA1, disrupts expression of heroin contextually conditioned suppression of NO. Experimental timeline **(A)**. DREADD (mCherry) expression and spread. **(B)**. Effect of CNO administration on expression of heroin contextually conditioned suppression of plasma nitrate/nitrite concentration **(C)** and splenic iNOS mRNA **(D)** and protein **(E)** expression. CS = conditioned stimulus or heroin-paired context, HC = home cage; *, statistically significant difference relative to respective HC control. ‡, statistically significant difference relative to Vehicle/CS group.

due to insufficient plasma collection (final n = 7-8). No subjects were dropped from iNOS qPCR and ELISA analyses (final n = 8).

Plasma nitrate/nitrite concentration during the test for the expression of heroin contextually conditioned suppression of NO following chemogenetic inhibition of dHpc outputs is shown in **Figure 1C**. A 2 x 2 ANOVA revealed significant differences in plasma nitrate/nitrite concentration between treatment groups ($F_{(3,27)} = 14.264$, $p < .001$). There was a significant interaction between exposure regimen (CS or HC) and injection (CNO or Veh) on plasma nitrate/nitrite ($F_{(1,27)} = 4.725$, $p = .039$). There were also significant main effects of exposure ($F_{(1,27)} = 29.321$, $p < .001$) and injection ($F_{(1,27)} = 8.002$, $p = .009$) on this measure. In vehicle-

treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less nitrate/nitrite in response to LPS than animals that remained in their home cage ($p < .001$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, confirming expression of the conditioned effect. In CNO-treated animals, a planned comparison also revealed that CS-exposed animals demonstrated significantly less nitrate/nitrite in response to LPS than animals that remained in their home cage ($p = .027$). This result would seem to indicate that in CNO-treated animals, there was still significant conditioned suppression of NO, yet post-hoc analysis showed that CNO-treated CS-exposed animals showed significantly higher nitrate/nitrite concentration than vehicle-treated CS-exposed animals ($p < .001$) which we interpret to be indicative of partial attenuation of conditioned suppression of nitrate/nitrite when dHpc output inhibition preceded CS exposure.

Splenic iNOS mRNA expression during the test for the expression of heroin contextually conditioned suppression of NO is shown in **Figure 1D**. A 2 x 2 ANOVA revealed significant differences in iNOS mRNA expression between treatment groups ($F_{(3,28)} = 8.060$, $p = .001$). There were significant main effects of exposure (CS or HC; $F_{(1,28)} = 13.178$, $p = .001$) and injection (CNO or Veh; $F_{(1,28)} = 7.346$, $p = .011$) on this measure but no significant interaction between exposure and injection ($F_{(1,28)} = 3.117$, $p = .088$). In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less iNOS mRNA expression in response to LPS than animals that remained in their home cage ($p = .001$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, confirming expression of the conditioned effect in vehicle-treated animals. In CNO-treated animals, a planned comparison revealed that CS-exposed animals showed similar iNOS mRNA expression in response to LPS compared to animals that remained in their home cage ($p = .181$).

Furthermore, post-hoc analysis showed that CNO-treated CS-exposed animals showed significantly higher iNOS mRNA expression than vehicle-treated CS-exposed animals (p = .018). These results indicate that there was no significant conditioned suppression of NO when dHpc output inhibition preceded CS exposure.

Splenic iNOS protein expression during the test for the expression of heroin contextually conditioned suppression of NO is shown in **Figure 1E**. A 2 x 2 ANOVA revealed significant differences in iNOS protein expression between treatment groups ($F_{(3,28)} = 13.838$, $p < .001$). There was a significant interaction between exposure regimen (CS or HC) and injection (CNO or Veh) on iNOS protein expression ($F_{(1,28)} = 9.145$, $p = .005$). There were also significant main effects of exposure ($F_{(1,28)} = 25.081$, $p < .001$) and injection ($F_{(1,28)} = 7.288$, $p = .012$) on this measure. In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less iNOS protein expression in response to LPS than animals that remained in their home cage ($p < .001$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, confirming expression of the conditioned effect in vehicle-treated animals. In CNO-treated animals, a planned comparison revealed that CS-exposed animals showed similar iNOS protein expression in response to LPS compared to animals that remained in their home cage ($p = .172$). Furthermore, post-hoc analysis showed that CNO-treated CS-exposed animals showed significantly higher iNOS mRNA expression than vehicle-treated CS-exposed animals ($p = .002$). These results indicate that there was no significant conditioned suppression of NO when dHpc output inhibition preceded CS exposure.

Overall, the results from this experiment supports the conclusion that chemogenetic inhibition of dHpc output subregions dSub and dCA1 results in either partial or full attenuation

of conditioned suppression of measures of NO production after exposure to a heroin-paired context.

Discussion

Research has shown that one role of the dorsal hippocampus (dHpc) is to process contextual information that can help guide future behavior. In addiction, context reliably influences and prompts drug taking and seeking behavior, but context can also prompt other changes in behavior unrelated to the rewarding aspects of drug use. With heroin, since it is a potent modulator of immune function, these effects can be recapitulated through exposure to a heroin-paired context. Our laboratory has repeatedly shown that these conditioned immune responses to a heroin-paired context require the dHpc. However, little is known about how the contextual processes of the dHpc ultimately lead to changes in behavior.

The study described in this chapter represents the first step to understanding dHpc processing at both the local and circuit level during the expression of heroin contextually conditioned immune modulation. Chemogenetic inhibition of dorsal subiculum (dSub) and dorsal CA1 (dCA1), two of the main output regions of the dHpc, disrupted the expression of heroin contextually conditioned immune suppression, as determined by measures of nitric oxide production in response to LPS. This experiment is the first to manipulate specific populations of dHpc neurons in this paradigm and provide empirical evidence that this chemogenetic technique can be used to ask questions about the broader circuitry of contextually influenced behaviors that require the dHpc.

These results complement substantial evidence that Ca^{2+}/calmodulin-dependent protein kinase II alpha (CAMKIIα) expressing neurons in the dHpc are crucial to formation of hippocampal-dependent memory. In fact, since dCA1 neurons are the predominant model used to

study long-term potentiation (LTP), a cellular mechanism of learning and memory through synaptic plasticity (Takeuchi et al., 2014), an abundance of evidence exists to demonstrate the importance of dHpc CAMKIIα to both pre-synaptic (Hojjati et al., 2007) and post-synaptic (Incontro et al., 2018; Lisman et al., 2012) plasticity. Perturbations in CAMKIIα and subsequent interference with LTP has also been linked with deficits in contextual and spatial memory. Mice with a disrupting mutation or deletion of the CAMKIIα gene from birth or at the time of training show impaired dCA1 LTP as well as impaired spatial and contextual learning (Achterberg et al., 2014; Giese et al., 1998; Silva et al., 1992a; Silva et al., 1992b).

Furthermore, there is evidence that CAMKIIα mechanisms are important to drug contextual memory. Chronic opiate administration was shown to elevate CAMKIIα mRNA expression in dCA1 (Chen et al., 2008), and given it's important role in synaptic plasticity, this may represent encoding of drug associations. CAMKII inhibition in the dHpc impaired acquisition and drug-primed reinstatement of morphine conditioned place preference (Lu et al., 2000). Interestingly, these studies also implicated dHpc CAMKII in the development of morphine dependence as well, suggesting that the dHpc may be involved in drug synaptic plasticity beyond simply encoding contextual associations. Less evidence supports the role of CAMKIIα expressing neurons in contextual memory recall, that is, during *expression* of a previously learned hippocampal-dependent contextual task. This is likely due to the fact that few investigate the role of CAMKIIα expressing neurons themselves and are instead looking at the role of CAMKIIα expression by these neurons. In the case of CAMKIIα, this gene seems to have more of a reconsolidation or extinction (new learning) role at the time of memory recall than a role in memory retrieval itself (Vigil and Giese, 2018). Cao et al found that transient CAMKIIα expression disrupts recall not by disrupting the retrieval process but by actively erasing the fear

memories (Cao et al., 2008). Thus, the distinction between CAMKIIα expression and CAMKIIα expressing neurons is important in studies like this one which investigates the global role of the neurons themselves, without necessarily looking at that gene's expression by those neurons. In the context of this chapter, the overwhelming evidence of CAMKIIα's importance to dHpc function and memory formation merely provides evidence that these neurons might be sites of important *previous* plasticity that is relied on during expression of heroin contextually conditioned immune modulation.

This experiment targeted prominent hippocampal output regions with the aim of beginning to elucidate important Hpc projections. However, it is altogether possible that inhibiting CAMKIIα expressing neurons perturbed local hippocampal signaling and this resulted in impaired expression instead of the intended inhibition of outgoing projections. This possibility could be ruled out by specifically inhibiting these CAMKIIα-DREADD expressing neurons at their projection terminals in areas outside of the hippocampus. A site-directed CNO infusion at an area receiving projections from CAMKIIα-DREADD expressing neurons would demonstrate conclusively that signals from dHpc outputs are required for the expression of heroin contextually conditioned immune modulation. Chapter 4 will describe the results from such an experiment. Additionally, concerns of CNO action non-specifically through DREADD-independent mechanisms have gained much attention recently (Gomez et al., 2017; Mahler and Aston-Jones, 2018). These concerns are not specific to Chapter 2's experiment and so will be discussed at length in Chapter 5: General Discussion.

This experiment targeted prominent hippocampal output region, dSub, but virus expression was not specific only to this region. Most notably, virus also spread to dCA1. Indeed many tracing studies who targeted the dSub saw similar labelling of proximal dCA1 (Witter,

2006) so this spread is not unique to our DREADD delivery system. Additionally, dCA1 is also considered an output region of the dHpc and so spread to this region does not interfere with testing the question about dHpc outputs and connectivity. However, DREADD expression was also seen in other regions other than dCA1 and dSub within and outside of the hippocampus, and this represents a major limitation of this study. Deep layers of cortex, including retrosplenial and visual cortex, and post-subiculum also consistently expressed the mCherry tag. The contributions of these areas to the observed effect could be ruled out using a site-directed CNO infusion in these areas. However, given the close proximity of these areas to the intended dHpc targets, which is likely why DREADD expression spread to these areas in the first place, it would be challenging to ensure that CNO action was restricted to these areas in such an experiment. The same limitation would also apply to a site-directed CNO infusion directly into the dHpc. Given these issues, a more promising way to determine specificity of the effect to inhibition of dHpc outputs is to specifically target dHpc projection areas that do not receive inputs from these overlying extra-hippocampal regions in follow-up experiments. Not only will experiments such as these confirm specificity of the effects seen here to inhibition of dHpc output regions, but also will help to understand the larger circuitry of heroin contextually conditioned immune modulation.

Our previous work has implicated the dHpc in the expression of heroin contextually conditioned immune modulation. The current results build on past data to implicate a specific population of neurons, those expressing CAMKIIα in the dSub and dCA1 regions of the dHpc. The logical next step is to determine which specific projections from these two regions are required. Collectively, dSub and dCA1 project to numerous targets including the entorhinal cortex, retrosplenial cortex, medial mammillary nucleus, lateral septum, and nucleus accumbens

core. The potential role of each of these projections in heroin contextually conditioned immune modulation is discussed in more detail in Chapters 4 and 5. This experiment demonstrates that CAMKIIα-DREADDs can be successfully used to investigate how the dHpc might relay contextual information to regulatory brain regions in both reward and immune paradigms with drugs of abuse.

CHAPTER 3: VENTRAL HIPPOCAMPAL OUTPUT REGIONS ARE NOT REQUIRED FOR THE EXPRESSION OF HEROIN'S CONTEXTUALLY CONDITIONED IMMUNE EFFECTS

Introduction

Chapter 2 focused on the role of the dorsal hippocampus (dHpc) in the expression of heroin contextually conditioned immune modulation. This chapter will focus on the role of the ventral hippocampus (vHpc) in this paradigm. Like the dHpc, the vHpc also processes information serially. Serial pathways route information ultimately to ventral subiculum (vSub) and ventral CA1 (vCA1) (Knierim, 2015; O'Mara, 2005) which then send projections to other regions of the brain. Both vSub and vCA1, mirroring their dorsal counterparts, are considered the main output regions from the vHpc (Witter, 2006). Unlike the role of the dHpc in processing mostly spatial and contextual information, the vHpc has been thought to process mostly emotional (i.e. stress and reward) information. Most relevant to this dissertation is the fact that the vHpc has direct connections with all of the brain regions implicated in both conditioned immune and conditioned reward behaviors including the medial nucleus accumbens shell (medial NAc shell), ventral tegmental area (VTA), and basolateral amygdala (BLA). Anatomical connectivity with important brain regions alone would not necessarily imply a role of the vHpc outputs in heroin contextually conditioned immune modulation. Yet, more questions than answers arise when reviewing relevant behavioral evidence.

There is some functional evidence that activity in these output regions is relevant to context and drugs of abuse. Unlike with the dCA1, which has been extensively used to study synaptic plasticity, the vCA1 subregion has been much less thoroughly investigated.

