Drugs and bugs: The role of toll-like receptor 4 in nucleus accumbens synaptic physiology and reward behavior

By

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DEDICATION

To black-haired Obaachan, who made the best ramen

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LIST OF ACRONYMS USED

$1/CV^2_{N:A}$: Ratio of $1/CV^2_{NMDAR}$ to $1/CV^2_{AMPAR}$
2-AG: 2-arachidonoylglycerol
ACSF: Artificial cerebral spinal fluid
A/N: AMPAR/NMDAR (ratio)
AEA: Anandamide
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
asEPSC: Asynchronous excitatory post-synaptic current
BAC: Bacterial artificial chromosome
BDNF: Brain-derived neurotrophic factor
BLA: Basolateral amygdala
CaMK: Calcium/calmodulin-dependent protein kinase
CaMKII: Calcium/calmodulin-dependent protein kinase II
CB1: Cannabinoid receptor 1
CNS: Central nervous system
ChAT: Choline acetyltransferase
CPP: Conditioned place preference
CR3: Complement receptor 3
CV: Coefficient of variance
DREADD: Designer receptors exclusively activated by designer drugs
DSE: Depolarization induced suppression of excitation
dSub: Dorsal subiculum of hippocampus
EAAT: Excitatory amino acid transporter
eCB: Endocannabinoid
EPSC: Excitatory post-synaptic current
EVOO: Extra-virgin olive oil
FSI: Fast-spiking interneuron
FST: Forced-swim test
GABA: γ-aminobutyric acid
GAT: γ-aminobutyric acid transporter
GFAP: Glial fibrillary acidic protein
GPCR: G-protein coupled receptor
HFS: High-frequency stimulation
i/o: Input/output
I-V: Current-voltage
ISI: Interstimulus interval
IfL: Infralimbic cortex
IFN: Interferon
IL: Interleukin
LFS: Low-frequency stimulation
LPS: Lipopolysaccharide
LTD: Long-term depression
LTP: Long-term potentiation
LTSI: Low-threshold spiking interneuron

MCAO: Middle cerebral artery occlusion
MD2: Myeloid differentiation factor 2
MDD: Major depressive disorder
mEPSC: Miniature excitatory post-synaptic current
mGluR: Metabotropic glutamate receptor
MiR-124: MicroRNA-124
MPLA: Monophoshoryl lipid A
mPFC: Medial prefrontal cortex
mThal: midline/intralaminar nuclei of thalamus
MSN: Medium spiny neuron
NAc: Nucleus accumbens
NMDAR: N-methyl-D-aspartate receptor
PAM: Positive allosteric modulator
PICK1: Protein interacting with C-kinase 1
PPR: Paired-pulse ratio
PrL: Prelimbic cortex
PSD: Post-synaptic density
PV: Parvalbumin
RI: Rectification index
RGC: Retinal ganglion cell
SNc: Substantia nigra pars compacta
sEPSC: Spontaneous excitatory post-synaptic current
$T_{1/2}$: Time to half-peak
TLR4: Toll-like receptor 4
TLR4.KO: Toll-like receptor 4 knockout
TNFα: Tumor necrosis factor α
TRPV1: Transient receptor potential cation channel subfamily V member 1
TST: Tail-suspension test
TTX: Tetrodotoxin
vHipp: Ventral hippocampus
VP: Ventral pallidum
VTA: Ventral tegmental area
vSub: Ventral subiculum of hippocampus
WD: Withdrawal day
WT: Wild-type

CHAPTER I

INTRODUCTION

I-1. Overview

Our ability to respond to stimuli, learn, and remember hinges on properly regulated communication between neurons. Such communication occurs through connections called synapses. In addition to serving as a site of information relay, synapses also play a role in information storage. Synaptic plasticity, or changing the strength of connections between particular neurons, is thought to form the basis of learning and memory.

Whereas proper regulation of synaptic strength is an essential component of everyday function, its dysregulation leads to a variety of diseases. In particular, synaptic dysregulation in the reward circuit is one of the root causes for many neurologic and psychiatric pathologies afflicting society. This includes schizophrenia, substance use disorders, mood disorders, and many others with an economic and healthcare burden in the hundreds of billions of dollars (National Institute of Mental Health, 2002; National Institute on Drug Abuse, 2017a). The reward circuit consists of a series of interconnected brain structures that regulate responses to both rewarding and aversive stimuli (Russo and Nestler, 2013). Central to the reward circuit is the nucleus accumbens (NAc). Part of the ventral striatum, this is a structure receives input from several brain structures to integrate information on motivation and reward.

Though much research over the past half-century focused on neuronal changes in response to experience, emerging evidence implicates the immune system playing an important role in shaping behavior and synaptic physiology.

This chapter will outline our knowledge of NAc circuitry and physiology, discuss immunologic regulation of synapses, and describe immunologic influence of the reward circuit and associated pathologies. Chapter 2 will discuss basal differences in NAc synaptic physiology and behavior as a consequence of knocking out the immune-associated protein toll-like receptor 4 (TLR4; TLR4.KO). Chapter 3 examines differences in NAc synaptic physiology between wild type (WT) and TLR4.KO mice in the context of exposure to cocaine. Chapter 4 examines the behavioral and synaptic consequences of activating TLR4 in a model of sickness. Finally, Chapter 5 expands upon these discussions and suggests future experiments and directions.

I-2. Cells and circuitry of the Nucleus accumbens (NAc)

Simultaneously vexing from a clinical perspective yet fascinating from a research perspective, the NAc is a heterogeneous structure comprised of multiple inputs, cell types, and projections modulated in unique ways to shape behavior. Further complicating the anatomy, the NAc is sub-divided into 2 regions—the NAc shell and NAc core (Voorn *et al*, 2004). These subregions receive distinct inputs and modulate different aspects of behavior (Sesack and Grace, 2010). The NAc shell functions to encode unconditioned reinforcing/aversive effects of behavior whereas the NAc core is important for instrumental learning and control (Voorn *et al*, 2004).

I-2a. Medium spiny neurons

The majority of neurons of the NAc core and shell are γ-aminobutyric acid (GABA) -ergic medium spiny neurons (MSN). These cells comprise 90-95% of the neurons in this region and are subdivided into 2 populations based on projection targets and gene expression patterns: 1) those projecting to ventral tegmental area (VTA)/substantia nigra pars compacta (SNc) and ventral pallidum (VP) expressing the D1 dopamine receptor (D1 MSN), and 2) those projecting to the VP and expressing the D2 dopamine receptor (D2 MSN) (Figure 1) (Grueter *et al*, 2012). These two populations of MSNs have roughly opposing effects on reward behavior with D1 MSNs canonically believed to be “pro-reward” and D2 MSNs being “anti-reward/pro-aversion” (Lobo *et al*, 2010). These cell types are also differentiated based on expression of other peptides and receptors with neuromodulatory properties. D1 MSNs also express A1 adenosine receptors, M4 muscarinic receptors, and release the peptides dynorphin and substance P. D2 MSNs express A2a adenosine receptors and release enkephalin and neurotensin (Kauer and Malenka, 2007; Steiner and Gerfen, 1998). Normally quiescent, D1 and D2 MSNs receive glutamatergic/excitatory inputs from multiple brain regions. NAc shell MSNs receives such inputs from the infralimbic cortex (IfL), ventral subiculum of the hippocampus (vSub), basolateral amygdala (BLA), and midline/intralaminar nuclei of the thalamus (mThal) (Joffe and Grueter, 2016; Sesack and Grace, 2010). NAc core MSNs receive glutamatergic inputs from the prelimbic cortex (PrL), dorsal subiculum of the hippocampus (dSub), BLA, and thalamic nuclei (Sesack and Grace, 2010). In addition to glutamatergic inputs, the NAc receives modulatory dopaminergic inputs from the VTA and SNc, serotonin from the dorsal raphe nuclei, norepinephrine from the locus

coeruleus, and several hormones from the hypothalamus (Sesack and Grace, 2010). The in addition to dopamine, the VTA also sends GABAergic and glutamatergic inputs into the NAc (Brown *et al*, 2012; Qi *et al*, 2016).

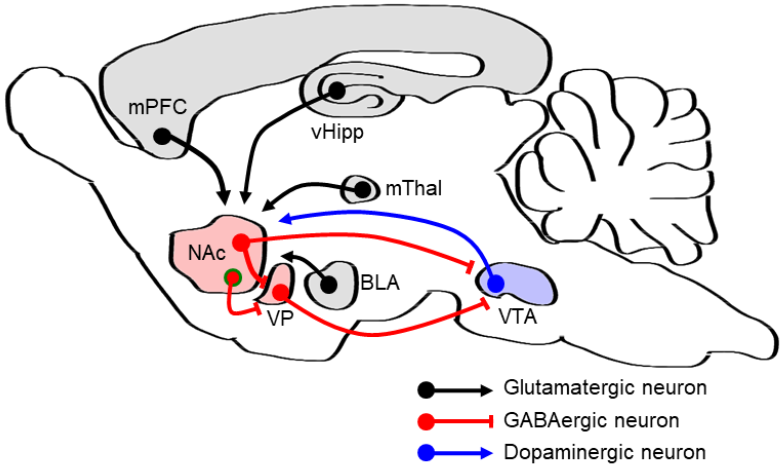

Figure 1. Overview of NAc circuitry.
Sagittal representation of mouse brain depicting general inputs and main outputs of the NAc (both core and shell subregions). The NAc primarily consists of medium spiny neurons (MSN) expressing either D1 dopamine receptors (outlined in red) or D2 dopamine receptors (outlined in green). These cells integrate glutamatergic inputs from the medial prefrontal cortex (mPFC), ventral hippocampus (vHipp), medial thalamic nuclei (mThal), and basolateral amygdala (BLA). There is also dopaminergic input from the ventral tegmental area (VTA). Specific sub-divisions of these inputs differentially innervate the NAc core and shell subregions. D1-receptor expressing MSNs project to the ventral pallidum (VP) as well as back to midbrain dopaminergic areas including the VTA. D2-receptor expressing MSNs only project to the VP.