Comparatively, there is more evidence implicating the vSub in drug-context associations and behaviors. Several studies have shown that exposure to a cocaine-associated context increases activity in vCA1 and vSub neurons (Franklin and Druhan, 2000; Neisewander et al., 2000), although some of these effects were modest and did not differ significantly from unpaired controls where the context was not associated with cocaine. Reversible lidocaine-mediated lesions of vCA1/vSub has been shown to block context-induced reinstatement of cocaine seeking (Atkins et al., 2008). GABAergic inhibition of the vSub, but surprisingly not vCA1, reduced context-induced reinstatement of heroin seeking (Bossert and Stern, 2014). The vSub was also shown to be important for context-induced reinstatement of ethanol seeking (Marchant et al., 2016a). Moreover, the role of these regions seems to be due to their projections to the nucleus accumbens. For example, stimulating the vSub alone is enough to both increase dopamine release in the nucleus accumbens and increase reinstatement of drug-seeking behavior with cocaine (Vorel et al., 2001) and d-amphetamine (Taepavarapruk and Phillips, 2003). Pathway specific inhibition of projections from the vSub to nucleus accumbens also reduces context-induced reinstatement of heroin seeking (Bossert et al., 2016). Overall, these data indicate that the vHpc, especially vSub, is required across different drugs of abuse for contextual influences on drug behaviors and that this role is likely due in part to its modulation of dopamine signaling in the nucleus accumbens.

So far, the studies summarized here imply that these vHpc output regions are important for reward-motivated drug behaviors involving a context. What is unclear is what aspects of re-exposure and subsequent drug seeking are being encoded by these regions – reward associations generally, context-reward associations, or contextual associations *per se*. It has been proposed that both the dHpc and vHpc could contribute to spatial/contextual memory, but that the vHpc is

less involved in these tasks (Vann et al., 2000). The vCA1 possesses place cells that could be capable of contextual processing, though they are fewer in number and have much lower spatial resolution (e.x. larger place fields) than cells in dCA1 (Jung et al., 1994). Floresco and colleagues found that inhibition of the vCA1 and vSub disrupted acquisition of escape behavior in the Morris Water Maze, a spatial memory task (Floresco et al., 1996). Notably this manipulation did not affect performance during a subsequent test after temporary inhibition of vCA1/vSub had passed, indicating that the spatial information necessary to perform the task was encoded independent of these vHpc outputs. However, spatial memory in these tasks is more navigational than contextual. More convincingly, Atkins and colleagues demonstrated that inhibition of vCA1/vSub causes a deficit in the ability to discriminate between saline- and cocaine-paired contexts (Atkins et al., 2008). This study also showed that this manipulation blocked context-induced, but not discrete cue-induced reinstatement of cocaine seeking, which would seem to indicate a role for the vHpc output areas in contextually influenced drug behaviors.

No study to date has investigated the role of the vHpc, much less specific output subregions, in heroin contextually conditioned immune modulation. The current experiment aimed to chemogenetically inhibit neurons in vSub and vCA1 just prior to re-exposure to a heroin-paired context in order to test their hypothesized role in mediating the expression of heroin contextually conditioned immune modulation.

Materials & Methods

Animals

Adult, male Lewis rats weighing initially 225-250 g (N = 41) were purchased from Charles River Laboratories (Kingston, NY, USA). Rats were housed individually on a reversed,

12-h light-dark cycle and all experimental procedures took place during the animals' active dark period (7 am – 7 pm). Food and water were provided *ad libitum* in home cages and animals were handled regularly. All experimental procedures were conducted in accordance with federal guidelines and with approval from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC).

Drugs and Delivery

Heroin (diacetylmorphine hydrochloride) was procured from the National Institute on Drug Abuse (NIDA) drug supply program, dissolved in sterile 0.9% saline to produce a 1.0 mg/mL solution, and stored at 4°C. During each conditioning session, rats were administered 1.0 mg/kg heroin subcutaneously. This dosage was based on our experiments showing that it reliably alters measures of nitric oxide (NO) in spleen and blood plasma following endotoxin immune challenge (Lysle and How, 2000; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Lipopolysaccharide (LPS, derived from *E. coli*, serotype O55:B5, Cat# L2880, MilliporeSigma, St. Louis, MO, USA) was dissolved in sterile 0.9% saline the day before use to produce a 1 mg/mL solution, which was then stored at 4°C. Following the test session, LPS was injected subcutaneously at a dose of 1.0 mg/kg which produces sickness behavior and production of NO measures. We have used this particular LPS serotype to previously investigate heroin-and conditioning-induced changes in immune response. Replications of these experiments should employ the same serotype, if possible, as activity between serotypes can vary (Caroff et al., 2002). The synthetic DREADD agonist clozapine-*N*-oxide (CNO, NOCD-135, NIDA Drug Supply Program, Bethesda, MD, USA) was prepared as a solution on the same day as it was used while also protecting this light-sensitive reagent from light. CNO first dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with sterile 0.9% saline to a final concentration of

3.0 mg/mL CNO and 0.5% DMSO. CNO was injected subcutaneously at a dose of 3.0 mg/kg. All injected reagents stored at 4°C were allowed to come to room temperature just before use.

Surgical Procedure

To deliver a designer receptors exclusively activated by designer drugs (DREADD) containing virus *in vivo* to ventral hippocampus (vHpc), rats underwent intracranial surgery. Rats were anesthetized with 1.0 mL/kg of a 9:1 (vol:vol) mixture of ketamine hydrochloride (100 mg/mL) and xylazine (100 mg/mL) injected intraperitoneally. Head-shaven animals were placed in a stereotaxic apparatus, the surgical site sterilized, and an incision was made in the skin covering the skull. Holes were drilled into the exposed skull at bilateral coordinates for ventral subiculum (vSub). Coordinates relative to bregma for vSub were AP -6.0 mm, ML ±4.6 mm, DV -8.5 mm (Paxinos and Watson, 2007). Injectors (33 gauge, Plastics One, Roanoke, VA, USA) containing virus and connected with tubing to syringes and a microinfusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) were lowered at a 0° lateral angle to the appropriate DV coordinate but were raised 0.1 mm DV to create a pocket before virus infusion. The virus was infused at the received titer of $4.4x10^{12}$ GC/mL, volume of 0.7 µL per hemisphere, and a rate of 0.05 µL/min. Injectors were then left in place for 10 min to allow for diffusion away from the injection site. Injectors were raised slowly (over 1-2 min) to limit spread of the virus up the injection tract. All animals received DREADD virus. The incision was closed using 4-0 nylon monofilament non-absorbable suture. Animals were given at least two weeks to recover prior to undergoing conditioning.

DREADD Virus and Incubation

DREADDs are delivered *in vivo* using viruses. In these experiments, the DREADD plasmid CAMKIIα-hM4D(G_i)-mCherry, a gift from Bryan Roth, was purchased pre-packaged in

an adeno-associated virus serotype 5 (AAV5) from Addgene (Viral prep 50477-AAV5; http://n2t.net/addgene:50477; RRID:Addgene_50477, Cambridge, MA, USA). The Ca^{2+}/calmodulin-dependent protein kinase II alpha (CAMKIIα) promoter element was chosen based on pilot studies showing that virus with this promoter exhibited stronger expression and less dorsal-ventral spread relative to virus with the human synapsin 1 (hSyn) promoter (unpublished data). Furthermore, the CAMKIIα promoter element may allow for the DREADD to be expressed preferentially in excitatory neurons, especially CA1 pyramidal neurons (Achterberg et al., 2014; Guo et al., 2010; Johansen et al., 2010; Liu and Jones, 1996; Tsien et al., 1996), which is one of our main target populations. The mCherry reporter element produced fluorescent signal in transduced cells that allowed for localization of DREADD-expressing cells postmortem. Following virus delivery, the virus was allowed to incubate to promote DREADD expression for at least two weeks prior to the initiation of any training. By the time animals were treated with CNO, total incubation time was at least four weeks.

Conditioning & Testing Procedure

All rats in these studies were Pavlovian conditioned using five, 60-min sessions every 48 hours where they received an injection of heroin, the unconditioned stimulus (US), and were immediately placed in a distinct context, the conditioned stimulus (CS). This training regimen has repeatedly produced a conditioned immunomodulatory response to the heroin-paired context alone in our laboratory (Lebonville et al., 2016; Paniccia et al., 2018; Szczytkowski et al., 2011; Szczytkowski et al., 2013; Szczytkowski and Lysle, 2010). The CS was a standard operant chamber (BRS/LVE, Laurel, MD, USA; W 30.5 cm x H 26.7 cm x D 24.1 cm) that was enclosed by a sound and light attenuating outer chamber (W 50.8 cm x H 36.8 cm x D 34.3 cm). To distinguish these chambers from any home cage stimuli, the conditioning chambers were housed

in a separate room from the vivarium and contained distinct auditory (noise-masking house fan), tactile (metal footshock bar floor), visual (metal side walls), and olfactory (cedar bedding) cues. Between animals, the chambers were thoroughly cleaned with Roccal-D Plus (Zoetis, Kalamazoo, MI).

Six days after the final conditioning session (day 15), animals were tested for the expression of a conditioned immune response by being re-exposed to the CS (conditioning chamber) for 60 min. Thirty-minutes before CS re-exposure, animals received either an injection of CNO (experimental) or vehicle (control). As a behavioral control representing a typical immune response to LPS, another group of animals remained in home cage instead of being re-exposed to the CS before LPS challenge. Prior work in our laboratory has demonstrated that the LPS response of these heroin-conditioned home-cage control animals are not different than unmanipulated animals, saline conditioned controls, or animals that received heroin and CS-exposure in an unpaired manner (Lysle and Ijames, 2002). Collectively, these results indicate not only that immunomodulation to a heroin-paired context is a conditioned response to the CS and not ancillary effects of conditioning procedures or heroin dosing, but also that the use of only one of these equivalent control groups in future experiments is valid. We believe the heroin-conditioned home-cage control to be the most important and thus it is used here.

Blood and Spleen Tissue Collection

Immediately after CS (or home cage control) exposure, all animals received an LPS immune challenge and were sacrificed by cervical dislocation without anesthesia 6 h later for brain, blood, and spleen collection. This time point is optimized to detect measures of NO production in spleen and blood plasma. Blood was collected in heparinized syringes, transferred to tubes, and spun at 2000 g and 4°C for 20 min. Plasma was collected and stored at -80°C.

Spleen tissue has shown robust expression of iNOS in response to LPS in multiple immune cell types (Bandaletova et al., 1993) and has reliably demonstrated opioid-conditioned immunomodulation in our studies (Lebonville et al., 2016; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Spleen tissue was dissected out postmortem and cut into approximately 100 mg pieces for RT-qPCR and ELISA. For RT-qPCR analysis, tissue was stored in RNA*later* (ThermoFisher Scientific, Waltham, MA, USA) for two days at 4°C and then -80°C. For ELISA analysis, spleen tissue was stored in protease inhibitor buffer (Pierce™, ThermoFisher Scientific) at -80°C.

Brain Histology and DREADD Expression Analysis

Whole brains were extracted and post-fixed in 4% paraformaldehyde at 4°C for 48 hours with agitation. Then brains were cryoprotected at 4°C in 30% sucrose containing 0.1% sodium azide until sunk (from 6-8 days). Brains were next embedded in frozen section compound (VWR, Radnor, PA, USA), frozen in a -23 to -25°C freezing microtome, covered with aluminum foil, and stored at -80°C. Brains were allowed to warm to -20 to -21°C before being sliced into 40 µm coronal sections on a cryostat (Leica CM 3050 S, Leica Microsystems, Buffalo Grove, IL, USA). Free-floating slices were stored in a cold solution of ethylene glycol and polyvinylpyrrolidone at -20°C. Desired sections were slide mounted onto charged glass slides (FisherBrand Superfrost, ThermoFisher Scientific, Waltham, MA, USA), allowed to air dry under dark conditions, and then coverslip mounted using HardSet VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescent microscopy (Leica DM6000 B widefield light microscope, Leica Microsystems, Buffalo Grove, IL, USA) was used to verify positive bilateral DREADD expression in the hippocampal region of interest

though localization of the mCherry fluorescent tag. Any animals that did not show bilateral mCherry expression in vSub were removed from all subsequent analyses.

Nitrate/nitrite Assay

The byproducts of NO degradation in plasma, nitrate and nitrite, were measured using a Greiss reagent assay as previously described (Szczytkowski and Lysle, 2007). Recovery of nitrate is greater than 95% using this assay. Briefly, in a 96-well plate in triplicate, 12 µL plasma, 38 µL dH_2O, 10 µL nitrate reductase (1.0 U/mL), 20 µL of 0.31 M phosphate buffer (pH 7.5), 10 µL 0.86 mM NADPH, and 10 µL 0.11 mM FAD were incubated in the dark at room temperature for 90 min. Next, 200 µL of Greiss reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride in dH_2O) was added and color allowed to develop for 10 min at room temperature. Bubbles were popped using a syringe needle and the bottom of the plate was wiped clean before measuring absorbance at 550 nm in a spectrophotometer. Total nitrate/nitrite concentration was determined from a concurrently run known standard dilution series with a 4-parameter logistic curve fit.

RNA Extraction & RT-qPCR

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was performed on spleen samples to measure iNOS messenger ribonucleic acid (mRNA) expression. First, spleen tissue free of residual RNA*later* was homogenized in 1 mL TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) using a bead mill homogenizer (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France) and the following parameters: 7500 rpm, 30 s, 30 s pause, 6 cycles, cooling samples on ice between every three cycles. RNA was purified using TRI-Reagent's manufacturer protocols with the following modifications: performed optional homogenization step to remove debris, added 100 µL RNAse Free H_2O in conjunction

with 100 µL of BCP to reduce the density of the homogenate and aid phase separation in PhaseLock Gel Tubes (Heavy formulation, 5Prime/Quantabio, Beverly, MA, USA), and conducted three RNA pellet washes with 75% ethanol. The purified RNA pellet was dissolved by 55-60°C incubation in 150 µL of RNase Free H_2O.

RNA purity and concentration were assessed by spectrophotometry (Take3 microdot plate and the Epoch™ spectrophotometer, BioTek Instruments Inc., Winooski, VT). RNA was diluted 1:20 in 1xTE (pH = 7.5) for spectrophotometric readings. A260 nm/280 nm ratios were used to assess RNA purity. A260 nm values were used to determine RNA concentration. RNA quality was assessed using the RNA 6000 Nano kit on a 2100 Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). A minimum RNA integrity number (RIN) of 8.0 was considered indicative of high quality, intact RNA. All samples demonstrated A260 nm/280 nm values close to 2.1, indicating high purity.

cDNA synthesis was performed on a Veriti 96 Well Fast Thermal Cycler (Applied Biosystems, ThermoFisher Scientific) using the Advantage RT-for-PCR Kit, according to the manufacturer protocol (Clontech/Takara Bio, Mountain View, CA, USA). Priming for the RT reaction was carried out using Oligo(dT) primers. Input RNA concentration was equalized across samples (1 µg). Undiluted cDNA from each sample was pooled into a single sample of which five serial 1:10 dilutions were made to evaluate qPCR efficiency. Remaining cDNA from each sample was then diluted 1:5 in PCR-grade H_2O.

qPCR was run on a QuantStudio™ 6 Flex system (AP Biosystems, ThermoFisher Scientific) using TaqMan™ Fast Advanced Master Mix (AP Biosystems) and predesigned fluorescein (FAM) assays for iNOS (NOS2, gene of interest, Assay ID: Rn00561646_m1, ThermoFisher Scientific) and L13A (Rpl13a, reference gene, Assay ID: Rn01475911_g1,

ThermoFisher Scientific). Individual reactions containing 1.5 µL cDNA template, 3.0 µL of PCR-grade water, 5.0 µL master mix, and 0.5 µL assay were run in triplicate on a 384-well plate. Cycling parameters were 50°C for 2 min for degradation of any qPCR product contamination, 90°C for 20 s for polymerase activation, and 45 cycles of 95°C for 1 s and 60°C for 20 s for target amplification. Fluorescent data was collected at the end of each of each cycle. Efficiency was estimated by plotting the dilution number (5, 4, 3…with 5 being the most concentrated) by the Ct and fitting a linear regression to the data. The slope of the line was then used to estimate the efficiency using an online calculator (Agilent Genomics: Tools – Bio Calculators) which used the equation: Efficiency = $-1+10^{(-1/\text{slope})}$. For all qPCR assays, iNOS and L13A amplification efficiency of cDNA pools were at least 92% and roughly equivalent between the two genes, two important prerequisites for relative qPCR analysis. The comparative delta delta Ct method (ΔΔCt) was used for data analysis (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Any Ct value in a triplicate that differed by 0.5 or more from the other two was removed from analysis. The validity of L13A as a reference gene was verified using a 2 x 2 ANOVA to show that L13A expression did not differ in any way by group ($F_{(3,23)} = .899$, $p = .457$). The first normalization (ΔCt) was to the reference gene, L13A, while the second normalization was to an average of iNOS ΔCt for the experiment, since the experimental design did not have a single control group. ΔΔCt values were linearly transformed into $2^{-\Delta\Delta Ct}$ values for graphical representation.

Protein Extraction & iNOS ELISA

Spleen tissue was thawed and homogenized on ice in sterile, glass, Dounce grinders. Cells were lysed using two freeze-thaw cycles, where the protein was released into supernatant (protease inhibitor buffer). The homogenate was then centrifuged and the supernatant containing

isolated protein collected. Total protein was quantified by Bradford Assay as previously described (Lebonville et al., 2016). To quantify iNOS protein, 38 µg of total protein from each sample was run in triplicate in a rat iNOS sandwich ELISA (Cat #: abx256135, Abbexa Ltd., Cambridge, UK) following the manufacturer's protocol. An automatic plate washer (EL 403, BioTek Instruments Inc., Winooski, VT, USA) was used to uniformly wash the plate. Absorbance of the finished ELISA was read at 450 nm. Technical replicates greater than 2 standard deviations from the triplicate mean were removed from analysis. The amount of iNOS protein per sample was determined from the 4-parameter logistic curve fit to a dilution series of the included standard. Quantity of iNOS protein is reported in pg per 38 µg total protein.

Statistical Analysis

A 2 x 2 analysis of variance (ANOVA) was performed on all data sets using statistical software (SPSS Statistics 24 and 25, IBM, Armonk, NY, USA). For all tests, the level of significance was set to $p \leq .05$. The validity of using an ANOVA with the current data was tested using Shapiro-Wilk Test for normality and Levene's Test for homogeneity of variance (HOV). Two violations of these assumptions are reported in the results, but all other assumptions were supported. Planned contrasts were performed with the CS and HC groups within each drug treatment (vehicle or CNO) to test for a conditioned effect with exposure to the CS. Any ancillary effects were probed using Tukey's Honestly Significant Difference (HSD) post-hoc test. The presence of statistical outliers was probed using Grubb's test. Statistical outliers were removed from final analysis. For RT-qPCR data, statistical analysis was performed with ΔΔCt values (without linear transformation) because these values tend to better meet the assumptions of an ANOVA in our experience.

Results

This experiment tested the effect of DREADD-mediated inhibition of ventral hippocampal (vHpc) outputs on the expression of heroin contextually conditioned suppression of NO. The experimental design is displayed in **Figure 2**. Virally-transduced cells, as determined by mCherry staining, were present in multiple areas of the ventral/intermediate hippocampus (DG, CA1, CA3, and Sub) and adjacent cortex (**Figure 2B**). The most intense and consistent staining was present in vSub and vCA1 subregions. Intense staining was also seen in the adjacent cortical and cortical-like regions which included dorsolateral entorhinal cortex, amygdalopiriform transition area, and amygdalohippocampal area. More caudally, staining was also seen in the intermediate entorhinal cortex, medial entorhinal cortex, parasubiculum and

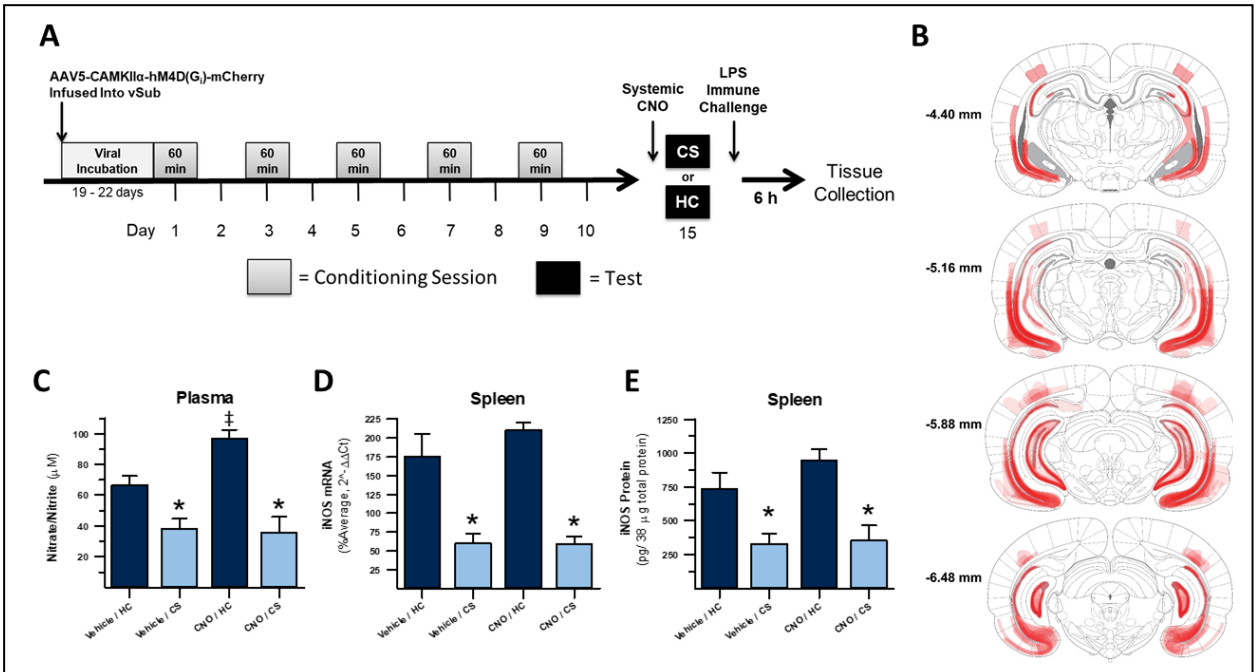

Figure 2. Inactivation of vHpc output regions, including vSub and vCA1, does not disrupt expression of heroin contextually conditioned suppression of nitric oxide. Experimental timeline **(A)**. DREADD (mCherry) expression and spread **(B)**. Effect of CNO administration on expression of heroin contextually conditioned suppression of plasma nitrate/nitrite concentration **(C)** and splenic iNOS mRNA **(D)** and protein **(E)** expression. CS = conditioned stimulus or heroin-paired context, HC = home cage; *, statistically significant difference relative to respective HC control. ‡, statistically significant difference relative to Vehicle/HC group.

presubiculum. Nine subjects were dropped from all analyses due to non-bilateral virus expression in vSub. One additional subject was dropped from nitrate/nitrite analysis due to being identified as a statistical outlier (final n = 6-8). Two additional subjects were dropped from iNOS qPCR analysis due to being identified as statistical outliers final (n = 5-8). No additional subjects were dropped from iNOS ELISA analysis (final n = 6-8).

Two ANOVA assumption violations were seen in this experiment. Specifically, iNOS qPCR data violated the assumption of HOV ($p = .014$) and iNOS ELISA data violated the assumption of normality for the vehicle-treated CS-exposed group only ($p = .015$). ANOVA are considered robust to violations in these assumptions, and the results are not likely to be due to these violations as the nitrate/nitrite data which meets these assumptions also support our conclusions from this data.

Plasma nitrate/nitrite concentration during the test for the expression of heroin contextually conditioned suppression of NO following chemogenetic inhibition of vHpc outputs is shown in **Figure 2C**. A 2 x 2 ANOVA revealed significant differences in plasma nitrate/nitrite concentration between treatment groups ($F_{(3,24)} = 16.091$, $p < .001$). There was a significant interaction between exposure regimen (CS or HC) and injection (CNO or Veh) on plasma nitrate/nitrite ($F_{(1,24)} = 5.361$, $p = .029$). There was also a significant main effect of exposure ($F_{(1,24)} = 38.893$, $p < .001$) but a marginally non-significant main effect of injection ($F_{(1,24)} = 3.839$, $p = .062$) on this measure. In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less nitrate/nitrite in response to LPS than animals that remained in their home cage ($p = .008$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, confirming expression of the conditioned effect in vehicle-treated animals. In CNO-treated animals, a planned comparison

also revealed that CS-exposed animals demonstrated significantly less nitrate/nitrite in response to LPS than animals that remained in their home cage ($p < .001$). This result indicates that in CNO-treated animals, there was still significant conditioned suppression of NO. Follow-up post-hoc analysis showed that CNO-treated CS-exposed animals did not differ in nitrate/nitrite concentration compared to vehicle-treated CS-exposed animals ($p = .994$) which does not indicate any attenuation of conditioned suppression of nitrate/nitrite when CNO treatment precedes CS exposure, unlike with Chapter 2 results for this measure. The interaction seems to be driven by a significant difference in nitrate/nitrite concentration in the two HC groups. In HC-exposed animals, CNO-treated animals exhibited significantly higher nitrate/nitrite than vehicle-treated animals. The implications of these results are discussed later.

Splenic iNOS mRNA expression during the test for the expression of heroin contextually conditioned suppression of NO is shown in **Figure 2D**. A 2 x 2 ANOVA revealed significant differences in iNOS mRNA expression between treatment groups ($F_{(3,23)} = 9.556$, $p < .001$). There was a significant main effect of exposure (CS or HC; $F_{(1,23)} = 26.310$, $p < .001$) but no significant main effect of injection (CNO or Veh; $F_{(1,23)} = 1.143$, $p = .296$) or interaction between exposure and injection ($F_{(1,23)} = .036$, $p = .851$). In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less iNOS mRNA expression in response to LPS than animals that remained in their home cage ($p = .001$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, confirming expression of the conditioned effect in vehicle-treated animals. In CNO-treated animals, a planned comparison revealed that CS-exposed animals also showed less iNOS mRNA expression in response to LPS compared to animals that remained in their home cage ($p < .001$). Furthermore, post-hoc analysis showed that CNO-treated CS-exposed animals showed similar

iNOS mRNA expression compared to vehicle-treated CS-exposed animals (p = .929). These results indicate that there was still significant conditioned suppression of NO when vHpc output inhibition preceded CS exposure.

Splenic iNOS protein expression during the test for the expression of heroin contextually conditioned suppression of NO is shown in **Figure 2E**. A 2 x 2 ANOVA revealed significant differences in iNOS protein expression between treatment groups ($F_{(3,25)} = 8.711$, $p < .001$). There was a significant main effect of exposure (CS or HC; $F_{(1,25)} = 24.059$, $p < .001$) but no significant main effect of injection (CNO or Veh; $F_{(1,23)} = 1.339$, $p = .258$) or interaction between exposure and injection ($F_{(1,25)} = .845$, $p = .367$). In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less iNOS protein expression in response to LPS than animals that remained in their home cage ($p = .006$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, confirming expression of the conditioned effect in vehicle-treated animals. In CNO-treated animals, a planned comparison revealed that CS-exposed animals showed similar iNOS protein expression in response to LPS compared to animals that remained in their home cage ($p = .001$). Furthermore, post-hoc analysis showed that CNO-treated CS-exposed animals showed similar iNOS mRNA expression compared to vehicle-treated CS-exposed animals ($p = .998$). These results indicate that there was still significant conditioned suppression of NO when vHpc output inhibition preceded CS exposure.