I-2b. Interneurons

In addition to the MSNs, the neuronal population of the dorsal striatum and by extension, the NAc, also includes several types of interneurons. These include cholinergic (ChAT), somatostatin/neuropeptide Y-expressing low-threshold spiking (LTSI), and parvalbumin (PV)-expressing fast-spiking interneurons (FSI) (Kawaguchi *et al*, 1995; Straub *et al*, 2016). These interneurons form microcircuits to help regulate

MSN activity (Straub *et al*, 2016; Wright *et al*, 2016). Whereas striatal MSNs form occasional monosynaptic connections with neighboring MSNs, individual FSIs form robustly inhibitory connections onto neighboring MSNs and other FSIs to but not LTSIs or cholinergic cells (Gittis *et al*, 2010). On the other hand, LTSIs form sparse connections on all neighboring cell types suggesting they modulate dendritic excitability (Gittis *et al*, 2010). Inputs onto striatal interneurons differ depending on cell identity. PV FSIs, somatostatin LTSIs, and ChAT cells receive glutamatergic input from the cortex. ChAT interneurons are also innervated by the thalamus, and local MSNs (Kawaguchi *et al*, 1995). Finally, PV FSIs are also innervated by glutamatergic VTA projections (Qi *et al*, 2016). Though comparatively less work has been performed in the NAc, the research published shows similarity to the striatum as a whole. Namely, NAc FSIs form robust connections with neighboring MSNs while MSNs form sparse connections with one another (Wright *et al*, 2016). From the behavioral perspective, VTA glutamatergic projections onto NAc shell innervate PV interneurons. Optogenetic stimulation of these glutamatergic *terminals* in the NAc is aversive as assessed through conditioned place aversion (Qi *et al*, 2016). Interestingly, stimulation of the *soma* of these VTA glutamatergic cells is rewarding as assessed through real-time place preference. It was found that in addition to projections to NAc PV INs, these cells also form asymmetric synapses on neighboring VTA neurons to cause their firing (Wang *et al*, 2015). Subsequent release of dopamine into brain regions such as the NAc likely mediates the reward underlying somatic stimulation. In contrast, VTA GABAergic projections to the NAc innervate ChAT interneurons. Activation of these inputs silences NAc ChAT interneurons and enhances stimulus-outcome learning in a rodent model of aversion

(Brown *et al*, 2012). NAc ChAT interneurons also play a role in cocaine reward learning (Lee *et al*, 2016) (see below).

I-3. Circuit changes in behavior and pathology

Changes in the strength of synaptic connections underlie learning and memory (Kessels and Malinow, 2009; Malenka and Bear, 2004). One of the primary means to alter synaptic strength is through regulation of excitatory transmission mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR). Although this plays an important role in our ability to navigate and survive the world around us, maladaptive alterations/regulations of this process manifest as neurologic or psychiatric disorders. For example, pathologies of reward and motivation such as depression and substance use disorders involve alterations in NAc excitatory synaptic physiology (Francis and Lobo, 2016; Kauer and Malenka, 2007). Importantly, exogenous manipulation of excitatory synaptic properties is sufficient to alter NAc-associated learning, memory, and behavior (Ma *et al*, 2014; Pascoli *et al*, 2012, 2014).

Broadly, modulation of synaptic strength occurs through one of two ways: Hebbian plasticity and homeostatic scaling. Hebbian plasticity is associative and involves activity-dependent changes occurring between specific synapses. Homeostatic scaling involves global alterations affecting the gain of the system (Turrigiano and Nelson, 2004). Over the past 40 years, we gained significant insight into the mechanisms of such changes. Though the hippocampus remains the best-understood mammalian central nervous system (CNS) structure in relation to learning and memory

(Malenka and Bear, 2004), significant progress has been made in understanding the NAc.

I-3a. Synaptic plasticity in the NAc: Hebbian plasticity

The past 3 decades brought increased understanding of excitatory plasticity mechanisms and their consequences on the NAc and related behaviors. Regarding Hebbian plasticity, experiments performed in the early 1990s confirmed the existence of long-term potentiation (LTP) occurring on NAc MSNs dependent on N-methyl-D-aspartate receptors (NMDAR) (Kombian and Malenka, 1994; Pennartz *et al*, 1993). Such studies used *ex vivo* rodent brain slices and found that high-frequency stimulation (HFS) of afferent fibers results in the strengthening of NAc MSN synapses. This is due to calcium passage through NMDARs leading to increased AMPAR currents (Thomas and Malenka, 2003). Studies performed primarily in the hippocampus later established a downstream signaling cascade requiring calcium/calmodulin-dependent protein kinase II (CaMKII) for LTP induction (Malenka and Bear, 2004). Expression of NMDAR LTP is thought to be post-synaptic with increased AMPAR function through phosphorylation at Ser831 as well as increased delivery/clustering of AMPAR at the synapse (Malenka and Nicoll, 1999). In the NAc shell, HFS-LTP is inducible at both D1 and D2 MSNs (Pascoli *et al*, 2012).

Almost a decade later, a Thomas et al. characterized a NMDAR-dependent form of synaptic long-term depression (LTD) in the NAc shell (Thomas *et al*, 2000). This study demonstrated that low-frequency stimulation (LFS) of afferent fibers combined with post-synaptic depolarization causes a reduction in synaptic strength. This LTD was

blocked by application of the NMDAR antagonist APV suggesting a NMDAR-dependent induction mechanism. Subsequent research suggests that LFS of afferents results in modest current through NMDARs leading to AMPAR endocytosis (Brebner *et al*, 2005). Similar to NMDAR-dependent LTP, NMDAR-dependent LTD in the NAc also involves CaMKII and is expressed post-synaptically.

In addition to NMDAR forms of plasticity, the past few decades characterized several other forms of Hebbian plasticity in the NAc dependent on metabotropic glutamate receptors (mGluR). These are G-protein coupled receptors (GPCR) categorized into 3 groups. mGluR1/5 make up Group I, mGluR2/3 make up Group II, and mGluR4/6/7/8 make up Group III (Conn *et al*, 2005). In the NAc, all 3 groups of mGluRs functionally alter excitatory synaptic transmission (Manzoni *et al*, 1997). Since this initial discovery, experiments examined induction and downstream expression mechanisms of mGluR-dependent LTD. Studies performed in the early 2000s initially found functional expression of cannabinoid receptor 1 (CB1) in the NAc core (Robbe *et al*, 2001). This was next linked to a 10-13 Hz afferent stimulation protocol the post-synaptic synthesis of the endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG) acting on presynaptic CB1 to decrease presynaptic release probability (Robbe *et al*, 2002). As mGluR5 antagonism or intracellular calcium chelation blocks this LTD, it was concluded that induction of this form of plasticity is dependent on mGluR5 and intracellular calcium (Robbe *et al*, 2002). With the advent of fluorescent reporter mice allowing identification MSN identity, it was found that this 10 Hz LTD occurs on D2 but not D1 MSNs (Grueter *et al*, 2010). In addition to 2-AG/CB1, 10 Hz LTD was also found to involve synthesis of the eCB anandamide (AEA) acting on post-synaptic transient receptor potential cation

channel subfamily V member 1 (TRPV1) to further depress evoked responses (Grueter *et al*, 2010).

I-3b. Synaptic plasticity in the NAc: Homeostatic scaling

In addition to synapse-specific changes falling under the category of Hebbian plasticity are global alterations occurring as part of homeostatic plasticity to prevent circuit over- or underactivity (Turrigiano and Nelson, 2004). The best-studied form of homeostatic plasticity in the nervous system is synaptic scaling (Turrigiano, 2012). This was first demonstrated in 1998 with work in cortical cultures showing post-synaptic regulation of AMPAR activity compensating for prolonged activity blockade or enhancement (Turrigiano *et al*, 1998). Importantly, these adaptations preserve the relative strength of synaptic inputs which preserves information storage/processing (Turrigiano, 2012).

Molecular mechanisms underlying synaptic scaling have been characterized using cortical and hippocampal slice cultures. In response to prolonged exposure to the voltage-gated sodium channel blocker tetrodotoxin (TTX) or AMPAR antagonism, cortical neurons upregulate synaptic AMPARs downstream of decreased calcium-calmodulin kinase (CaMK) IV (Ibata *et al*, 2008). Other studies implicate a variety of factors contributing towards increased AMPAR activity including brain-derived neurotrophic factor (BDNF), tumor necrosis factor α (TNFα), major histocompatibility complex I, β3 integrins, protein interacting with C-kinase 1 (PICK1), post-synaptic density (PSD) 93 and 95, and the immediate early gene *Arc* (Turrigiano, 2012).

More recent work showed that prolonged increases in synaptic activity as a result of prolonged optogenetic stimulation results in the downregulation of AMPAR activity

dependent on CaMKIV (Goold and Nicoll, 2010). These results point to CaMKIV being an activity-dependent switch for synaptic scaling. Other contributory factors for scaling down synapses include EphA4, plk-CD5 signaling, and the immediate early gene Homer1A (Turrigiano, 2012).

Initial studies examining synaptic scaling assessed synaptic properties from cultured brain slices following prolonged exposure to pharmacologic agents or optogenetic stimulation which perturb network activity (Goold and Nicoll, 2010; Ibata *et al*, 2008; Stellwagen and Malenka, 2006; Turrigiano *et al*, 1998). These studies focused on glutamatergic structures where many of the excitatory synapses remain intact following tissue procurement and culturing. In contrast, less is known about synaptic scaling in the NAc. Unlike the hippocampus or cortex, the NAc is a GABA-ergic structure with glutamatergic connections originating exclusively from other brain regions. As such, slice procurement leads to de-afferentation of many excitatory connections. This muddles the assessment of processes that occur in response to prolonged changes in network activity. One way investigators worked around this issue is through co-culturing NAc MSNs with cortical neurons to establish excitatory connections (Reimers *et al*, 2014). Using this assay, it was found that BDNF scales down excitatory connections through decreasing surface AMPAR expression, an effect opposite to the cortex (Reimers *et al*, 2014). Further highlighting differences between the NAc and other brain regions, the cytokine TNFα which scaled up synaptic strength in the hippocampus following prolonged activity deprivation (Stellwagen and Malenka, 2006), decreased the synaptic strength onto NAc D1 MSNs (Lewitus *et al*, 2016). Future studies may circumvent the problem of de-afferentation in studying NAc synaptic scaling

by taking advantage of in vivo manipulations in the form of designer receptors exclusively activated by designer drugs (DREADD) or optogenetics to induce homeostatic changes before preparing acute slices for recording.

I-4. NAc physiology and substance use disorders

Substance use disorders affect over 8% of the US population (SAMHSA, 2014). Beyond effects on the individual are those felt by family and friends. From the economic standpoint, this problem costs the nation hundreds of billions of dollars every year in direct hospital costs as well as through lost productivity (National Institute on Drug Abuse, 2017a). Broadly, substance use disorders are thought of as continued use of substances despite adverse consequences. More specifically, diagnosis involves patients meeting a minimum of 2 in 12 criteria spanning categories of impaired control over use, social impairment, risky use, and pharmacological (DSM V, Table 1).

Criterion A: Impaired control
1) Individual takes substance in larger amounts or over longer period than originally intended
2) Individual expresses a persistent desire to cut down or regulate substance use and/or reports multiple unsuccessful efforts to decrease or discontinue use
3) Individual spends a great deal of time obtaining the substance, using the substance, or recovering from its effects
4) Individual exhibits craving (Intense desire or urge for the drug that may occur at any time)
Criterion B: Social impairment
5) Individual fails to fulfill major role obligations at work, school, or home because of substance use
6) Individual continues substance use despite persistent/recurrent social or interpersonal problems caused or exacerbated by the effects of the substance
7) Individual reduces or gives up important social, occupational, or recreational activities because of substance use
Criterion C: Risky use
8) Individual continues substance use in situations where it is physically dangerous
9) Individual continues substance use despite knowledge of having persistent/recurent physical or psychological problem caused/exacerbated by substance
Criteria D: Pharmacologic
10) Individual exhibits pharmacologic tolerance to substance
11) Individual exhibits withdrawal symptoms from substance

Table 1. Diagnostic criteria for substance use disorder.
Individual must have problematic pattern/use of substance leading to clinically significant impairment/distress with 2 or more of the 11 criteria within a 12-month period [adapted from DSM-V (American Psychiatric Association, 2013)].

Starting in the 1980s, studies suggested that despite differing pharmacology, drugs of abuse all affect the NAc and VTA (Di Chiara and Imperato, 1988; Nestler, 2004; Volkow and Morales, 2015). Since then, research focused on the synaptic changes that occur in response to drug experience as well as their behavioral ramifications. In examining synaptic adaptations to drugs of abuse, many of these studies focused on cocaine as it is highly addictive and unlike other drugs of abuse, does not produce physical dependence (Nestler, 2004). Though the bulk of studies examining NAc synaptic physiology is centered on rodents, it is worth noting that drugs of abuse also alter this brain region in more “advanced” species including non-human primates (Lyons *et al*,

1996) and humans (London *et al*, 1990) in addition to the well-studied rats (Porrino *et al*, 1988) and mice (Zocchi *et al*, 2001).

In the NAc, drugs such as cocaine alter glutamatergic and dopaminergic transmission onto MSNs raising the possibility of a “common pathway” for addiction (Nestler, 2005). In support of this idea, virtually all drugs of abuse cause NAc upregulation of the protein ΔFosB (Robison and Nestler, 2011). However, the specific synaptic changes were found to differ based on a variety of factors including drug identity, administration paradigm, and timing (Nestler, 2005). More recent work addressed this discrepancy at the circuit level with literature suggesting that despite causing different synaptic adaptations in the NAc, exposure to different drugs of abuse (including alcohol, morphine, and cocaine) bias the relative strength of excitatory inputs towards the “pro-reward” D1 MSNs (Cheng *et al*, 2016a; Graziane *et al*, 2016; Wright and Dong, 2017).

The link between drug experience and NAc synaptic plasticity was established in the early 2000s. Thomas et al. found that 5 days of non-contingent cocaine administration followed by forced abstinence and a challenge dose resulted in a decrease in NAc MSN synaptic strength. This change was associated with a loss of NMDAR-dependent LTD attributed to occlusion (Thomas *et al*, 2001). Subsequent studies explored differential effects on NAc synapses based on drug identity, region (core vs. shell), administration protocol (contingent vs. non-contingent), time-point, and cell type painting a complicated picture of synaptic plasticity in relation to drug experience. More recently, the advent of optogenetics and DREADDs permitted study of input-specific synaptic changes occurring as a result of drug exposure. However, some

caution should be taken with DREADD studies (Gomez *et al*, 2017). Here, synaptic changes on NAc MSNs and associated behavior will be summarized with a focus on cocaine. Findings are grouped based on drug administration paradigm and NAc subregion. Observations with interneurons will be noted when relevant.

I-4a. Single exposure cocaine

Several groups reported synaptic changes in the NAc following a single injection of cocaine. In the NAc shell, it was originally reported that single exposure to 15 mg/kg as well as a high dose of 40 mg/kg cocaine causes no change in post-synaptic glutamatergic properties as assessed through AMPAR/NMDAR (A/N) ratio both 24 h and 7 days injection (Kourrich *et al*, 2007). Interestingly, another group found that a single cocaine injection followed by a 7 day wait results in increased miniature excitatory post synaptic current (mEPSC) amplitudes on D1 MSNs suggesting increased post-synaptic strength (Pascoli *et al*, 2012). This finding was associated with attenuation (likely occlusion) of HFS-LTP and an exaggeration of medial prefrontal cortex (mPFC) to NAc shell D1 MSN 1 Hz LTD. Furthermore, *in vivo* optogenetic 1 Hz stimulation of mPFC to NAc afferents reduces mEPSC amplitude and blunts the locomotor response to a second cocaine injection (Pascoli *et al*, 2012). A single cocaine injection with a 7-10 day wait is also associated with increased AMPAR rectification and sensitivity to the GluA2-lacking AMPAR antagonist philanotoxin in shell D1 MSNs (Terrier *et al*, 2015). This suggests the strengthening of synapses over the week following cocaine exposure is due to insertion of GluA2 lacking AMPARs. GluA2-lacking AMPARs flux larger unitary currents and are permeable to calcium ions making them

important in synaptic plasticity, development, and excitotoxicity (Bellone and Lüscher, 2012).

In the neighboring NAc core, a single injection of cocaine followed by a 24 hour wait abolishes 10 -13 Hz mGluR5/eCB LTD as assessed through field potentials. This loss of plasticity was confirmed in whole-cell recordings from D2 MSNs (Grueter *et al*, 2010). However, this change in plasticity was found to be temporary as 1 week following a cocaine injection, this form of LTD was restored (Fourgeaud *et al*, 2004). Single exposure to cocaine had no effect on LTD induced by a group II mGluR agonist (Fourgeaud *et al*, 2004). Importantly, input- and cell-type specific changes have not been investigated in the NAc core following single cocaine exposure.

I-4b. Repeated non-contingent cocaine/locomotor sensitization

Repeated psychostimulant injections result in locomotor sensitization dependent on context. Each drug injection results in an increase in locomotor activity compared to the prior exposure (Thomas *et al*, 2001). With forced withdrawal/abstinence, NAc synapses continue remodeling leading to further increases in locomotor activity when given a challenge dose of drug in a similar context (Whitaker *et al*, 2016). Multiple non-contingent injections of cocaine, withdrawal, and cocaine challenge result in distinct sets of NAc neuronal and synaptic changes throughout the administration paradigm.

I-4b-i. Repeated cocaine: Early (1-3 day) withdrawal

In the NAc shell, 5 days of cocaine followed by a 1-3 day wait results in decreased MSN intrinsic excitability (Kourrich *et al*, 2013; Kourrich and Thomas, 2009). If the cocaine

injections are given in the context of the animals' home cage, this change is seen on D1 but not D2 MSNs (Kim *et al*, 2011). Synaptically, 5 day cocaine injections in a non-home-cage environment leads to NAc shell MSN having decreased A/N ratios without change in AMPAR rectification (Kourrich *et al*, 2007). Home cage injections increase dendritic spine density on D1 MSNs but do not change the A/N ratio or presynaptic release probability either MSN type (Kim *et al*, 2011). This time point is also associated with an increase in "silent synapses," or connections containing NMDAR without AMPAR. Such structures are an important substrate of meta-plasticity (Huang *et al*, 2009). With cocaine, these connections are associated with GluN2B NMDARs and are generated de novo on shell D1 MSNs (Graziane *et al*, 2016). Interestingly, this contrasts with morphine exposure which causes the internalization of AMPARs on D2 MSNs (Graziane *et al*, 2016). When examined in an input-specific manner, 5 days of cocaine followed by a 3 day wait was found to strengthen the relative ratio BLA inputs onto D1 MSNs compared to D2 MSNs (D1/D2 ratio) while decreasing this ratio from the ventral hippocampus (vHipp) (MacAskill *et al*, 2014). Through use of strontium to assess asynchronous EPSCs (asEPSC) and dye-filling of MSNs to assess dendritic spines, this group found that the increased D1/D2 ratio from BLA inputs is likely due to increased synapse number onto D1 MSNs while the decreased D1/D2 ratio from vHipp inputs is likely due in part to decreased AMPAR transmission at each synapse. Importantly, inhibiting BLA activity during cocaine exposure prevents the increase in D1/D2 ratio of BLA inputs onto the NAc shell and decreases the locomotor response to cocaine (MacAskill *et al*, 2014). In the core sub-region, 5 days of cocaine followed by a 1-3 day wait results in increased intrinsic excitability of NAc MSNs (Kourrich and Thomas,

2009). This time point was also associated with an increased A/N ratio in D1 but not D2 MSNs (Lewitus *et al*, 2016). Whether this change is due to decreased AMPAR transmission or increased NMDAR transmission is unknown.

I-4b-ii. Repeated cocaine: Late (10+ days) withdrawal

Roughly mimicking forced abstinence from drugs of abuse, a prolonged delay period following non-contingent cocaine exposure results in additional changes in the NAc. This drug paradigm causes preferential upregulation in activity of a subset of MSNs (Koya *et al*, 2009). When sampling across all MSNs, a 5 day exposure to cocaine followed by 10-14 days of withdrawal results in increased A/N ratio, mEPSC amplitude, and mEPSC frequency when compared to saline treated animals (Kourrich *et al*, 2007). These investigators found no change in AMPAR rectification, paired-pulse ratio (PPR), NMDAR mEPSC or NMDAR decay kinetics. Prolonged withdrawal (35-49 days) following 8 days of cocaine injections does not alter AMPAR rectification (McCutcheon *et al*, 2011b). In total, these results point to an increase in synapse number as well as AMPAR quantity at each synapse. The lack of change in rectification argues against changes in AMPAR subunit composition. Importantly, these experiments did not differentiate between D1 and D2 MSNs. To reconcile this, a later study presented similar experiments in a cell-type specific manner and found increased A/N ratio and AMPAR rectification in NAc shell D1s following 5 days of cocaine with a 7-10 day withdrawal (Terrier *et al*, 2015). No synaptic changes were observed in D2 MSNs. Interestingly, this study found that even when sampling NAc shell MSNs in a non-specific manner, cocaine exposure and withdrawal significantly increased AMPAR

rectification. Despite the discrepancy in AMPAR rectification, these studies point towards increased post-synaptic strength on at least D1 MSNs. Similar to acute withdrawal, non-cell-type specific current clamp experiments also reveal a compensatory decrease in MSN intrinsic excitability associated with sigma-1 receptors and Kv1.2 (Kourrich *et al*, 2013; Kourrich and Thomas, 2009).