Overall, the results from this chapter's experiment support the conclusion that chemogenetic inhibition of vHpc outputs results in neither partial nor full attenuation of conditioned suppression of measures of NO production after exposure to a heroin-paired context.

Discussion

In addiction, context reliably prompts drug taking and seeking behavior. Context can also cause other changes in behavior perhaps unrelated to the rewarding aspects of drug use. With heroin, a potent modulator of immune function, immunomodulatory effects can be recapitulated through exposure to a heroin-paired context. Our laboratory has repeatedly shown that these conditioned immune responses to a heroin-paired context require the dorsal hippocampus (dHpc). However, nothing is known about the contribution of the ventral hippocampus (vHpc) to the expression of this effect. This question is important to investigate because of overwhelming evidence that the dHpc and vHpc are functionally distinct.

Research has shown that the vHpc, especially the ventral subiculum (vSub), is involved in context-induced reinstatement to drug seeking behavior. The study described in this chapter represents the first step to understanding vHpc processing during other modes of conditioned drug behaviors. Chemogenetic inhibition of ventral subiculum (vSub) and ventral CA1 (vCA1), two of the main output regions of the vHpc, had no effect on the expression of heroin contextually conditioned immune suppression of measures of nitric oxide (NO) production in response to LPS. This experiment is the first to manipulate vHpc neurons in this paradigm and provides the first empirical evidence that only the dHpc within the hippocampus proper is required for immune modulation by a heroin-paired context.

Stimulating the vSub alone is enough to both increase dopamine release in the nucleus accumbens and increase reinstatement of drug-seeking behavior with cocaine (Vorel et al., 2001) and d-amphetamine (Taepavarapruk and Phillips, 2003). It is tempting to conclude from this evidence that stimulation of the vSub “reactivates” a specific drug-context association which results in engagement in drug-seeking behavior relevant to that context. However, the specific

ensemble encoding of contexts makes it unlikely that a simple stimulation of the vSub conveys associations of a specific context to the reward circuitry. It is much more likely that vSub stimulation is related to creating a general arousal or affective state that encourages such behaviors (Taepavarapruk and Phillips, 2003; Vorel et al., 2001). Support for this context-independent role of the vSub comes from evidence that the vSub is also required for drug- and discrete cue-induced reinstatement of drug seeking (Sun, 2003). It seems that, while theoretically capable of encoding spatial context due to the presence of place cells, the vHpc may be more involved in processing the context of an emotional arousal state, rather than spatial context (see Chapter 5: General Discussion).

In fact, most studies that are known to rely on the vHpc have an emotional component to them (e.g. fear conditioning). Drugs of abuse certainly have emotional components (reward, stress) that might additionally engage the vHpc and cause it to be required when recalling a previously emotionally aroused state. It is unclear what the emotional component of our paradigm might be and whether this emotional component would be then required for conditioned immune modulation. In fact, recent evidence suggests that the conditioned rewarding and immune aspects with heroin, and perhaps with other drugs of abuse, are dissociable in mechanism. For example, in the dHpc, either antagonism of the proinflammatory cytokine interleukin-1 or activation of G_i signaling in astrocytes disrupted the expression of heroin contextually conditioned immune modulation, but neither manipulation had any effect on the expression of heroin conditioned place preference, a measure of reward conditioning to a context. If the neural mechanisms governing expression of heroin's conditioned reward/motivational effects and the conditioned immune modulating effects are dissociable even

within the dHpc, it does not seem far-fetched that the dHpc and vHpc play a different role in each type of conditioning.

The lack of an effect with chemogenetic inhibition of the vHpc is still somewhat surprising in light of the extent of virus spread in this experiment. Virus expression extended dorsally into the intermediate hippocampus – which could have functions that more closely resemble those of the dHpc, and yet even still, no effect on conditioned immune modulation to a context was detected. Oddly, there seemed to be a significant effect of CNO in the home cage control group, but not in the CS-exposed group. It is possible that inactivating the vHpc alone had some influence on the subsequent immune response to LPS. There is some support for vHpc modulation of immune function. Lesions of the vHpc have been shown to influence immunity by decreasing antibody titers in response to an antigen (Devi et al., 2004), increasing splenocyte proliferation (Devi and Namasivayam, 1990), and increasing leucocyte migration (Devi and Namasivayam, 1991). It is unknown whether vHpc manipulation affects NO production and why an increase would not have also been seen in CS-exposed animals. To address the latter point, perhaps contextually conditioned immune suppression of NO was strong enough to overcome modest increases due to vHpc inhibition. Future experiments should look at the mechanism of changes in immunity due to manipulation of the vHpc and see if previously described effects on immunity extend to NO production.

In summary, the present results indicate that the vHpc is not required for the expression of heroin contextually conditioned immune modulation and point to a specific role of the dHpc in this learning paradigm.

CHAPTER 4: A PROJECTION FROM THE DORSAL HIPPOCAMPUS TO THE RETROSPLENIAL CORTEX IS NOT REQUIRED FOR THE EXPRESSION OF HEROIN'S CONTEXTUALLY CONDITIONED IMMUNE EFFECTS

Introduction

Experiments from Chapters 2 and 3 indicate that the dorsal hippocampus (dHpc), but not the ventral hippocampus (vHpc) plays an important role in the ability of a heroin-paired context to modulate immune function. In particular, these studies point to a role of two main outgoing projections from the dHpc, dorsal subiculum (dSub) and dorsal CA1 (dCA1). The overarching hypothesis of this thesis is that the hippocampus encodes context within the heroin contextually conditioned immune modulation paradigm. If this hypothesis is correct, then likely the hippocampus must convey current contextual information to other brain regions in order to elicit conditioned immune modulation (see Chapter 1 for more on these underlying hypotheses). One benefit of using chemogenetic manipulations of cellular populations is that you can get additional information on where these cells project to from axonal trafficking of the fluorescent reporter proteins (Smith et al., 2016). The Chapter 2 experiment using a G_i-coupled DREADD expressed within dSub and dCA1, allowed us to identify several potentially important projection targets of these regions.

The dHpc does not have any direct connections to other brain regions known to be required for expression of heroin contextually conditioned immune modulation (e.g. BLA, NAc). However, one of its projection targets, the retrosplenial cortex (RSC), is implicated in contextual learning and memory. The RSC is activated by tasks that require contextual information or contextual associations and may be involved in transforming a particular contextual experience

into a general representation of that context (Bucci and Robinson, 2014; Kveraga et al., 2011; Todd et al., 2017). In fact, like the dHpc, the RSC possesses “place cells” that encode locations within a context and that might support ensemble encoding of context in a similar way (Mao et al., 2017). The dHpc has reciprocal connections with the RSC and it is possible that this connection supports the hippocampus’ processing of context and context associations or even acts as a relay for contextual reactivation of relevant memories (Mao et al., 2017). Lesions of the RSC impair the expression of contextual fear, but have no effect on fear to a discrete cue (Keene and Bucci, 2008a, b) . Furthermore, disruption of glutamatergic signaling within the RSC also disrupts recall of contextual fear (Corcoran et al., 2011).

Surprisingly, no work was found that looked at the role of the RSC in drug contextually conditioned behaviors. In fact, only a few studies to date have made a connection at all between drugs of abuse and the RSC. One study identified the RSC as a region that shows enhanced activity during morphine withdrawal in rats as measured by manganese-enhanced magnetic resonance imaging (Niu et al., 2017). Their investigation sought to answer which brain regions might contribute to the cognitive deficits seen during states of morphine withdrawal. Their results indicate that within the RSC, enhanced activity was particularly elevated in a specific vertical band in retrosplenial cortex, granular area b/c (RSCgb). In the Chapter 2 experiment using designer receptors exclusively activated by designer drugs (DREADDs) for inhibition of dHpc outputs, this same specific band of RSC was also intensely and consistently labeled with mCherry in a manner that indicates that this band receives strong projections from the dHpc output regions manipulated. This connection and its specific topography is also supported anatomically through several retrograde tracing studies (Van Groen and Wyss, 2003; Wyss and Van Groen, 1992).

Due to strong theoretical, anatomical, and technical relevance to the current dissertation, the experiment in this chapter sought to inhibit the specific projection from the dHpc to RSCgb using chemogenetics. As in Chapter 2, an inhibitory DREADD was infused into the dSub where it expressed in both dSub and dCA1, the main output regions of dHpc. However, instead of administering a systemic injection of the DREADD agonist clozapine-*N*-oxide (CNO), CNO was delivered directly to dHpc projection terminals in the RSCgb. The hypothesis that the dHpc to RSCgb projection is specifically required for the expression of heroin contextually conditioned immune modulation was tested by inhibiting this projection just before re-exposure to a heroin-paired context.

Materials & Methods

Animals

Adult, male Lewis rats weighing initially 225-250 g (N = 46) were purchased from Charles River Laboratories (Kingston, NY, USA). Rats were housed individually on a reversed, 12-h light-dark cycle and all experimental procedures took place during the animals' active dark period (7 am – 7 pm). Food and water were provided *ad libitum* in home cages and animals were handled regularly. All experimental procedures were conducted in accordance with federal guidelines and with approval from the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC).

Drugs and Delivery

Heroin (diacetylmorphine hydrochloride) was procured from the National Institute on Drug Abuse (NIDA) drug supply program, dissolved in sterile 0.9% saline to produce a 1.0 mg/mL solution, and stored at 4°C. During each conditioning session, rats were administered 1.0 mg/kg heroin subcutaneously. This dosage was based on our experiments showing that it reliably

alters measures of nitric oxide (NO) in spleen and blood plasma following endotoxin immune challenge (Lysle and How, 2000; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Lipopolysaccharide (LPS, derived from *E. coli*, serotype O55:B5, Cat# L2880, MilliporeSigma, St. Louis, MO, USA) was dissolved in sterile 0.9% saline the day before use to produce a 1 mg/mL solution, which was then stored at 4°C. Following the test session, LPS was injected subcutaneously at a dose of 1.0 mg/kg which produces sickness behavior and production of NO measures. We have used this particular LPS serotype to previously investigate heroin-and conditioning-induced changes in immune response. Replications of these experiments should employ the same serotype, if possible, as activity between serotypes can vary (Caroff et al., 2002). The synthetic DREADD agonist clozapine-*N*-oxide (CNO, NOCD-135, NIDA Drug Supply Program, Bethesda, MD, USA) was prepared as a solution on the same day as it was used while also protecting this light-sensitive reagent from light. CNO was dissolved in sterile 0.9% saline to a final concentration of 1 mM (FW 342.82 g/mol). All injected reagents stored at 4°C were allowed to come to room temperature just before use.

Surgical Procedure

To deliver a designer receptors exclusively activated by designer drugs (DREADD) containing virus *in vivo* to the dorsal hippocampus (dHpc) output regions, rats underwent intracranial surgery. Rats were anesthetized with 1.0 mL/kg of a 9:1 (vol:vol) mixture of ketamine hydrochloride (100 mg/mL) and xylazine (100 mg/mL) injected intraperitoneally. Head-shaven animals were placed in a stereotaxic apparatus, the surgical site sterilized, and an incision was made in the skin covering the skull. Holes were drilled into the exposed skull at bilateral coordinates for dorsal subiculum (dSub). Coordinates relative to bregma for dSub were AP -6.0 mm, ML ±2.8 mm, DV -3.5 mm (Paxinos and Watson, 2007). Injectors (33 gauge,

Plastics One, Roanoke, VA, USA) containing virus and connected with tubing to syringes and a microinfusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) were lowered at a 0° lateral angle to the appropriate DV coordinate but were raised 0.1 mm DV to create a pocket before virus infusion. The virus was infused at the received titer of $4.4x10^{12}$ GC/mL, volume of 0.7 µL per hemisphere, and a rate of 0.05 µL/min. Injectors were then left in place for 10 min to allow for diffusion away from the injection site. Injectors were raised slowly (over 1-2 min) to limit spread of the virus up the injection tract. All animals received DREADD virus. Then, in the same surgical session, guide cannula (26-gauge, Plastics One, Roanoke, VA) aimed at retrosplenial cortex, granular areas b/c (RSCgb) were implanted. Coordinates relative to bregma for RSC was AP -3.4 mm, ML ±1.3 mm, DV -0.8 mm, with a 20° lateral angle (Paxinos and Watson, 2007). Cannula were secured to the skull with cranial screws, cyanoacrylate adhesive, and dental acrylic. Dummy injectors with no projection were inserted in the cannula to keep the cannula viable. Animals were given at least two weeks to recover prior to undergoing conditioning.

DREADD Virus and Incubation

DREADDs are delivered *in vivo* using viruses. In these experiments, the DREADD plasmid CAMKIIα-hM4D(G_i)-mCherry, a gift from Bryan Roth, was purchased pre-packaged in an adeno-associated virus serotype 5 (AAV5) from Addgene (Viral prep 50477-AAV5; http://n2t.net/addgene:50477; RRID:Addgene_50477, Cambridge, MA, USA). This virus and delivery was identical to that used in the experiments in Chapters 2 and 3. The mCherry reporter element produced fluorescent signal in transduced cells that allowed for localization of DREADD-expressing cells postmortem. Following virus delivery, the virus was allowed to incubate to promote DREADD expression for at least two weeks prior to the initiation of any

training. By the time animals were treated with CNO, total incubation time was at least four weeks.

Conditioning & Testing Procedure

All rats in these studies were Pavlovian conditioned using five, 60-min sessions every 48 hours where they received an injection of heroin, the unconditioned stimulus (US), and were immediately placed in a distinct context, the conditioned stimulus (CS). This training regimen has repeatedly produced a conditioned immunomodulatory response to the heroin-paired context alone in our laboratory (Lebonville et al., 2016; Paniccia et al., 2018; Szczytkowski et al., 2011; Szczytkowski et al., 2013; Szczytkowski and Lysle, 2010). The CS was a standard operant chamber (BRS/LVE, Laurel, MD, USA; W 30.5 cm x H 26.7 cm x D 24.1 cm) that was enclosed by a sound and light attenuating outer chamber (W 50.8 cm x H 36.8 cm x D 34.3 cm). To distinguish these chambers from any home cage stimuli, the conditioning chambers were housed in a separate room from the vivarium and contained distinct auditory (noise-masking house fan), tactile (metal footshock bar floor), visual (metal side walls), and olfactory (cedar bedding) cues. Between animals, the chambers were thoroughly cleaned with Roccal-D Plus (Zoetis, Kalamazoo, MI).

Six days after the final conditioning session (day 15), animals were tested for the expression of a conditioned immune response by being re-exposed to the CS (conditioning chamber) for 60 min. Approximately 5 minutes before CS re-exposure, animals received either an intracranial microinfusion of CNO (experimental) or vehicle (control) through bilateral cannula aimed at the RSCgb. CNO and vehicle were infused at a volume of 0.5 µL and rate of 0.25 µL/min. Following the infusion, injectors were allowed to sit for 1 min to allow for diffusion away from the injection site. As a behavioral control representing a typical immune

response to LPS, another group of animals remained in home cage instead of being re-exposed to the CS before LPS challenge. Prior work in our laboratory has demonstrated that the LPS response of these heroin-conditioned home-cage control animals are not different than unmanipulated animals, saline conditioned controls, or animals that received heroin and CS-exposure in an unpaired manner (Lysle and Ijames, 2002). Collectively, these results indicate not only that immunomodulation to a heroin-paired context is a conditioned response to the CS and not ancillary effects of conditioning procedures or heroin dosing, but also that the use of only one of these equivalent control groups in future experiments is valid. We believe the heroin-conditioned home-cage control to be the most important and thus it is used here.

Blood and Spleen Tissue Collection

Immediately after CS (or home cage control) exposure, all animals received an LPS immune challenge and were sacrificed by cervical dislocation without anesthesia 6 h later for brain, blood, and spleen collection. This time point is optimized to detect measures of NO production in spleen and blood plasma. Blood was collected in heparinized syringes, transferred to tubes, and spun at 2000 g and 4°C for 20 min. Plasma was collected and stored at -80°C. Spleen tissue has shown robust expression of iNOS in response to LPS in multiple immune cell types (Bandaletova et al., 1993) and has reliably demonstrated opioid-conditioned immunomodulation in our studies (Lebonville et al., 2016; Lysle and Ijames, 2002; Szczytkowski and Lysle, 2007). Spleen tissue was dissected out postmortem and cut into approximately 100 mg pieces for RT-qPCR and ELISA. For RT-qPCR analysis, tissue was stored in RNA*later* (ThermoFisher Scientific, Waltham, MA, USA) for 4° for about a month and then at -80°C. For ELISA analysis, spleen tissue was stored in protease inhibitor buffer (Pierce™, ThermoFisher Scientific) at -80°C.

Brain Histology and Injector/DREADD Expression Analysis

Whole brains were extracted and post-fixed in 10% formalin at 4°C for 48 hours with agitation. Then brains were cryoprotected at 4°C in 30% sucrose containing 0.1% sodium azide at least until sunk. Brains were next embedded in frozen section compound (VWR, Radnor, PA, USA), frozen in a -23 to -25°C freezing microtome, covered with aluminum foil, and stored at -80°C. Brains were allowed to warm to -20 to -25°C before being sliced into 40 µm coronal sections on a cryostat (Leica CM 3050 S, Leica Microsystems, Buffalo Grove, IL, USA). Desired sections were slide mounted onto charged glass slides (FisherBrand Superfrost, ThermoFisher Scientific, Waltham, MA, USA), and allowed to air dry under dark conditions. A subset of slides were coverslip mounted using HardSet VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescent microscopy (Leica DM6000 B widefield light microscope, Leica Microsystems, Buffalo Grove, IL, USA) was used to verify positive bilateral DREADD expression in the dorsal subiculum (dSub) though localization of the mCherry fluorescent tag. Any animals that did not show bilateral mCherry expression in dSub were removed from all subsequent analyses. Injectors directed at the rostral retrosplenial cortex, granular areas b/c (RSCgb) was also verified and any animal that did not show correct bilateral placement in this area was dropped from analysis. Finally, if dHpc projections in the RSCgb were not visible at the site of the injector, the animal was removed from analysis. Thus, remaining animals in the analysis show both bilateral dSub DREADD expression and proper cannula placement directed at the RSCgb at the site of dHpc projections.

Nitrate/nitrite Assay

The byproducts of NO degradation in plasma, nitrate and nitrite, were measured using a Greiss reagent assay as previously described (Szczytkowski and Lysle, 2007). Recovery of

nitrate is greater than 95% using this assay. Briefly, in a 96-well plate in triplicate, 12 µL plasma, 38 µL dH_2O, 10 µL nitrate reductase (1.0 U/mL), 20 µL of 0.31 M phosphate buffer (pH 7.5), 10 µL 0.86 mM NADPH, and 10 µL 0.11 mM FAD were incubated in the dark at room temperature for 90 min. Next, 200 µL of Greiss reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride in dH_2O) was added and color allowed to develop for 10 min at room temperature. Bubbles were popped using a syringe needle and the bottom of the plate was wiped clean before measuring absorbance at 550 nm in a spectrophotometer. Total nitrate/nitrite concentration was determined from a concurrently run known standard dilution series with a 4-parameter logistic curve fit.

RNA Extraction & RT-qPCR

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was performed on spleen samples to measure iNOS messenger ribonucleic acid (mRNA) expression. First, spleen tissue free of residual RNA*later* was homogenized in 1 mL TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) using a bead mill homogenizer (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France) and the following parameters: 7500 rpm, 30 s, 30 s pause, 6 cycles, cooling samples on ice between every three cycles. RNA was purified using TRI-Reagent's manufacturer protocols with the following modifications: performed optional homogenization step to remove debris, added 100 µL RNAse Free H_2O in conjunction with 100 µL of BCP to reduce the density of the homogenate and aid phase separation in PhaseLock Gel Tubes (Heavy formulation, 5Prime/Quantabio, Beverly, MA, USA), and conducted three RNA pellet washes with 75% ethanol. The purified RNA pellet was dissolved by 55-60°C incubation in 150 µL of RNase Free H_2O.

RNA purity and concentration were assessed by spectrophotometry (Take3 microdot plate and the Epoch™ spectrophotometer, BioTek Instruments Inc., Winooski, VT). RNA was diluted 1:20 in 1xTE (pH = 7.5) for spectrophotometric readings. A260 nm/280 nm ratios were used to assess RNA purity. A260 nm values were used to determine RNA concentration. RNA quality was assessed using the RNA 6000 Nano kit on a 2100 Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). A minimum RNA integrity number (RIN) of 8.0 was considered indicative of high quality, intact RNA. All samples demonstrated A260 nm/280 nm values close to 2.1, indicating high purity.

cDNA synthesis was performed on a Veriti 96 Well Fast Thermal Cycler (Applied Biosystems, ThermoFisher Scientific) using the Advantage RT-for-PCR Kit, according to the manufacturer protocol (Clontech/Takara Bio, Mountain View, CA, USA). Priming for the RT reaction was carried out using Oligo(dT) primers. Input RNA concentration was equalized across samples (1 µg). Undiluted cDNA from each sample was pooled into a single sample of which five serial 1:10 dilutions were made to evaluate qPCR efficiency. Remaining cDNA from each sample was then diluted 1:5 in PCR-grade H_2O.

qPCR was run on a QuantStudio™ 6 Flex system (AP Biosystems, ThermoFisher Scientific) using TaqMan™ Fast Advanced Master Mix (AP Biosystems) and predesigned fluorescein (FAM) assays for iNOS (NOS2, gene of interest, Assay ID: Rn00561646_m1, ThermoFisher Scientific) and L13A (Rpl13a, reference gene, Assay ID: Rn01475911_g1, ThermoFisher Scientific). Individual reactions containing 1.5 µL cDNA template, 3.0 µL of PCR-grade water, 5.0 µL master mix, and 0.5 µL assay were run in triplicate on a 384-well plate. Cycling parameters were 50°C for 2 min for degradation of any qPCR product contamination, 90°C for 20 s for polymerase activation, and 45 cycles of 95°C for 1 s and 60°C for 20 s for

target amplification. Fluorescent data was collected at the end of each of each cycle. Efficiency was estimated by plotting the dilution number (5, 4, 3…with 5 being the most concentrated) by the Ct and fitting a linear regression to the data. The slope of the line was then used to estimate the efficiency using an online calculator (Agilent Genomics: Tools – Bio Calculators) which used the equation: Efficiency = $-1+10^{(-1/slope)}$. For all qPCR assays, iNOS and L13A amplification efficiency of cDNA pools were at least 92% and roughly equivalent between the two genes, two important prerequisites for relative qPCR analysis. The comparative delta delta Ct method (ΔΔCt) was used for data analysis (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Any Ct value in a triplicate that differed by 0.5 or more from the other two was removed from analysis. The validity of L13A as a reference gene was verified using a 2 x 2 ANOVA to show that L13A expression did not differ in any way by group ($F_{(3,24)} = .836$, $p = .487$). The first normalization (ΔCt) was to the reference gene, L13A, while the second normalization was to an average of iNOS ΔCt for the experiment, since the experimental design did not have a single control group. ΔΔCt values were linearly transformed into $2^{-\Delta\Delta Ct}$ values for graphical representation.

Protein Extraction & iNOS ELISA

Spleen tissue was thawed and homogenized on ice in sterile, glass, Dounce grinders. Cells were lysed using two freeze-thaw cycles, where the protein was released into supernatant (protease inhibitor buffer). The homogenate was then centrifuged and the supernatant containing isolated protein collected. Total protein was quantified by Bradford Assay as previously described (Lebonville et al., 2016). To quantify iNOS protein, 38 µg of total protein from each sample was run in triplicate in a rat iNOS sandwich ELISA (Cat #: abx256135, Abbexa Ltd., Cambridge, UK) following the manufacturer's protocol. An automatic plate washer (EL 403,

BioTek Instruments Inc., Winooski, VT, USA) was used to uniformly wash the plate. Absorbance of the finished ELISA was read at 450 nm. Technical replicates greater than 2 standard deviations from the triplicate mean were removed from analysis. The amount of iNOS protein per sample was determined from the 4-parameter logistic curve fit to a dilution series of the included standard. Quantity of iNOS protein is reported in pg per 38 µg total protein.

Statistical Analysis

A 2 x 2 analysis of variance (ANOVA) was performed on all data sets using statistical software (SPSS Statistics 24 and 25, IBM, Armonk, NY, USA). For all tests, the level of significance was set to $p \leq .05$. The validity of using an ANOVA with the current data was tested using Shapiro-Wilk Test for normality and Levene's Test for homogeneity of variance (HOV). Violations of these assumptions for this experimental data is reported in the results. Planned contrasts were performed with the CS and HC groups within each drug treatment (vehicle or CNO) to test for a conditioned effect with exposure to the CS. Any ancillary effects were probed using Tukey's Honestly Significant Difference (HSD) post-hoc test. The presence of statistical outliers was probed using Grubb's test. Statistical outliers were removed from final analysis. For RT-qPCR data, statistical analysis was performed with ΔΔCt values (without linear transformation) because these values tend to better meet the assumptions of an ANOVA in our experience.