In the core subregion, a 5 day exposure to cocaine followed by a 10-14 day withdrawal period results in increased A/N ratios and mEPSC amplitudes without change in mEPSC frequency (Jedynak *et al*, 2016). Additionally, an 8 day exposure followed by a prolonged withdrawal of 35-42 days yields no change in AMPAR rectification (McCutcheon *et al*, 2011b). Similar to the shell, this suggests a strengthening of NAc core MSN synapses through increased AMPAR transmission. A recent optogenetic interrogation of PFC and medial thalamic nuclei (mThal) inputs onto NAc core MSNs revealed further insights. For PFC inputs, a 5 day exposure to cocaine with 10-14 day withdrawal decreased the A/N ratio onto D1 MSNs without affecting asEPSCs or NMDAR stoichiometry (Joffe and Grueter, 2016). This constellation of data points to a decrease in A/N ratio due to upregulated NMDAR function in PFC to NAc core D1 MSN synapses. This cocaine experience caused no change in PFC to D2 MSN synapses. For mThal inputs, this cocaine exposure paradigm increased asEPSC amplitude and caused upregulation of GluN2C containing NMDARs on D1 MSNs (Joffe and Grueter, 2016). This was associated with an unmasking of 1 Hz LTD of mThal inputs. On D2 MSNs, cocaine exposure and withdrawal appears to increase the number of silent synapses. No changes in intrinsic excitability were observed when sampling in a non-cell type specific manner (Kourrich and Thomas, 2009).

I-4b-iii. Repeated cocaine: Withdrawal and cocaine challenge

A challenge dose of cocaine following exposure and withdrawal roughly mimics relapse and causes rapid changes in NAc synaptic physiology. Using mice with the neuronal-activity dependent promoter c-Fos linked to expression of fluorescent protein, it was shown that cocaine experience activates an “ensemble” of NAC MSNs (Koya *et al*, 2009, 2012). Pharmacologic inhibition of this MSN ensemble attenuates locomotor sensitization following withdrawal (Koya *et al*, 2009). When comparing neurons activated during the cocaine experience to the remaining MSNs following a challenge dose of cocaine after withdrawal, it was found that the active neurons have a significant reduction in A/N ratios and silent synapses without difference in AMPAR rectification (Koya *et al*, 2012). Importantly, this alteration is context-dependent—no upregulation in silent synapses was observed from activated neurons following a cocaine challenge in a novel environment (Whitaker *et al*, 2016).

NAc recordings made the day following a challenge dose caused a decrease in the A/N ratio of NAc shell MSNs compared to animals receiving saline during sensitization period (Thomas *et al*, 2001). A/N ratios remain elevated for animals receiving saline during the challenge time point. Additionally, no differences were observed for AMPAR rectification (Kourrich *et al*, 2007). These observations are associated with an attenuation in LFS-LTD (Thomas *et al*, 2001) suggesting occlusion. Similar to the shell, a challenge dose of cocaine drops the A/N ratios down to control levels in the NAc core (Jedynak *et al*, 2016).

I-4c. Conditioned place preference

Conditioned place preference tests associative reward learning. In this assay, animals are typically given the freedom to explore 2 spatially distinct settings. Over 3-10 days, one setting is paired with a drug injection. If the animal finds the drug rewarding and can associate the experience with a spatial context, then it will spend more time on the drug paired side when given a choice. Similar to locomotor sensitization, drug administration is performed in a non-contingent manner. Through this assay, the opposing roles of NAc shell D1 and D2 MSNs on drug reward behavior was demonstrated (Lobo *et al*, 2010). Specifically, *in vivo* optogenetic stimulation of NAc D1 MSNs induced place preference to a sub-threshold dose of cocaine while stimulation of D2 MSNs decreased preference to a cocaine dose that normally elicits preference. This effect was attributed to BDNF/TrkB signaling as cell-type specific deletion of TrkB mimicked *in vivo* optogenetic stimulation on cocaine conditioned place preference (Lobo *et al*, 2010). *In vivo* calcium imaging of NAc core MSNs further support the D1/D2 dichotomy on drug reward. Cocaine increases D1 MSN activity while decreasing D2 activity (Calipari *et al*, 2016). After establishing cocaine conditioned place preference, it was found that D1 MSNs increase firing while D2 MSNs decrease firing just prior to entering a drug-paired chamber. Furthermore DREADD-mediated inhibition of D1 MSNs during cocaine pairing sessions or during choice test prevents place preference (Calipari *et al*, 2016).

From a synaptic perspective, changes in NAc MSNs do not cleanly match the behavioral output. With a 10-day drug/context pairing followed by withdrawal periods of various times, it was found that drug/context pairing causes an increase in silent synapses of NAc shell MSNs similar to what is observed with locomotor sensitization.

Over 20 days of withdrawal, this number decreases while AMPAR rectification increases suggesting the “unsilencing” of synapses through insertion of GluA2-lacking AMPARs (Shukla *et al*, 2017). Place preference for the drug-paired context was retained beyond withdrawal day (WD) 20. However, results from mice with mutations in post-synaptic proteins dissociate this synaptic phenomenon from behavior. Animals lacking PSD95 exhibit a preference for a drug-paired context on WD 1 but not at WD 20. These mice exhibit the increase in silent synapse number at WD 1 which does not drop by WD 20 suggesting that maturation of silent synapses are important for persistence of drug reward learning. However, SAP102 knockout mice learn and maintain cocaine conditioned place preference despite a lack of maturation of silent synapses (Shukla *et al*, 2017). Furthermore, administration of the mGluR1 positive allosteric modulator (PAM) SYN119 in WT mice prior to a WD 20 preference test reversed synaptic changes occurring during the withdrawal period but did affect persistence of drug-context associations. These results suggest that maturation of NAc shell silent synapses are dispensable for context/reward associations.

I-4d. Self-administration

Though non-contingent drug administration protocols provided many important insights into the actions of drugs on the brain, they lack much of the anticipatory stimulus-response associations present in human subjects (Jacobs *et al*, 2003). In this sense, operant/contingent drug administration models may provide better face validity (Suska *et al*, 2013) and are associated with distinct changes in the NAc. The past decade of research revealed distinct synaptic changes in the NAc following drug administration

contingent on operant (lever presses or nose pokes) behavior. Studies using cocaine self-administration typically use a training paradigm of short access (1-3 h/day) or long access (6 h/day) for 5-10 days followed by one or more periods of abstinence, extinction training, or reinstatement. Extinction training involves exposure to operant cues (lever/nose poke hole) without associated drug administration leading to a decrease in the behavioral response. Reinstatement mimics relapse and similar to human subjects, involves exposure to one of stress, drug-associated cues, or the a priming dose of the drug itself (Wolf and Ferrario, 2010).

I-4d-i. Self-administration: Short-access

In the NAc shell, short-access cocaine self-administration followed by 28+ days abstinence results in increased A/N ratio and AMPAR rectification in D1 MSNs from mice (Terrier *et al*, 2015). No changes were observed in D2 MSNs similar to non-contingent cocaine exposure and withdrawal. When examined in an input-specific manner, differential effects on mPFC, BLA, and vHipp to D1 MSN synapses were found (Pascoli *et al*, 2014). Short access cocaine and withdrawal increased AMPAR rectification and decreased the A/N ratio of mPFC inputs while increasing the A/N ratio of vHipp inputs. These changes were associated with differential effects of input-specific LFS LTD. In mPFC inputs, cocaine experience attenuates 1 Hz LTD and exaggerates 13 Hz LTD. The latter normalizes AMPAR rectification and A/N ratio. In vivo application of this LTD results in decreased cocaine seeking and cue discrimination as assessed through an extinction test where the mouse is exposed to an “active” and “inactive” lever. For the vHipp input, withdrawal from short-access cocaine self-administration

causes an attenuation of 13 Hz LTD without affecting 1 Hz LTD. However, 1 Hz LTD normalized the A/N ratio from this input. *In vivo* application of 1 Hz vHipp LTD decreased active lever pressing while 13 Hz LTD diminished cue discrimination. No synaptic changes were observed from BLA inputs (Pascoli *et al*, 2014).

In rats, input-specific interrogation of synaptic changes revealed a time course of synaptic changes occurring during withdrawal from short-access cocaine self-administration. On WD 1, BLA inputs were found to have an increase in silent synapses (Lee *et al*, 2013). Over a subsequent 45 days of withdrawal, these silent synapses were "unsilenced" through insertion of GluA2-lacking AMPARs. Additionally, optogenetic LFS-LTD (3 min., 5 Hz x 3) of BLA to NAc shell synapses in cocaine withdrawn rats reverses this process by internalizing GluA2-lacking AMPARs. When performed *in vivo*, this stimulation protocol reverses incubation of cocaine craving as assessed through an extinction test. However, this depotentiation of BLA to NAc shell synapses is temporary as within 1 day after *in vivo* LTD, GluA2-lacking AMPARs returned along with cocaine craving/seeking behavior. Environmental enrichment prolongs the synaptic and behavioral effects of *in vivo* BLA to NAc shell LTD by at least 20 days (Ma *et al*, 2016). IfL to NAc shell synapses also upregulate silent synapses which mature through insertion of GluA2-lacking AMPARs following short-access self-administration and 45 day withdrawal (Ma *et al*, 2014). Similar to the BLA, an optogenetic LFS-LTD (10 min., 1 Hz) of this input also acts on GluA2-lacking AMPARs. However, in vivo administration of this LTD was found to increase cocaine craving/seeking at WD 45 (Ma *et al*, 2014). In the NAc core, projections from PrL also show an increase in silent synapse number which declines through withdrawal from short-access self-administration. However,

unlike the BLA or IfL inputs onto the NAc shell, PrL inputs do not exhibit upregulation of GluA2-lacking AMPARs (Ma *et al*, 2014). *O*ptogenetic LFS-LTD (10 min., 1 Hz) of this input at WD 45 decreases cocaine craving/seeking showing bi-directional effects of PFC synapses onto the NAc shell vs. core.

I-4d-ii. Self-administration: Short-access with extinction +/- reinstatement

Extinction training following short-access cocaine self-administration itself a form of learning producing distinct synaptic changes (Wolf and Ferrario, 2010). In rats, short-access self-administration, extinction-training in a new context, and cue/setting-induced reinstatement upregulates neuronal activity in a subset of neurons in the NAc shell and core (Cruz *et al*, 2014). Pharmacologic inhibition of cocaine-activated MSNs activity in the shell attenuates cue/setting-induced reinstatement.

Though similar inhibition of the cocaine-activated MSNs in the NAc core produced no such attenuation, other studies point towards this subregion's importance in drug-induced extinction learning. In mice trained for short-access cocaine self-administration and extinction, either chemogenetic activation of D1 MSNs or inhibition of D2 MSNs in the NAc core augments cue-induced reinstatement (Heinsbroek *et al*, 2017). *In vivo* field potential recordings from the NAc core of rats suggests a loss of "metaplasticity" as extinction training blocks HFS-LTP (2 sec., 50 Hz x 2) and LFS-LTD (3 min., 5 Hz x 3) when stimulating the PFC (Moussawi *et al*, 2009). The ability to induce HFS-LTP/LFS-LTD was restored with an injection of N-acetyl cysteine, a compound that also reduced drug-induced and cue-induced reinstatement behavior. This effect is mediated by mGluRs: *in vivo* administration of a mGluR2/3 antagonist

prevented N-acetyl cysteine's rescue of impairments in HFS-LTP whereas the mGluR5 antagonist MPEP prevented rescue of LFS-LTD (Moussawi *et al*, 2009). *Ex vivo* slice electrophysiology experiments from the NAc core of saline/naïve rats revealed a bidirectional effect of N-acetyl cysteine on presynaptic release probability. A low dose (0.5μM) decreased release probability whereas a high dose (500μM) increased release probability. These changes in release probability corresponded with low-dose N-acetyl cysteine decreasing evoked excitatory post-synaptic current (EPSC) amplitudes and high dose increasing amplitudes. mGluR2/3 antagonism reversed the effects of low-dose N-acetyl cysteine whereas mGluR5 antagonism reversed the high-dose effects (Kupchik *et al*, 2012). Whether these presynaptic changes underlie differences in meta-plasticity following extinction training remains to be seen. At the behavioral level, MPEP alone reduces drug-induced reinstatement while strengthening mGluR5 signaling through PAM blocks N-acetyl cysteine's effect on reinstatement (Moussawi *et al*, 2009). mGluR2/3 antagonism blunts cue-induced drug seeking behavior (Kupchik *et al*, 2012). Short-access cocaine self-administration followed by extinction training also decreases the expression of the glutamate transporter GLT-1 and the catalytic subunit of the cysteine-glutamate exchanger, xCT (Knackstedt *et al*, 2010). N-acetyl cysteine as well as the brain-permeable antibiotic ceftriaxone restore GLT-1 and xCT function and attenuate cue-induced reinstatement (Knackstedt *et al*, 2010). Short-access cocaine self-administration with extinction, as well as cue-induced reinstatement also increases the A/N ratio and dendritic spine density in the NAc core (Smith *et al*, 2014). This was associated with an upregulation of matrix metalloproteases (MMP)-2 and MMP-9 expression in the NAc core but not shell. Inhibition of MMP-2 after extinction training

prevented these changes and inhibited cue-induced reinstatement. MMP-9 inhibition prevented changes in the A/N ratio following reinstatement and attenuated both cue- and drug-induced reinstatement. These results point to the importance of matrix metalloproteases in synaptic remodeling and behavior following drug experience.

I-4d-iii. Self-administration: Extended access

Extended-access (6+ hours/day) cocaine self-administration and 5+ week withdrawal produces NAc synaptic adaptation partially overlapping with those observed with other administration paradigms. In the NAc shell, A 5-7 week withdrawal period causes upregulation of GluA2-lacking AMPARs (McCutcheon *et al*, 2011b). In the NAc core, extended-access self-administration initially decreases surface AMPAR expression (Conrad *et al*, 2008). However, over 45 days of withdrawal, there is a significant increase in both surface and intracellular AMPAR expression suggesting decreased degradation or increased synthesis. This time-course is also associated with decreased surface/intracellular ratio of GluA2 and increased AMPAR rectification signifying an increase in GluA2-lacking AMPAR function (McCutcheon *et al*, 2011a, 2011b). Pharmacologic inhibition of GluA2-lacking AMPAR before an extinction test reduced cocaine seeking behavior signifying their importance in this behavior (Conrad *et al*, 2008). Pharmacologic agonism of mGluR1 given systemically or into the NAc just prior to an extinction test also reduces cocaine seeking behavior (Loweth *et al*, 2014). *Ex vivo* slice physiology experiments from the NAc core revealed that mGuR1 agonism normalized AMPAR rectification associated with incubation of cocaine craving. However, 2 days following *in vivo* mGluR1 agonism, AMPAR rectification and cocaine

seeking behavior returned. It was then demonstrated that withdrawal from long-access cocaine self-administration also reduces mGluR1 expression and chronic mGluR1 agonism during withdrawal prolongs attenuation of seeking behavior beyond 5 days (Loweth *et al*, 2014). These findings led to the idea that mGluR1 acts as a "brake" for accumulation of GluA2-lacking AMPARs necessary for cocaine seeking behavior. Many of these findings were also repeated with amphetamine suggesting a shared mechanism of synaptic adaptations for psychostimulants (Scheyer *et al*, 2016).

Though different drug administration protocols result in differential NAc synaptic adaptations, a reoccuring finding is the strengthening of glutamatergic connections onto MSNs following drug exposure. For many of these paradigms, investigator-induced reversal of such synaptic alterations attenuates drug-associated behaviors pointing to the importance of these changes. Examination into the cellular/molecular factors mediating these synaptic adaptations may prove useful in developing treatments for cocaine and other substance use disorders.

I-5. Down with the sickness: Behavior, plasticity, and the innate immune system

With the goal of better-understanding substance use disorders, the majority of research is devoted to a region-specific, neuron-centric examination of drug effects. Though this approach revealed many important insights on mechanisms of synaptic plasticity relating to drug use, there is growing evidence implicating the brain's innate immune system in several aspects of synaptic change. Unique to the brain are microglia and astrocytes. These 2 cell types are associated with the innate immune system but do not have a direct homolog in the periphery (Ransohoff and Brown, 2012). In addition to their

roles in pathogen detection, these cells play an essential role in maintaining functional circuits through neuronal homeostasis and regulation of synaptic strength. With a focus on the NAc when possible, this section will discuss these cells' roles in regulating synaptic physiology and behavior.

I-5a. Astrocytes: The "stars" of the CNS

Astrocytes are glial cells responsible for a multitude of functions important for synaptic and neuronal health. Broadly, these include neurotrophic support, synaptogenesis/maturation/maintenance, synaptic pruning, blood flow, and uptake/secretion of a variety of factors necessary for neuronal health (Liddelow and Barres, 2015). The number, size, and complexity of astrocytes increases along the CNS complexity: they make up 20% of cells in invertebrate CNSs and up to 50% of cells in humans (Khakh and Sofroniew, 2015; Liddelow and Barres, 2015).

Based on morphologically and protein expression, these cells may be divided in up to 9 different types/categories in rodents and possibly more in humans (Ben Haim and Rowitch, 2017). These include "proteoplasmic," "fibrous," and a variety of region-specific phenotypes. Proteoplasmic astrocytes are found in the gray matter and have extensively branched processes with terminal structures known as end feet which encase blood vessels and synapses (Khakh and Sofroniew, 2015). This maintains the blood brain barrier and provides trophic support to synaptic connections (Liddelow and Barres, 2015). Fibrous astrocytes reside in white matter, are less branched, and function to repair damaged tissue and provide support to nodes of Ranvier (Liddelow and Barres, 2015). Similar to microglia, astrocytes respond to injury and disease states

through changing transcriptional profiles and morphology and become “reactive” astrocytes. Depending on the stimulus, these cells adopt “A1” or “A2” identities corresponding to pro- or anti-inflammatory effectors, respectively (Liddelow *et al*, 2017).

I-5a-i. Stars as the supporting cast: Astrocytes and neurotransmission

Astrocytes communicate with neurons through multiple mechanisms. These cells exhibit regional both within as well as between different brain regions (Ben Haim and Rowitch, 2017).

One of the well-known interactions between astrocytes and neurons is through their function in taking up neurotransmitters such as glutamate and GABA to terminate their synaptic action (Attwell *et al*, 2010). Glutamate uptake into astrocytes depends on excitatory amino acid transporters (EAAT)-1 and -2 (Hertz and Rodrigues, 2014). Once taken up, astrocytes convert glutamate to glutamine via glutamine synthase. Glutamine is transported back to axon terminals for reconversion to glutamate and vesicle packaging (Hertz and Rodrigues, 2014). GABA clearance is mediated by GABA transporter (GAT) and has been functionally demonstrated in olfactory bulb astrocytes (Doengi *et al*, 2009). Glutamate and GABA clearance by astrocytes is also associated with regulation of local blood flow (Attwell *et al*, 2010; Doengi *et al*, 2009). Beyond maintaining existing synapses, astrocytes also play a role in synaptogenesis and maturation. Work from the using slice cultures and media swaps found that astrocytes release a variety of factors controlling pre- and post-synaptic development and function. These include BDNF, cholesterol, ephrins, glypicans, hevin, SPARC, and thrombospondins (Clarke and Barres, 2013). Cholesterol increases the number of

excitatory synapses in retinal ganglion cells (RGC) possibly through providing more structural substrate. In the hippocampus, astrocytic ephrin A3 interacts with dendritic Eph A4 on pyramidal cells to cause spine retraction (Murai *et al*, 2003). Glypican 4/6 is secreted by astrocytes and strengthens glutamatergic synapses through clustering AMPARs and forming new synapses in RGC cultures (Allen *et al*, 2012). Hevin is also secreted from astrocytes and connects neurexin-1a with neuroligin-1 thalamocortical synapses (Singh *et al*, 2016). SPARC is also secreted and seems to specifically antagonize hevin (Kucukdereli *et al*, 2011). Finally, astrocytic thrombospondin-1/2 induces silent synapse formation in cultured RGCs while accelerating the formation of synapses in cultured hippocampal cells (Xu *et al*, 2010).

Astrocytes have also been implicated in synaptic pruning. Whereas microglia use the complement cascade to facilitate phagocytosis of synaptic elements (Stevens *et al*, 2007), astrocytes use the phagocytic receptors MERTK and MEGF10 in a manner independent of complement (Chung *et al*, 2013). These receptors were found necessary for pruning in the developing retinogeniculate system, and separately, astrocytes were found to engulf glutamatergic- and GABAergic synapses in the adult rodent cortex.

I-5a-ii. Astrocytic role in drug use and the NAc

With such diverse yet important roles in neuronal/synaptic physiology, it is not surprising that astrocytes are implicated in certain aspects of drug-reward behavior. In rodents, short-access cocaine self-administration and extinction is associated with changes in NAc core astrocyte morphology including decreased astrocyte volume, decreased co-

localization with synaptic markers, and decreased staining for glial fibrillary acidic protein (GFAP), a common marker for astrocytes (Scofield *et al*, 2015b). Moreover, activation of G_q signaling cascade using a hM3D DREADD in these astrocytes increases extracellular glutamate and blunts cue-induced reinstatement in a mGluR2/3-dependent manner (Scofield *et al*, 2015a).

Astrocytes also express CB1s which respond to eCBs. However, unlike neuronal presynaptic terminals where CB1 is coupled to G_o signaling, astrocytic CB1s are associated with G_q signaling cascades which increase intracellular calcium leading to release of factors that may modulate neurotransmission (Lutz *et al*, 2015). Given activation of G_q DREADDs in NAc core astrocytes is sufficient to blunt cue-induced reinstatement to cocaine self-administration, astrocytic CB1 modulation may be one mechanism behind the observation that cannabis use in humans reduces crack cocaine intake among illicit drug users (Socías *et al*, 2017).