Results

This experiment tested the effect of DREADD-mediated inhibition of dorsal hippocampal (dHpc) projections to the retrosplenial cortex, granular areas b/c (RSCgb) on the expression of heroin contextually conditioned suppression of NO. The experimental design is displayed in **Figure 3A**. Virally-transduced cells, as determined by mCherry staining, were present in

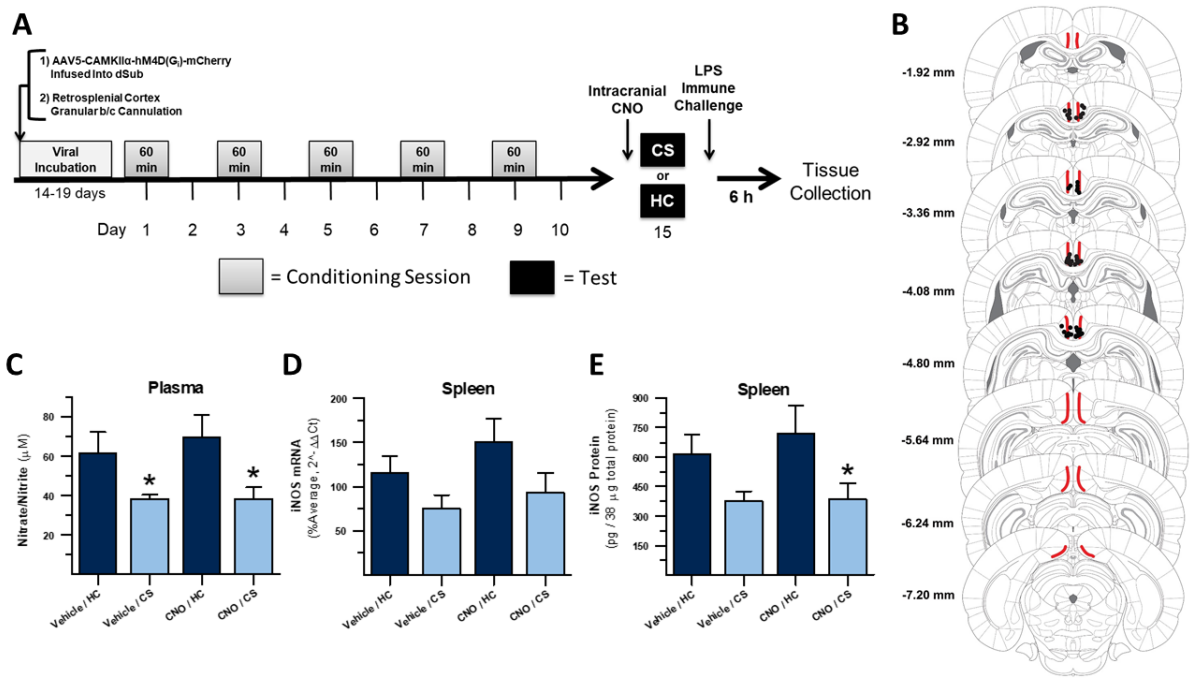

Figure 3. Inactivation of dHpc projections to RSCgc does not disrupt expression of heroincontextually conditioned suppression of NO. Experimental timeline **(A)**. Representative DREADD (mCherry) projection expression. **(B)**. Effect of CNO administration on expression of heroin contextually conditioned suppression of plasma nitrate/nitrite concentration **(C)** and splenic iNOS mRNA **(D)** and protein **(E)** expression. CS = conditioned stimulus or heroin-paired context, HC = home cage; *, statistically significant difference relative to respective HC control.

multiple areas, but most prominently in the dSub and dCA1 (see Chapter 2 for a more thorough description of viral expression using this construct and delivery method).

Ten subjects were dropped from all analyses due to non-bilateral virus expression, improper injector placement outside the RSCgb, or injector placement at a region that did not show mCherry labeled projection fibers (final n = 6-8). One additional subject was dropped from nitrate/nitrite analysis due to a technical problem in collecting plasma. No additional subjects were dropped from iNOS qPCR or ELISA analysis (final n = 6-8).

One ANOVA assumption violation was seen in this experiment. Specifically, nitrate/nitrite concentration data violated the assumption of HOV (p = .007). ANOVA are considered robust to violations in these assumptions, and the results are not likely to be due to

these violations as the qPCR and ELISA data which meets these assumptions also support our conclusions from this data.

Plasma nitrate/nitrite concentration during the test for expression of heroin contextually conditioned suppression of NO following chemogenetic inhibition of dHpc to RSCgb projections is shown in **Figure 3C**. A 2 x 2 ANOVA revealed significant differences in plasma nitrate/nitrite concentration between treatment groups ($F_{(3,23)} = 4.167$, $p = .017$). There was no significant interaction between exposure regimen (CS or HC) and injection (CNO or Veh) on plasma nitrate/nitrite ($F_{(1,23)} = .266$, $p = .611$). There was a significant main effect of exposure ($F_{(1,23)} = 11.578$, $p = .002$) but no significant main effect of injection ($F_{(1,23)} = .254$, $p = .619$) on this measure. In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less nitrate/nitrite levels in response to LPS than animals that remained in their home cage ($p = .05$). This comparison shows that exposure to the CS dampened the production of NO to LPS stimulation, indicative of expression of the conditioned effect in vehicle-treated animals. In CNO-treated animals, a planned comparison revealed that CS-exposed animals demonstrated significantly less nitrate/nitrite levels in response to LPS than animals that remained in their home cage ($p = .012$). This result indicates that in CNO-treated animals, there was still significant conditioned suppression of NO. Follow-up post-hoc analysis showed that CNO-treated CS-exposed animals did not differ in nitrate/nitrite concentration compared to vehicle-treated CS-exposed animals ($p = 1.000$) which does not indicate any attenuation of conditioned suppression of nitrate/nitrite when CNO treatment precedes CS exposure.

Splenic iNOS mRNA expression during the test for the expression of heroin contextually conditioned suppression of NO is shown in **Figure 3D**. A 2 x 2 ANOVA did not reveal

significant differences in iNOS mRNA expression between treatment groups ($F_{(3,24)} = 2.393$, $p = .093$). However, there was a significant main effect of exposure (CS or HC; $F_{(1,24)} = 6.383$, p .019) and no significant main effect of injection (CNO or Veh; $F_{(1,24)} = .477$, $p = .496$) or interaction between exposure and injection ($F_{(1,24)} = .042$, $p = .839$). In both vehicle- and CNO-treated animals, planned comparisons revealed that CS-exposed animals demonstrated non-significantly less iNOS mRNA expression in response to LPS than animals that remained in their home cage (Veh, $p = .116$; CNO, $p = .064$). Taking into account the strong main effect of CS exposure and visual inspection of the data, there is no indication that CNO treatment attenuated iNOS mRNA expression. We believe that there was conditioned suppression of iNOS mRNA in both vehicle and CNO-treated CS-exposed animals, but that there was not quite enough power for this to come out as statistically significant due to the fact that too many animals had to be dropped due to the rigorous standards of virus and injection placement. These results seem to indicate that there was still conditioned suppression of NO when inhibition of dHpc output to RSCgb preceded CS exposure.

Splenic iNOS protein expression during the test for the expression of heroin contextually conditioned suppression of NO is shown in **Figure 3E**. A 2 x 2 ANOVA revealed significant differences in iNOS protein expression between treatment groups ($F_{(3,24)} = 3.180$, $p = .042$). There was a significant main effect of exposure (CS or HC; $F_{(1,24)} = 8.661$, $p = .007$) but no significant main effect of injection (CNO or Veh; $F_{(1,24)} = .363$, $p = .552$) or interaction between exposure and injection ($F_{(1,24)} = .237$, $p = .631$). In vehicle-treated animals, a planned comparison revealed that CS-exposed animals demonstrated non-significantly less iNOS protein expression in response to LPS than animals that remained in their home cage ($p = .097$). This comparison shows that exposure to the CS likely dampened the production of NO to LPS stimulation, but

that there was not quite enough power for this to come out as statistically significant. This was likely due to the fact that too many animals had to be dropped due to the rigorous standards of virus and injection placement. In CNO-treated animals, a planned comparison revealed that CS-exposed animals showed significantly less iNOS protein expression in response to LPS compared to animals that remained in their home cage (p = .023). These results indicate that there was still significant conditioned suppression of NO when inhibition of dHpc output to RSCgb preceded CS exposure.

Overall, the results from this chapter's experiment support the conclusion that chemogenetic inhibition of dHpc projections to RSCgb results in no attenuation of conditioned suppression of measures of NO production after exposure to a heroin-paired context.

Discussion

The retrosplenial cortex (RSC) has been implicated in contextual processing. Heroin contextually conditioned immune modulation requires the dorsal hippocampus (dHpc), a region with significant connectivity with the RSC. In the experiment described in this chapter, chemogenetic inhibition of projections from the dHpc to the RSC granular area b/c (RSCgb) does not interfere with the expression of contextually conditioned suppression of nitric oxide (NO), an important immune regulator. These results are surprising, given the strong evidence that the RSC may help form neural representations of context in the brain.

Our hypothesis is that the hippocampus serves to index the recall of relevant drug associated memories after receiving sensory information allowing identification of the context. There is evidence that this is the role of the hippocampus in contextual reward where the hippocampus may signal other areas to reactivate the appropriate memory engram and contextually relevant motivational states (Destexhe et al., 2015; Lansink et al., 2009). The

proposed role of the RSC is in contextual/spatial and episodic memory, just like the hippocampus (Vann et al., 2009), but the contribution of this area to contextually conditioned drug behaviors where fine spatial processing is not necessarily required has not been demonstrated. In addition, perhaps the RSC supports the hippocampus in its role through its projections to the hippocampus, and the reverse hippocampal projections back to the RSC are not necessarily required for contextual recall. In fact, the RSC may receive early sensory information and send it to the hippocampus (Vann et al., 2009) and in fact disrupting function of the RSC has been shown to change spatial coding in the hippocampus (Cooper and Mizumori, 2001). Future studies should look at the role of RSC projections to the hippocampus in the expression of contextually conditioned behaviors.

Rostral RSCgb was targeted in this study because it demonstrated a densely stained vertical band (likely layers II-III) in the Chapter 2 experiment, anatomically it has been shown to receive dense projections from dSub (Van Groen and Wyss, 2003), and it is further removed from the areas expressing the DREADD, ensuring that site-specific CNO would be less likely to spread to these areas and non-specifically influence the conditioned effect. Additionally, rostral RSCgb is unlikely to receive strong projections from deep layers of visual cortex, which mostly innervates adjacent retrosplenial dysgranular cortex and caudal RSCgb (Vogt and Miller, 1983). One exception to this is that layer V cells of visual cortex (area 18b) do project to this rostral RSCgb band, although most of DREADD-expressing cells in this region were in layer VI. A general lack of connectivity between the target region and visual cortex is important because one of the secondary goals of this experiment was to rule out effect contributions from non-specifically stained regions of visual cortex from the Chapter 2 experiment.

The focus on solely rostral RSCgb may have actually contributed to the lack of effect seen in this experiment. The RSC is one of the largest areas in rodent cortex (Vann et al., 2009). Previous studies have shown that effects of RSC inhibition can depend on the physical extent of RSC inactivation. In fact several studies directly compared lesion size and resulting behavioral effects in spatial tasks and saw that more complete lesions of the RSC produced impairments not seen in so-called "standard" lesion sizes (Vann and Aggleton, 2002, 2004). This may be due to significant redundancy within the RSC or an ability for uninhibited cells to sufficiently support RSC function when only a small subset of RSC cells are affected [(Vann and Aggleton, 2004) and David Smith, Cornell University, personal communication)]. The dHpc to RSC projections seen in this experiment extend at least from -1.92 mm to -7.20 mm relative to bregma, or over 5 mm rostro-caudally. The injector sites spread from -2.92 mm to -4.80 mm (1.88 mm) and estimated CNO spread based on previous characterizations and the current dose and volume used is around 1.5 mm (Stachniak et al., 2014). Therefore, it is entirely likely that enough RSC function was spared to support contextual processing. Robinson et al used DREADD-mediated inhibition to silence the RSC to silence a much greater extent of the RSC than the current studies by injecting a DREADD directly into the RSC in several sites along the rostro-caudal axis and then inactivating the entire area using systemically delivered CNO (Robinson et al., 2014). Future studies could investigate if multiple injections of CNO along the rostro-caudal axis have a different effect than what was seen here with a single injection, or employ the method used by Robinson et al to target the RSC alone.

Only one study to our knowledge has investigated the role of specific subregions of the RSC, like RSCgb, in spatial memory (van Groen et al., 2004). These studies resulted in small impairments in spatial memory that were not as large as those seen in lesion studies, but

nevertheless indicated that RSCgb at least partially contributed to the acquisition of spatial memory. Regardless of the lack of an effect from chemogenetic inhibition of the RSCgb on heroin contextually conditioned suppression of NO, this study represents an important step in understanding RSC connectivity and the tools by which these connections could be modulated.

CHAPTER 5: GENERAL DISCUSSION

Primary Findings

The described experiments' primary finding is that dorsal hippocampal (dHpc), but not ventral hippocampal (vHpc), outputs are vital to the expression of heroin contextually conditioned immune effects. While this work did not identify which projection from the dHpc is important for the expression of this contextually elicited drug behavior, the work lays the foundation for future studies aimed at answering this question. These studies show the feasibility of using designer receptors exclusively activated by designer drugs (DREADD) expressed under a Ca^{2+}/calmodulin-dependent protein kinase II alpha (CAMKIIα) promoter to target projection neurons in the dHpc and vHpc. Furthermore, they demonstrate the value of using DREADD technology for basic functional inactivation where lesions or GABA agonists have been historically used. Following the experiment in Chapter 2 where a DREADD was infused into the dHpc output regions, dorsal subiculum (dSub) and dorsal CA1 (dCA1), several projection sites of interest were able to be identified without running additional pilot studies. Understanding the role of each of these projections in heroin contextually conditioned immune effects would provide valuable insight into hippocampal function and how the hippocampus can prompt memory recall by engaging other brain regions. Chapter 2 revealed many different projection targets that for various theoretical and practical reasons were not chosen as the primary target in Chapter 4. However, it would do each a disservice not to discuss them in greater detail here.

A Deeper Look at Dorsal Hippocampal Projection Targets

An unanswered question in both contextual reward and immune drug behaviors is how the dHpc prompts recall of relevant motivational and immunological memories, as there are no direct connections between this region and the regions implicated in studies with drugs of abuse, like the basolateral amygdala (BLA), nucleus accumbens (NAc), and ventral tegmental area (VTA). The literature investigating interactions between these regions are vastly confused by an underappreciation of hippocampal diversity in terms of the dorsal versus ventral aspects. Most studies showing an interaction between the dHpc and these regions cite literature directly manipulating or measuring connections between the vHpc and these areas instead, leaving the mechanism of this interaction a complete mystery and adding confusion to the field.