I-5b. Microglia: Small cells with big effects

Microglia are one of several macrophages of the central nervous system (Aguzzi *et al*, 2013; Prinz and Priller, 2014) (CNS). These cells make up 10% of the brain parenchyma (Kettenmann *et al*, 2013) and play a key role in mediating immune responses in this region (Joseph and Venero, 2013; Kettenmann *et al*, 2013). Under basal conditions, these cells adopt a “ramified” morphology consisting of a small soma and fine, motile cellular processes (Kettenmann *et al*, 2011). These processes scan the neuronal environment. Upon detection of altered CNS homeostasis including infections, ischemia, and altered neuronal activity, microglia lose these fine processes and take on

an amoeboid, “activated” morphology with an altered gene expression profile (Kettenmann *et al*, 2011). Both ramified and amoeboid microglia exhibit phagocytic activity (Kettenmann *et al*, 2013).

In addition to mediating immunologic responses, microglia modulate synaptic activity through physical and chemical interactions with neurons. Facial nerve injury (Kettenmann *et al*, 2013) or cortical injection of bacterial adjuvants (Trapp *et al*, 2007) induce synaptic stripping—where local activation of microglia results in the physical removal of synaptic inputs on the regional neurons (Kettenmann *et al*, 2013).

I-5b-i. Small talk: Microglial communication with neurons

One of the mechanisms by which microglia communicate with neurons to modulate synaptic physiology is through the use of the fractalkine/CX3CL1. This is a chemotactic cytokine constitutively expressed by forebrain neurons including the striatum (Paolicelli *et al*, 2014). In the CNS, microglia are the only cells known to express the fractalkine receptor, the G_i/G_o-coupled receptor CX3CR1 (Paolicelli *et al*, 2014). In acute hippocampal slices, one research group found that bath application of fractalkine prevents induction of HFS LTP in CA1 pyramidal cells in an adenosine receptor-3 and CX3CR1-dependent manner (Maggi *et al*, 2009, 2011). Interestingly, a separate research group found that hippocampal CA1 HFS LTP could not be induced in CX3CR1 knockout mice (Rogers *et al*, 2011). Though methodological differences between these studies may account for the discrepancy in results, it’s worth noting that both sets of researchers found hippocampal synaptic plasticity altered through perturbing a microglial signaling cascade.

In addition to direct interactions between neurons and their phagocytic neighbors, microglia also secrete a variety of chemical factors known to influence synaptic physiology (Kettenmann *et al*, 2011). Both TNFα (Pascual *et al*, 2012) and BDNF (Parkhurst *et al*, 2013) are released by microglia and are known mediators of synaptic scaling (Reimers *et al*, 2014; Stellwagen and Malenka, 2006). Microglial BDNF also regulates the chloride reversal potential in spinal cord neurons (Coull *et al*, 2005).

Investigators have also examined synaptic and behavioral effects of knocking out microglia. Parkhurst et al. created an elegant microglia-specific conditional knockout mouse to assess these cells' function independent of development (Parkhurst *et al*, 2013). These investigators found that microglial elimination in adult mice leads to behavioral deficits and synaptic changes. Specifically, these mice show a decreased fear conditioning response, decreased novel object recognition, and decreased performance-based improvements on the rotarod. At the cellular/synaptic level, these mice exhibited decreased spine formation, decreased AMPAR and NMDAR-dependent mEPSC frequency, and decreased BDNF mRNA. This same study also showed that the knocking out microglial BDNF is sufficient to induce the deficits in fear conditioning and rotarod performance.

Additional evidence pointing towards the importance of microglia in regulating synaptic physiology comes from neuronal changes observed as a result of exogenously activating microglia. Pascual et al. used lipopolysaccharide (LPS), an agonist of the pattern-recognition molecule TLR4, to activate microglia and assess changes in hippocampal synaptic physiology (Pascual *et al*, 2012). These researchers found that LPS bath application on acute mouse brain slices resulted in a temporary increase in

spontaneous EPSC frequency of hippocampal CA1 pyramidal cells. This phenomenon was found to depend on TLR4, mGluR5, and microglial ATP release acting on astrocytes. Interestingly, another group of investigators found LPS combined with hypoxia induces longer-lasting changes in the form of LTD in hippocampal CA1 pyramidal cells (Zhang *et al*, 2014) dependent on complement receptor 3 (CR3) but not TLR4. This change was associated with a decrease in mEPSC amplitude but not frequency suggesting a postsynaptic change. Behaviorally, peripheral LPS injections result in decreased food intake/weight, decreased social behavior, anhedonia, peripheral inflammation, and neural inflammation (Henry *et al*, 2008). The synaptic and behavioral changes occurring as a direct result of microglial perturbation demonstrates the importance of this cell population in regulating neuronal transmission. Additional insights from the spinal cord showed that stimulation of sensory afferents results in an increase in activated microglial markers (Xanthos and Sandkühler, 2014). This suggests neuronal activity may also activate microglia.

I-5b-ii. Microglia and the innate immune system in the NAc

Much of the foundational research examining the effects of microglia and the immune system on synaptic transmission largely focused on the spinal cord, hippocampus, and cortex (see above). Whether or not the same mechanisms of neuro-immune interactions exist in the NAc in relation to drug abuse is currently a subject of intense study. Although microglia display regional heterogeneity associated with geographically unique genetic and morphologic signatures (De Biase *et al*, 2017; Grabert *et al*, 2016), a broad feature shared across the brain may be a change in gene expression in response to

neuronal activity. This has been demonstrated in the NAc. Both chronic restraint stress and binge cocaine administration cause changes in microglial properties and/or protein expression (Tynan *et al*, 2010; Wang *et al*, 2017).

This NAc neuro-immune interaction is also bi-directional. For example, the cytokine TNFα is released by microglia and decreases synaptic strength on NAc core D1 MSNs (Lewitus *et al*, 2016). This counteracts synaptic changes caused by cocaine exposure and may be a form of homeostatic scaling. Interestingly, the synaptic changes observed in the NAc in response to microglial TNFα is opposite in direction to that seen in the hippocampus (Stellwagen and Malenka, 2006). It remains to be seen whether other cytokines associated with changing hippocampal physiology including interleukin (IL)-6, IL-1B, and interferon (IFN)-γ (Bilbo and Schwarz, 2012) also have opposite NAc effects.

I-5b-iii. The toll of drugs: TLR4 and other microglial proteins' effect on drug-reward

Other sets of studies show direct interaction between drugs of abuse and portions of the neuro-immune system linked to microglia. TLR4 is a pattern-recognition molecule of the innate immune system best studied for its ability to detect LPS, a component of gram-negative bacterial cell walls (Bohannon *et al*, 2013; O'Neill *et al*, 2013). In the NAc, TLR4 is primarily associated with microglia (Schwarz *et al*, 2013). Besides LPS, TLR4 also binds numerous endogenous ligands such as heat-shock proteins and saturated fatty acids (Trotta *et al*, 2014) suggesting role in brain homeostasis. Interestingly, TLR4 may also directly interact with some drugs of abuse. Specifically, the opioids morphine and remifentanil bind with TLR4 and its accessory protein, myeloid differentiation factor

2 (MD2) (Hutchinson *et al*, 2012; Wang *et al*, 2012). Furthermore, pharmacologic block or knocking out TLR4 reduced morphine conditioned place preference and dampened morphine-induced increases in NAc extracellular dopamine levels (Hutchinson *et al*, 2012). Although TLR4 expression is typically thought limited to microglia (Béchade *et al*, 2013; Pascual *et al*, 2012), there is some evidence suggesting that this receptor is also expressed on neurons (Leow-Dyke *et al*, 2012). TLR4 is also activated with ethanol and plays a role in alcohol-induced brain damage, inflammation, and behavioral changes in mice (Alfonso-Loeches *et al*, 2010; Pascual *et al*, 2011). Given the different behavioral responses elicited with opioids and ethanol compared to sickness and anhedonia induced by LPS, it will be interesting to see how TLR4 signaling causes these effects. Future studies may also resolve the discrepancy of where TLR4 is expressed as well as test whether other illicit drugs such as cocaine bind to TLR4/MD2.

In addition to TLR4, microglia express a wide variety of proteins and receptors previously studied as regulators of NAc MSN function or drug-reward. CB1 and TRPV1, mediators of mGluR-dependent LTD in the NAc (Grueter *et al*, 2010) and hippocampus (Chávez *et al*, 2010), are both expressed on microglia (Kettenmann *et al*, 2011; Kim *et al*, 2006). Microglia also express the cannabinoid-receptor CB2 which also modulates drug-reward behavior (Xi *et al*, 2011). Microglia also express NMDARs (Kaindl *et al*, 2012)—a key player in the eponymously-named NMDAR-dependent LTP and LTD. In culture, agonizing NMDAR causes release of cytokines akin to that of LPS (Kaindl *et al*, 2012). Highlighting regional differences in microglial properties (De Biase *et al*, 2017; Tay *et al*, 2017), fluorescent-activated cell sorting analysis of rat brains revealed that microglia from the NAc express D1 and D2 receptors that are absent from microglia of

the hippocampus (Schwarz *et al*, 2013). Microglial D2 receptors in the NAc have been implicated in mediating release of TNFα in response to cocaine (Lewitus *et al*, 2016). Understanding the function of microglial TRPV1, CB1, CB2, NMDAR, and D1 receptors in the context of normal and drug-induced NAc physiology remains an active area of study.

I-6. Summary

Illicit drug use and abuse remains a substantial burden to society. The last decade of research revealed that changes in NAc MSN synaptic physiology mediates some of the behavioral manifestations of drug exposure/addiction. More tellingly, reversal of drug-induced synaptic changes in this region mitigates or reverses drug-mediated behaviors. Meanwhile, research in other regions of the CNS point towards the importance of astrocytes, microglia, and the immune system in mediating synaptic physiology and behavior. Though there is some evidence in the context of adolescent drug exposure resulting in altered adult TLR4 expression in the NAc (Schwarz and Bilbo, 2013), there is a paucity of studies examining the role of microglia and neuro-immune system modulators on NAc MSN synaptic physiology. Future research needs to combine techniques used to study hippocampal and spinal cord microglial/immune system effects with drug-induced input and output-specific electrophysiology used in recent NAc studies. Such endeavors will better characterize changes occurring in the drug-altered brain and may provide novel therapeutic targets.