Retrosplenial and Medial Entorhinal Cortices

Perhaps there is an indirect connection with these regions via cortical intermediaries like the retrosplenial or entorhinal cortices. Chapter 4 explored the possibility that the retrosplenial cortex (RSC) was such an intermediary because it is implicated in contextual/spatial memory, but we saw no effect of dHpc to RSC chemogenetic inhibition on heroin contextually conditioned immune effects. The involvement of the RSC would not have easily solved the mystery around how the hippocampus relays contextual information to the reward circuit as it does not directly connect to these areas and would also require a relaying region to convey pertinent contextual information. One possible second relay that could serve this purpose is the periaqueductal gray (PAG) which receives input from the RSC and sends projections to the medial NAc shell (Brog et al., 1993; Van Groen and Wyss, 2003).

The entorhinal cortex on the other hand is an attractive region that is well positioned to serve as an intermediary between the dHpc and NAc shell. Particularly likely to serve this role is

the medial entorhinal cortex (mEC) which shows high connectivity to both the dHpc (Witter et al., 2017) and the medial NAc shell (Brog et al., 1993), the subregion of the NAc that, in particular, has been shown to be important for expression of contextually conditioned drug reward and immune effects (Bossert et al., 2006; Bossert et al., 2007; Szczytkowski et al., 2011). In support of this hypothesis, a dSub projection to the mEC has recently been found to be important for both contextual fear recall and expression of cocaine conditioned place preference (Roy et al., 2017).

In the experiment described in Chapter 2, we unfortunately did not see any labeled projections that would support the role of dHpc to mEC projections in expression of contextually conditioned drug behaviors. However, two factors may have influenced this. First, the mEC is more caudal relative to dSub and dCA1. It is likely that after only a short viral incubation period (4 weeks), there was not enough mCherry expression present to detect DREADD expression in terminals within the mEC. Sufficient time is needed to transport the viral genetic construct or its products to the most distal projection terminals, which would be necessary to identify a dHpc efferent to the mEC. Second, the brain slices collected for viral verification in this study were collected free floating and then slide mounted. This technique makes it challenging to collect the caudal-most slices of cortex and we likely did not collect enough tissue to be representative of this large region's known hippocampal input. Future studies should thoroughly investigate whether a dHpc to mEC projection can be identified. Some evidence against the involvement of the mEC comes from Chapter 3's experiment where the mEC non-specifically expressed DREADD to some extent when targeting the vHpc, yet inactivation of these neurons did not affect our measures. Whether the mEC was labeled in the correct area or to a sufficient extent is a topic for debate.

Nucleus Accumbens Shell

In the experiment from Chapter 2, the NAc was the first area examined for dHpc projections. There is evidence that dopaminergic signaling in this region is important in the unconditioned immune effects of opioids. For example, antagonism of D1 dopamine receptors in the medial NAc shell attenuates both heroin and morphine's unconditioned effects on natural killer cell activity, an important component of immunity (Saurer et al., 2006; Saurer et al., 2009). Heroin's unconditioned suppression of NO production also shows a reliance on D1-mediated dopamine signaling in the medial NAc shell (Saurer et al., 2009). D1 signaling in the medial NAc shell might be involved in regulation of the immune system even independent of drugs of abuse as D1 agonism in the medial NAc shell is sufficient to decrease natural killer cell activity (Saurer et al., 2006). The contextually conditioned immune effects of opioids might also be mediated through this dopaminergic mechanism since conditioned effects are thought to at least partially recapitulate the mechanisms of unconditioned effects. Indeed, D1 antagonism in the medial NAc shell also blocks expression of morphine contextually conditioned suppression of natural killer cell activity (Saurer et al., 2008). Glutamatergic signaling in the medial NAc shell was shown to be important for the expression of heroin contextually conditioned suppression of NO and proinflammatory cytokines (Szczytkowski et al., 2011), however dopamine signaling here has not been directly implicated in these particular immune effects. In fact, medial NAc shell D1 signaling was not involved in heroin's unconditioned decrease of lymphocyte proliferation (Saurer et al., 2009), indicating that mechanism may depend on the particular immune measure. D1 signaling in the medial NAc shell (but again not in the core) is also required for context-induced reinstatement of heroin seeking (Bossert et al., 2007), indicating

that this might be a common mechanism by which context can influence both contextual reward and immune effects of drugs of abuse.

Regardless of the mechanism, together, these studies provide strong evidence that the medial NAc shell is involved in both the conditioned and unconditioned immune effects of opioids, and perhaps also conditioned reward with opioids. However, the vHpc is the hippocampal region that has been documented to send strong projections to the medial NAc shell, and the experiment in Chapter 3 provides evidence that the vHpc is not involved in heroin's contextually conditioned suppression of NO. The dHpc, while shown to be important, has been previous reported to project mainly to the NAc core or *lateral* NAc shell (Brog et al., 1993; Groenewegen et al., 1987; Kelley and Domesick, 1982; Witter, 2006) which has been shown not to be involved in opioid unconditioned or conditioned immune effects (Saurer et al., 2006; Saurer et al., 2008) and is not thought to encode contextual incentives to seek drugs of abuse, but rather the incentives of discrete cues (Bossert et al., 2007; Chaudhri et al., 2010).

Initially, we looked in the NAc and did not see any labelled projection terminals from the dHpc in the NAc at all, in neither core nor shell. Recent pilot studies with a longer viral incubation time (8 weeks), however, revealed remarkably robust projections from the dHpc to the NAc that were restricted to only the most rostral region of this brain area. Interestingly, these projections were nearly absent at the rostro-caudal coordinate where all previous studies had targeted the medial NAc shell in both opioid unconditioned and conditioned immune effects. Additionally, at this "rostral pole" of the NAc, the core and shell are much harder to distinguish (Witter et al., 2017), and so many studies investigating the relative contributions of the core and shell areas are unlikely to look within rostral NAc.

Recent work, however, (just released for publication on March 7th, 2019) corroborates what we see here (Trouche et al., 2019). Trouche et al 2019 elegantly characterizes dHpc projections to rostral medial NAc shell (and also core), arising specifically from dCA1, and not dSub. These dCA1 pyramidal neurons coordinate the activity of NAc medium spiny neurons and this activity is crucial for the expression of contextually conditioned reward behavior. One caveat that might make these results less relevant to the current study is the fact that this study employed conditioned place preference, which is more likely to engage the navigational role of the hippocampus, instead of context representation *per se*. Even still, this is the first time that a dHpc to medial NAc shell connection has been described and characterized behaviorally which necessitates a future investigation of this connection in heroin contextually conditioned immune effects and other conditioned reward tasks.

Lateral Septum

The lateral septum (LS) receives massive input from the hippocampus (Risold and Swanson, 1997). While we did not initially see projections arising from the dHpc in the LS, recent pilot studies with a longer viral incubation period (8 weeks) revealed dense projections at this more distal region, similar to what was seen with the NAc. What is intriguing about this connection is that the LS in turn projects to the hypothalamus, NAc, BLA, and VTA. The LS, therefore, might represent a mechanism by which the dHpc can impact behavioral arousal, emotional states, and motivated behaviors (Risold and Swanson, 1997; Sheehan et al., 2004). Functionality of an indirect connection between the dHpc and VTA through the LS was established in studies showing that 86% of neurons in the VTA responded to dHpc stimulation and that inhibition of areas of the LS receiving dHpc input prevented these effects (Luo et al., 2011). Several studies have shown that disconnection of parts of the dHpc-LS-VTA circuit using

either GABA agonists or DREADD-mediated inhibition of dHpc projections in the LS prevents context-induced reinstatement to cocaine seeking (Luo et al., 2011; McGlinchey and Aston-Jones, 2018) whereas vHpc projections to the LS did not. Not only does this specific contribution of the dHpc mirror the effects seen in heroin contextually conditioned immune modulation, but this demonstrates that the LS is a likely relay by which the dHpc can affect dopaminergic signaling arising from the VTA.

If this connection shown later to be important in our paradigm, the modulation of VTA dopaminergic neurons via the LS could easily impact both BLA and NAc dopaminergic signaling, which play a role in conditioned and unconditioned immune effects of opioids. Interestingly, the source of dHpc input to the LS was shown to be dCA3, not dCA1 or dSub (McGlinchey and Aston-Jones, 2018), which is a departure from the canonical view of hippocampal processing. Under this model, the hypothesis that outputs from the dCA1 and dSub regions are important for relaying contextual information from the hippocampus would be challenged. In fact, if this dCA3 to LS connection were subsequently shown to be important for the expression of heroin contextually conditioned immune modulation, it may mean that our results from the current experiment may be explained by back projections from dCA1 to dCA3, bringing into question the role of dSub at all. Future studies should investigate this possibility and further delineate the contributions of dHpc subregions to heroin contextually conditioned immune modulation.

Conditioned Reward and Immune Behaviors – A Diverse Hippocampal Perspective

Generally, it is accepted that the dHpc is predominantly used for encoding purely spatial and navigational information while the vHpc is involved when there is an emotional component to the spatial task. Figuring out the potential role of the dHpc versus the vHpc when using a

contextual task can be difficult to predict. Place cells that could represent particular contexts exist both in the dHpc and vHpc (Jung et al., 1994) and determining whether a task has an emotional component is not always straight forward. Therefore, it is challenging to understand which paradigms will engage the dHpc, vHpc, or both during contextual recall.

One study investigated the respective roles of the dHpc and vHpc in a task where the context is a static signal that helps guide the appropriate behavioral response. After finding that the vHpc, but not the dHpc, was important for this task, the authors suggested that distal contextual (spatial) cues may be preferentially encoded by the dHpc while proximal contextual cues by the vHpc (Riaz et al., 2017). This is in line with thinking of the dHpc as necessary for navigation or complex spatial processing whereas the vHpc, with lower resolution place cells, would be involved perhaps when there are lesser spatial demands. However, this study also used an appetitive task where the context may be expected to have at least some emotional valence, thus preferentially engaging the vHpc.

There is extensive evidence suggesting a role for the vHpc in the expression of contextually conditioned fear, where an animal freezes or ceases movement in a context where a previously fearful experience has occurred. In these tasks, there are typically both proximal spatial cues (small behavioral chamber) and emotional valences (fear), so reliance predominantly on vHpc is to be expected. Nevertheless, some studies still show engagement of the dHpc in these paradigms during contextual recall (Matus-Amat et al., 2004; Roy et al., 2017). Likewise, in contextually conditioned reward (specifically context-induced reinstatement of drug seeking) and contextually conditioned immune behavior, a small behavioral chamber is used (proximal cues) and drugs of abuse are used (appetitive stimulus). Previous studies have shown that both dHpc and vHpc mechanisms are required for contextually conditioned drug reward behaviors

(Bossert et al., 2016; Bossert et al., 2013; Bossert and Stern, 2014; Fuchs et al., 2007a; Fuchs et al., 2005; Marchant et al., 2016a), but the results of the current experiments indicate that in contextually conditioned drug *immune* behaviors the dHpc plays a role but the vHpc does not.

It is not clear exactly why this might be the case. On one hand, this may represent a divergence in mechanism between the expression of contextually conditioned reward and immune behaviors with drugs of abuse. This divergence is supported by recent work showing that dHpc mechanisms which are necessary for the expression of heroin contextually conditioned suppression of NO are not required for the expression of heroin conditioned place preference (Paniccia et al., 2018). If expression of conditioned reward is likely to engage the vHpc, but conditioned immune is not, perhaps drug contextually conditioned immune behaviors do not require recall of the emotional component of the drug experience. Alternatively, in contextually conditioned reward with drugs of abuse, it is thought that the Hpc is responsible for recalling relevant behaviors that occurred previously in that context (that is, occasion setting) and/or that the Hpc recalls specific contextual-US associations that might influence motivation or craving. Contextually conditioned immune, however, is only thought only to be due to a recall of context-US associations and not occasion setting. Thus, the immune conditioning paradigm's independence of vHpc mechanisms may reflect the lack of occasion setting necessary for the conditioned effect, and not necessarily independence of drug reward. Perhaps a more systematic review of the composition of contextual stimuli, use of context as an occasion setter vs. a Pavlovian CS, and emotional valences (appetitive or aversive) of stimuli in contextual paradigms would better inform the relative roles of the dHpc and vHpc in processing contextual information during memory recall.

Limitations & Technical Concerns

While DREADDs are a powerful technique in neuroscience, they have certain unique challenges that traditional inactivation methodologies do not. The following section will address each of these concerns as it relates to the current experiments as well as discuss additional limitations within these experiments.

Neuron Identity

We chose to express the DREADD in CAMKIIα-expressing neurons (using the CAMKIIα promoter) which have been shown to be mostly excitatory, like CA1 pyramidal neurons in the hippocampus (Achterberg et al., 2014; Guo et al., 2010; Johansen et al., 2010; Liu and Jones, 1996; Tsien et al., 1996). The projections from both aspects of the hippocampus are glutamatergic (Britt et al., 2012; Kinnavane et al., 2018), and therefore this method was intended to specifically manipulate neurons that would project to other brain regions. However, the identity of these cells as solely glutamatergic was not formally verified. While CA1 pyramidal neurons are well classified as being glutamatergic and expressing CAMKIIα, dSub neurons are not as well characterized. In addition, there is a possibility that non-specific DREADD expression led to manipulation of cells that were not CAMKIIα-expressing. Thus, verification of this DREADD construct in our paradigm is needed to know for sure that either CAMKIIα-expressing or glutamatergic efferents of the dHpc are involved in heroin contextually conditioned immune modulation.

Insertion of DREADDs into Cellular Membranes

DREADD receptors, like other guanine nucleotide-binding protein (G-protein) coupled receptors, can show constitutive activity independent of ligand binding (Roth, 2016). The risk of constitutive activity is higher when the receptor is highly expressed. It is unclear whether the

expression of the G_i-coupled DREADD in these experiments can be considered high enough to result in inhibition of neurons even in the absence of clozapine-*N*-oxide (CNO), the DREADD activating ligand. To control for these possibilities, many use a control virus encoding for a simple fluorescent marker (e.g. enhanced green fluorescent protein, EGFP) where constitutive activity is not possible because there is no receptor expressed. So far, no constitutive activity has been reported with high levels of DREADD expression (Roth, 2016).

There is also the possibility of disrupting normal cellular function by overexpressing an exogenous receptor in the cellular membrane. For this reason, it has been suggested to use a control group where a DREADD receptor insensitive to CNO is expressed [e.g. κ-opioid receptor derived DREADD, KORD (Marchant et al., 2016b)]. This alternative receptor's expression would disrupt the cellular membrane to a similar degree but not be activated by the synthetic ligand. While this would control for membrane disruption, it would not control for constitutive activity.

Ultimately, we decided not to include groups specifically controlling for either of these two factors. The control group selected, that of vehicle-treated animals expressing the DREADD, we deemed sufficient to indirectly address these concerns. There were no noticeable effects on our primary measures in these DREADD-expressing, vehicle-treated groups as compared to previous experiments that did not use DREADDs. Therefore, we do not think either of these possibilities significantly affected the outcome of these experiments.

Non-Specific CNO Action

Recent work has highlighted a particular concern with CNO, the DREADD activating synthetic ligand (Gomez et al., 2017). This study demonstrates that CNO is metabolized into clozapine when administered systemically, and possibly directly into the brain as well. It is

clozapine, the authors argue, that is actually responsible for binding to and activating the DREADD since it more readily crosses the blood brain barrier. Furthermore, clozapine itself is psychoactive, unlike CNO which is supposed to be inert, and can have non-specific (DREADD-independent) effects. To control for this possibility, some have argued that a control group of animals not expressing a DREADD but receiving CNO should be used to investigate any DREADD-independent effects of CNO. However, there is still the possibility that CNO exerts both DREADD-dependent and DREADD-independent effects that interact in animals that do express the DREADD (Mahler and Aston-Jones, 2018). necessitating multiple control groups to rule out non-specific CNO effects entirely.

In our experiment showing that DREADD-mediated inhibition of dHpc outputs attenuated the expression of heroin contextually conditioned suppression of NO, we do not believe that CNO had any non-specific effects that contributed toward this effect. First, animals that expressed the same DREADD in another region, the vHpc, did not show attenuation of contextually suppressed NO, despite getting the same dose of systemically delivered CNO. Second, in our experiment with intracranially delivered CNO, we also did not see attenuation of this effect. While CNO is supposed to be inert, high enough concentrations (10 μM) have been shown to have non-specific effects at histamine, serotonin, muscarinic, and dopamine receptors (Gomez et al., 2017). Concentrations of this magnitude are much more likely with intracranial infusions of CNO, and our solution of CNO had a starting concentration 100 times higher than the concentration shown to exert these non-specific effects *in vitro* (1 mM vs. 10 μM). Yet, despite these risks, CNO infused at this dose into the brain did not cause any effects on our measures. The only remaining possibility is that CNO acted non-specifically and that this action interacted uniquely with specific DREADD mechanisms within the dHpc to cause the observed

effect. We view this possibility as fairly improbable, especially given that DREADD manipulation of the dHpc has been used in other paradigms with no evidence of such an interaction (López et al., 2016; Varela et al., 2016). Nevertheless, to rule out any remaining concern around non-specific CNO effects, future studies could replicate our experiments using Compound 21 as an alternative DREADD ligand, which, as-of-yet, has no documented non-specific effects (Mahler and Aston-Jones, 2018).

Functionality of DREADD

One final limitation of the current experiments is that we did not verify that the DREADD was functional in inhibiting DREADD-expressing neurons. This could be done using electrophysiological recordings from DREADD-expressing neurons *in vitro*, by imaging neuronal activity *in vivo*, or by conducting immunohistochemical staining for neuronal activity markers, like immediate-early gene Fos. This remains a significant limitation of the current experiments that should be addressed.

The magnitude of inhibition that could be measured using these methods is also important to interpreting the findings of our current experiments. DREADD mediated inhibition is thought to be less robust compared to traditional methods of neural inhibition using GABA agonists, for example (Smith et al., 2016). Not only is DREADD-mediated inhibition incomplete, but the DREADD used here was not expressed in every cell, although this is reportedly typical for DREADD expression (Smith et al., 2016). It is possible that the negative effects of DREADD-mediated inhibition seen in Chapters 3 and 4 with inhibition of vHpc and dHpc projections to RSC, respectively, reflect this incomplete inhibition. It is possible that with more complete inhibition of these targets, using GABA agonists, a role for the vHpc and dHpc

projection to the RSC would be revealed. Future studies should replicate these experiments with traditional inhibition methods to ensure that a lack of effect was not due to the method used here.

Other Limitations

A major limitation of the current experiments is the presence of DREADD expression non-specifically, outside of the dSub and dCA1 regions. It is therefore not certain whether it was specifically the inhibition of these dHpc projection regions that led to attenuation of NO suppression upon exposure to the heroin-paired context. This problem is unfortunately difficult to resolve. It is impractical to run a control experiment for every additional region the virus is expressed within because of the number of animals that would need to be used and the time each experiment requires. Furthermore, without careful characterization of the extent of CNO spread, the results of such experiments would present their own interpretation challenges. It was our hope that by subsequently manipulating projections within a region strongly documented to receive strong dHpc input, that this issue would be somewhat resolved. However, the experiment in Chapter 4 employing such a manipulation had no effect on our measures of contextually conditioned immune modulation.

Future studies will need to resolve this issue, perhaps by using a genetic approach. This could entail infusing into the dHpc a virus expressing the DREADD in a flipped reading orientation that does not allow for expression of the DREADD unless a recombinase is present. A second retrograde virus expressing recombinase could be infused into a projection target, which would express recombinase in any region projecting to this region and ultimately cause DREADD expression only in dHpc neurons that projected there. Unfortunately, no viruses like this exist using a CAMKIIα promoter, but these could be created fairly easily and have for other promotors like hSyn. Alternatively, it might be worthwhile to experiment with packaging the

DREADD in different viral serotypes (species) to see if more limited spread can be achieved. Different serotypes can result in surprising differences in patterns of expression (Smith et al., 2016). For example, the serotype 5 of adeno-associated virus (AAV) used in these experiments is a smaller viral particle allowing for more spread than larger viral particles from serotypes 1-4.

Chapter 2's experiment also does not allow for a distinction to be made between the contributions of dSub and dCA1. Whether the attenuating effect is due to either dSub, dCA1, or both has important implications. While dCA1 is a major input to the dSub, there is evidence that these two regions can function independently and they each have their own connections to other brain regions. For example, there is evidence that the dSub projection to mEC is not important for learning a behavioral response to a context but is important for recall of this behavior after learning, whereas with the dCA1 projection to mEC, the reverse is true (Roy et al., 2017). Furthermore, there is more evidence that dCA1 sends back projections to dCA3 which projects to the LS. If this connection is important, the contribution of dCA1 may be greater than that of dSub. To achieve manipulations of each of these regions independently requires more advanced genetic techniques than those employed here, for example, by using genetically engineered mice. Future studies should investigate these regions using these techniques to more thoroughly understand the role of these dHpc outputs in contextually conditioned drug behaviors.

While it was not mentioned previously in Chapters 2 and 3, it is worth pointing out here that both experiments from these chapters showed DREADD expression into the intermediate hippocampus, which shows a pattern of connectivity and function in between the dHpc and vHpc as a spatial gradient as you transition from one to the other. In the two experiments, different parts of the intermediate hippocampus showed DREADD expression. In the vHpc DREADD experiment, intermediate areas adjacent to the vHpc showed expression whereas in the dHpc

experiment, intermediate areas adjacent to the dHpc showed DREADD expression. These areas showed little to no overlap as you can see in Figures 1 and 2. However, it raises the possibility that perhaps the attenuation of contextually induced immune modulation see in Chapter 2 is due fully or in part to the labeled intermediate region of the Hpc, and not strictly the dHpc. Future studies should carefully dissect the function of the dorsal/intermediate hippocampus in this paradigm once suitable tools are available for such fine spatial resolution.

Broader Implications of Results

Our heroin contextually conditioned immune model is extremely integrative – drawing from theories and experimental evidence from the fields of drug addiction, learning and memory, and immunology. The benefit of existing at the intersection of these rich fields is that our results have far reaching implications and provide unique perspectives by which to investigate questions from these fields. In the realm of addiction, these results are relevant to any contextually conditioned behavior with abused drugs, as has been a focus of this dissertation. Additionally, both unconditioned and contextually conditioned immune altering effects are not limited to opioids (Kubera et al., 2008), so understanding neural mechanisms of contextually conditioned immune effects with opioids has the potential to call for and inform studies with other drugs of abuse. Understanding the role of context in relapse or health impairments with drugs of abuse will allow for the development of novel behavioral and pharmaceutical interventions to ease the financial and psychological cost of drug addiction. The results described here indicate that the dHpc might be a brain region of particular interest to blocking contextually conditioned reward and immune effects with a single intervention, but more studies are needed to determine the exact mechanisms that should be targeted.

In the learning and memory field, these results provide a new perspective on hippocampal processing of context. As was discussed previously, there is still much debate around the contribution of particular hippocampal subregions, in particular, the dorsal/ventral/intermediate aspects of the hippocampus, in contextual representations. There is also a gap in knowledge around how the hippocampus, especially the dorsal aspect, engages other brain regions leading to behavioral, endocrine, and immunological changes during contextual exposure. The results presented here are beginning to fill this gap, implicating the dHpc in particular for contextual control of immune status. Further investigation of particular dHpc efferents will build toward an understanding of these mechanisms and provide a unique perspective because our model is not widely used to study such mechanisms.

The contextual function of the hippocampus is also not limited to spatial context. In fact, many experts argue that the hippocampus processes much broader aspects of context including social, interoceptive, temporal, and cognitive context (Eichenbaum et al., 1999; Maren et al., 2013; Smith and Bulkin, 2014). They propose that the hippocampus encodes an episode consisting of a combination of both spatial and non-spatial stimuli and prompts relevant behaviors in these situations upon recurrence [The Memory Space Hypothesis, (Eichenbaum et al., 1999)]. This hypothesis proposes that the events encoded by the hippocampus are much more than a spatial landscape where there is relation between locations, but rather an experiential landscape where there is relation between all elements of an experience. If the hippocampus serves as a master contextual integrator to support indexing of memories and memory recall, then its role in maintaining cycles of substance abuse cannot be restricted to merely instances where there is clear spatial context. After all, drug use likely has social, interoceptive, temporal

contexts associated with it as well. Future studies should think about incorporating this view of the hippocampus and context into models of addiction and other psychopathological behaviors.

In immunology and health, these results help work toward understanding how the brain can control immune function in the peripheral organs. Conditioned immune responses are particularly valuable to investigations of these mechanisms because they originate in the brain. Conditioned immune responses have been demonstrated in rodent models using stimuli paired with distinct immunosuppressive agents outside of drugs of abuse, such as foot shock stress (Lysle et al., 1988; Perez and Lysle, 1995), cyclophosphamide (Ader and Cohen, 1975), and cyclosporine A (Exton et al., 1998a; Exton et al., 1998b; Exton et al., 1998c; von Horsten et al., 1998). Studies looking at the neurobiological mechanisms of the expression of taste conditioned immune modulation have implicated the insular cortex and ventromedial hypothalamic nucleus as important mediators (Pacheco-López et al., 2006; Vits and Schedlowski, 2014). To date, these brain regions' contribution to contextually conditioned immune modulation with opioids has not been investigated and represents a ripe area for future research. Likewise, the results from the current experiments can help inform the understanding of different ways that the hypothalamus or other afferents of the immune system can be engaged, especially when the paradigm involves the hippocampus. These and other forms of immune conditioning in rodents and humans has been primarily studied within the realm of placebo effects [for review (Pacheco-Lopez et al., 2006; Schedlowski et al., 2015)] with the goal of harnessing immune conditioning for therapeutic benefit in cases where immunosuppressant drugs are prescribed. An understanding of the neural mechanisms of immune conditioning more generally would help guide or even develop methods to enhance therapeutic immune conditioning. Many of these studies use a discrete stimulus as a CS to induce the desired immune effect. How might these strategies be

impacted by the context in which this conditioning takes place, and can context be used for added benefit? Results from the current experiments also help inform the health consequences of the increasing use of elicit opioids and opioid therapeutics alike. Our studies demonstrate the importance of considering not just whether opioid therapeutics are prescribed, but behaviorally how they can be administered in a way that reduces the impact of conditioned immune effects.

Conclusion

The context of previous drug use is a powerful stimulus that can influence health outcomes by regulating immunity and can simultaneously increase the risk of relapse by inducing craving and motivation to seek drug. The hippocampus is a brain region that encodes environmental context and drives the behavior behind these conditioned consequences. By understanding the mechanisms behind hippocampal engagement of other brain regions, we gain insight into how an environmental context can hijack immune function and motivational systems in drug abuse and beyond.

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