CHAPTER II

TLR4 AND BASAL NAc SYNAPTIC PHYSIOLOGY AND REWARD BEHAVIOR

II-1. Introduction

The integration of dopaminergic and glutamatergic signals within the NAc is key to processing motivation, reward, and goal-directed behavior (Sesack and Grace, 2010). Exposure to drugs of abuse leads to behavioral adaptations by recruiting molecular mechanisms of learning and memory within the reward system (Grueter *et al*, 2012; Joffe *et al*, 2014). Adaptations in NAc synaptic properties following exposure to drugs of abuse have been extensively characterized in a circuit-specific manner (Joffe and Grueter, 2016; Lee *et al*, 2013; Ma *et al*, 2014; Pascoli *et al*, 2012, 2014). While these studies revealed important insights into neuronal factors and alterations, they largely ignored the contribution of non-neuronal mechanisms to synaptic adaptations underlying drug-related behaviors. Recent studies have begun to elucidate the role of the innate immune system and more specifically, microglia in drug reward behavior and physiology (Lewitus *et al*, 2016; Sekine *et al*, 2008). However, many questions remain regarding the role of the innate immune system in supporting synaptic reorganization within the reward circuitry.

TLR4 is a pattern recognition molecule of the innate immune system linked to alcohol (June *et al*, 2015), morphine (Hutchinson *et al*, 2012), and recently cocaine-associated behaviors (Northcutt *et al*, 2015). However, the conclusions surrounding alcohol and cocaine have been disputed (Harris *et al*, 2017; Tanda *et al*, 2016). TLR4

recognizes gram-negative bacteria, “danger signals” released by damaged tissue, and free fatty acids (O’Neill, 2008; Shi *et al*, 2006). Beyond pathogen detection, TLR4 is associated with a wide range of behaviors including stress-induced depression (Cheng *et al*, 2016b), visceral pain (Tramullas *et al*, 2014, 2016), and opioid reward (Hutchinson *et al*, 2012). Despite the growing number of studies pointing to TLR4’s involvement in various motivated behaviors, there has been no examination into its role in glutamatergic synaptic physiology. Additionally, the localization of TLR4 within NAc subregions is unknown. To address these questions, we performed cell-type specific electrophysiology in the NAc core and shell subregions, field potential recordings, drug reward behavioral assays, and fluorescent *in-situ* hybridization. Our findings suggest TLR4 plays a role in basal NAc core synaptic physiology, plasticity, and drug reward behavior. We also confirm microglia as the primary cells expressing *Tlr4* in the NAc core.

II-2. Results

II-2a. TLR4.KO and wild-type mice exhibit synaptic differences in the NAc core but not shell

Within the NAc core and shell subregions, 90-95% of neurons are MSNs expressing D1 or D2 dopamine receptors (Sesack and Grace, 2010). Although similar in morphology, these MSNs differ in biochemistry, anatomical connectivity, and function (Grueter *et al*, 2010; Joffe *et al*, 2017; Kupchik *et al*, 2015; Lim *et al*, 2012; Tejeda *et al*, 2017). Furthermore, experience-dependent changes of glutamatergic synapses occur in a cell-type specific manner (Graziane *et al*, 2016; Hearing *et al*, 2016; Joffe and Grueter,

2016; Lim *et al*, 2012; Pascoli *et al*, 2014; Schwartz *et al*, 2014) and activation of these NAc MSN subtypes differentially regulates drug reward behavior (Lobo *et al*, 2010). Therefore, we addressed whether TLR4 influenced excitatory synaptic function within the NAc in a cell-type specific manner.

To assess cell-type specific NAc MSN physiology, we crossed WT and TLR4.KO mice to bacterial artificial chromosome (BAC) transgenic mice expressing the tdTomato fluorophore driven by the D1 dopamine receptor promotor. Whole-cell voltage clamp recordings were made from MSNs that expressed or lacked tdTomato fluorescence (D1(+)) and lacking (D1(-)) neurons. This defines D1 and D2 MSNs as described previously (Grueter *et al*, 2013; Joffe and Grueter, 2016). To determine the impact of TLR4 expression on excitatory synaptic properties in the NAc core, we assessed multiple pre- and postsynaptic properties. In NAc core D1(+) MSNs, TLR4.KO animals exhibit a significantly decreased A/N ratio compared to WT (Figure 2A). This suggests either a decrease in post-synaptic strength through reduced AMPAR transmission or an increase in NMDAR transmission. Altered AMPAR transmission may result from differential AMPAR stoichiometry or synaptic quantity. To test for differences in AMPAR stoichiometry, we assessed AMPAR current-voltage (I-V) relationships and rectification index (RI) (Figure 2C-D). We found no evidence for differences in AMPAR stoichiometry in D1(+) MSNs. To test for alterations in synaptic AMPAR function, we analyzed the amplitudes of mESPC and found no difference between WT and TLR4.KO D1(+) MSNs (Figure 3A-B). Not surprisingly, we also found no differences in spontaneous EPSC amplitudes (sEPSC; Figure 3C).

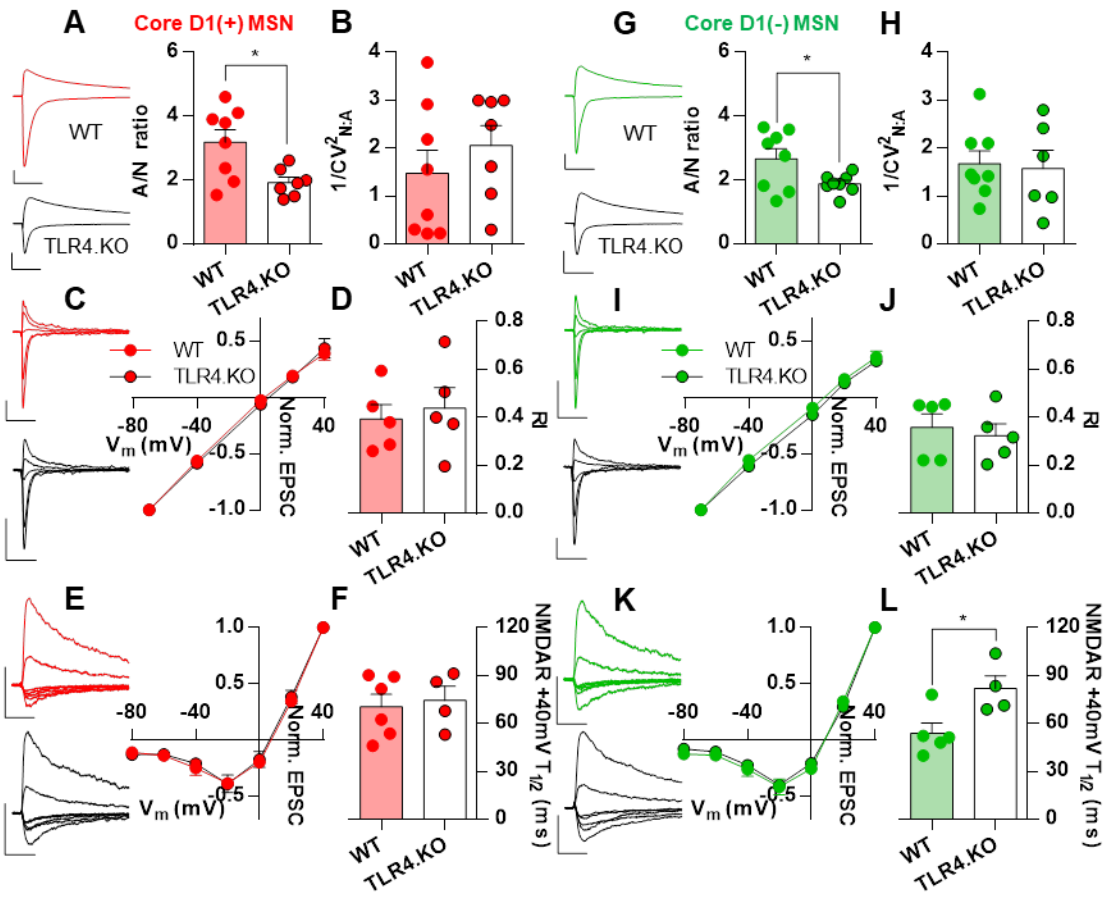

Figure 2. Altered synaptic properties in NAc core of TLR4.KO mice.
(A) (left) Representative -70 mV and +40 mV evoked current traces from D1(+) MSNs of WT (red) and TLR4.KO (black) animals. The peak current at -70 mV and the current magnitude 50 ms following current flow at +40 mV was used to calculate the AMPAR/NMDAR (A/N) ratio (WT $n_{(cells)}/N_{(mice)}$ = 8/4, TLR4.KO n/N = 7/4). *(right)* Summary plot of D1(+) A/N ratio. *(B)* Summary ratio of $1/CV^2_{NMDAR}$ to $1/CV^2_{AMPAR}$ ($1/CV^2_{N:A}$) in D1(+) MSNs (WT n/N = 8/4; TLR4.KO n/N = 7/4). *(C) (left)* Representative isolated AMPAR current traces recorded between -70 to +40 mV from D1(+) MSNs. *(right)* Mean AMPAR I-V plot from D1(+) MSNs. *(D)* Rectification index (RI; $I_{+40\ mV}$ / $I_{-70\ mV}$) (50 µM D-APV; n/N = 5/3 for both WT and TLR4.KO). *(E) (left)* Representative isolated NMDAR current traces recorded between -80 and +40 mV from D1(+) MSNs. *(right)* Mean NMDAR I-V plot from D1(+) MSNs. *(F)* Time to half-peak of +40 mV NMDAR currents (10 µM NBQX; WT n/N = 6/4, TLR4.KO n/N = 4/3). *(G-L)* Representative traces and summary plots of D1(-) MSNs for A/N ratio (WT = green, n/N = 8/4; TLR4.KO = black, n/N = 8/4), $1/CV^2_{N:A}$ (WT n/N = 8/4; TLR4.KO n/N = 5/4), AMPAR I-V and RI (n/N = 5/3 for both WT and TLR4.KO), and NMDAR I-V and NMDAR time to half-peak (WT n/N = 5/4, TLR4.KO n/N = 4/3). All recordings taken in the presence of picrotoxin (50 µM). Scale bars: 100 pA; 50 ms. *P < 0.05, unpaired t test.

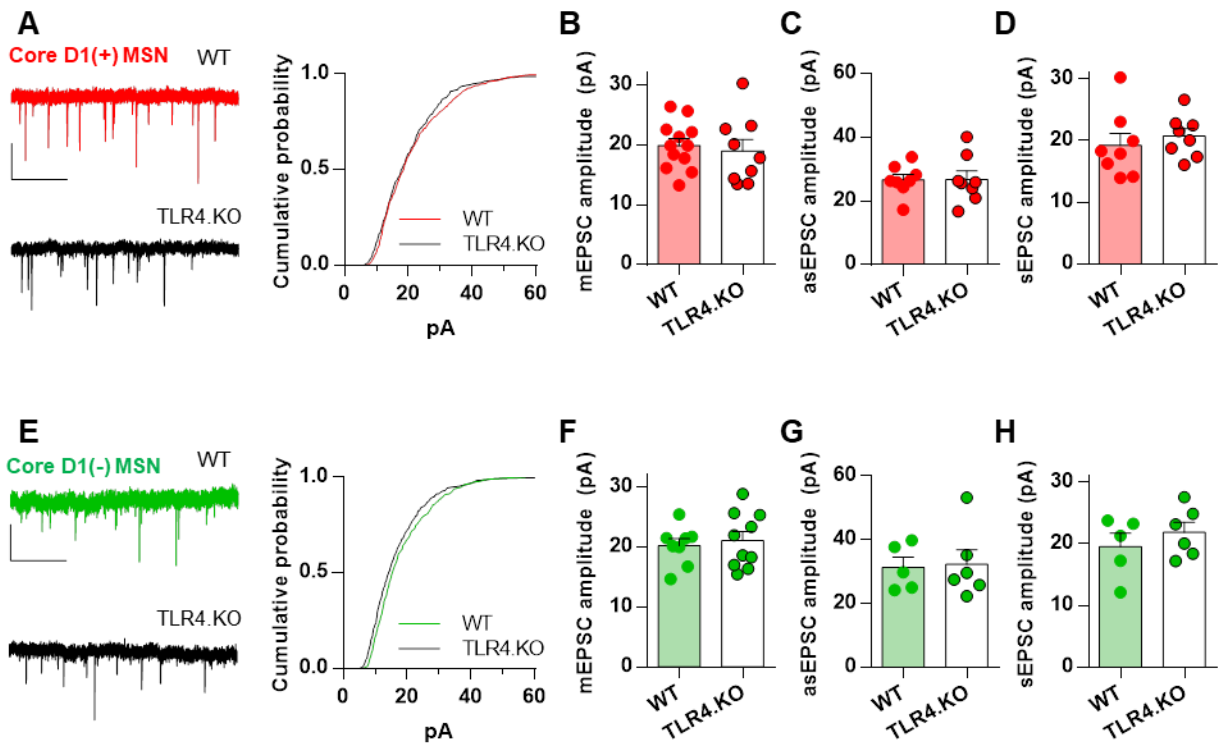

Figure 3. No significant differences in quantal AMPAR transmission onto NAc core. TLR4.KO MSNs *(A) (left)* Representative traces of mEPSCs from of WT (red) and TLR4.KO (black) D1(+) MSNs. *(right)* Cumulative probability plot of mEPSC amplitudes from D1(+) MSNs. *(B)* Summary plot of mEPSC amplitudes. *(C)* Summary plot of evoked asynchronous EPSC (asEPSC) amplitudes from D1(+) MSNs. *(D)* Summary plot of sEPSC amplitudes. *(E-H)* Representative traces, mEPSC amplitude cumulative probability and summary plot, asEPSC amplitude summary plot, and sEPSC amplitude summary plot for D1(-) MSNs (WT = green; TLR4.KO = black). All recordings taken in the presence of picrotoxin (50 μM). mEPSCs taken in the presence of tetrodotoxin (TTX; 1μM). asEPSCs and sEPSCs recorded using low Ca^{2+} ACSF with Sr^{2+}. n/N = 5-12 cells from 3-5 animals per group. Scale bars: 20 pA; 1 s. P > 0.05 for all comparisons, unpaired t test.

Whereas A/N ratios represent synaptic transmission from a subset of evoked afferents, mEPSCs and sEPSCs sample indiscriminately. To assess quantal events sampled from the evoked afferents used in A/N ratios, we used a Sr^{2+}-based artificial cerebral spinal fluid (ACSF) to record and analyze electrically-stimulated asEPSCs (Joffe and Grueter, 2016; Thomas *et al*, 2001). We found no differences in asEPSC amplitudes on D1(+)

MSNs (Figure 3D). Together these results suggest AMPAR transmission is not altered in TLR4.KO D1(+) MSNs. Therefore, the decreased A/N ratios are likely caused by altered NMDAR transmission.

Differences in NMDAR transmission may stem from receptor number, function, stoichiometry, or expression of NMDAR-only synapses. The latter are known as “silent” synapses and are an important substrate for meta-plasticity (Graziane *et al*, 2016). To assess potential differences in silent synapses, we calculated the ratio of $1/CV^2_{NMDAR}$ to $1/CV^2_{AMPAR}$ ($1/CV^2_{N:A}$) as described previously (Grueter *et al*, 2013). We found no evidence for differences in silent synapses between D1(+) MSNs from WT and TLR4.KO animals (Figure 2B). Alterations in NMDAR stoichiometry are associated with experience-dependent changes in NAc MSN physiology (Joffe *et al*, 2017). Therefore, we assessed NMDAR I-V relationships and decay kinetics for initial investigation into NMDAR stoichiometry as a potential cause of altered post-synaptic strength. We found no significant differences between WT and TLR4.KO animals in the NMDAR I-V relationship of D1(+) MSNs (Figure 2E). However, we observed a trend towards increased time to half-peak of +40mV dual component responses taken from the A/N ratios (Figure 4), to our surprise, we found no differences in isolated NMDAR decay kinetics in TLR4.KO D1(+) MSNs (Figure 2F).

In order to characterize presynaptic properties of TLR4.KO animals, we examined glutamate release probability using PPR and mEPSC frequency. PPR is inversely proportional to the presynaptic release probability. We observed that D1(+) MSNs from TLR4.KO animals have a decreased PPR at the 20 ms but not at 50, 100, 200, or 400 ms interstimulus intervals (ISI) (Figure 5A). However, this result was not

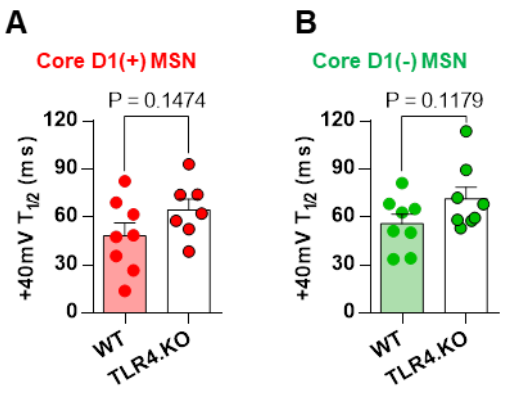

Figure 4. TLR4.KO mice trend towards increased decay kinetics of dual-component (AMPAR and NMDAR) currents.
(A) Time to half-peak of dual component currents at +40 mV from WT and TLR4.KO D1(+) MSNs (WT n/N = 8/4, TLR4.KO n/N = 7/4). *(B)* Time to half-peak of dual component currents at +40 mV from WT and TLR4.KO D1(-) MSNs (WT n/N = 8/4; TLR4.KO n/N = 8/4). P > 0.05 for all comparisons, unpaired t test.

corroborated by mEPSC frequency (Figure 5B-C; WT = 2.727 ± 0.2791 Hz; TLR4.KO = 3.367 ± 0.5189 Hz; P = 0.2598, unpaired t test). Together, these data suggest TLR4.KO animals have altered post-synaptic properties, possibly due to altered NMDAR transmission, in D1(+) MSNs in the NAc core.

Cell-type specific differences in NAc MSN synaptic physiology underlie behavioral differences in reward and motivation (Francis *et al*, 2015; Grueter *et al*, 2010; Lim *et al*, 2012; Lobo *et al*, 2010; Pascoli *et al*, 2014). Thus, we also assessed synaptic properties in NAc core D1(-) MSNs of TLR4.KO animals. We found that similar to the D1(+) cells, D1(-) MSNs exhibit a decreased A/N ratio (Figure 2G). In this population of MSNs, no differences were found for $1/CV^2_{N:A}$, AMPAR I-V or RI, mEPSC amplitude, sEPSC amplitude, asEPSC amplitude, PPR, mEPSC frequency, and NMDAR I-V (Figure 2G-L, Figure 3E-H, Figure 5D-F). However, we found that TLR4.KO D1(-) MSNs exhibit significantly slower NMDAR decay kinetics compared to WT (Figure 2L). These

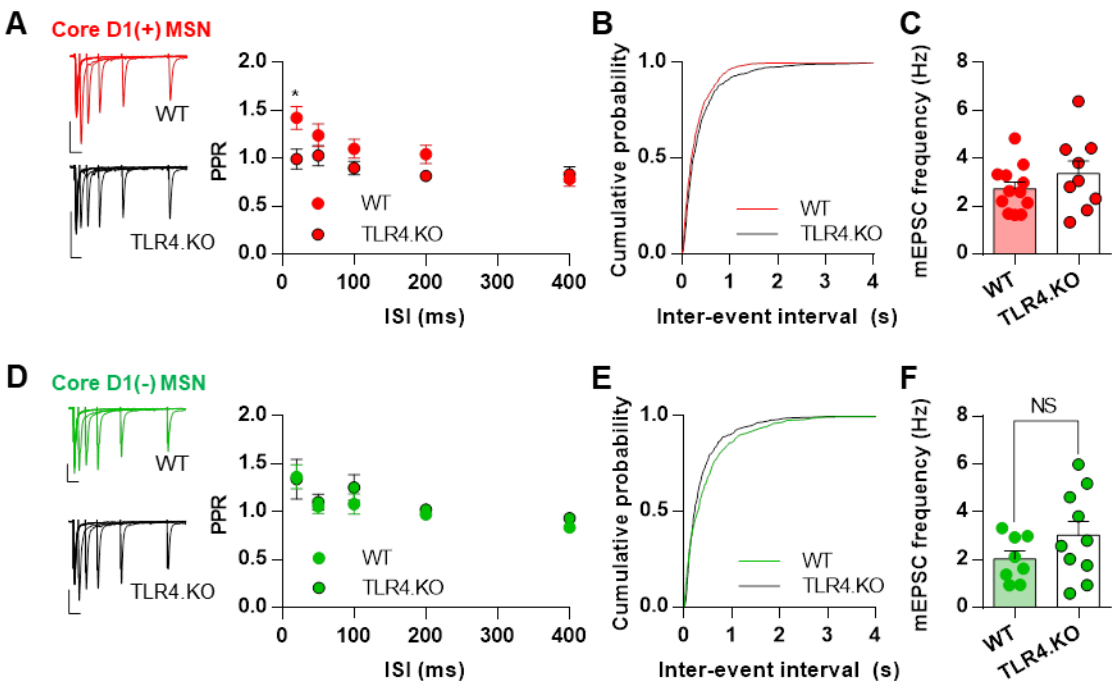

Figure 5. Presynaptic properties of NAc core TLR4.KO MSNs.
(A) (left) Representative paired pulse evoked EPSCs from WT (red; n/N = 15/6) and TLR4.KO (black; n/N = 7/4) MSNs. Interstimulus intervals (ISI) included 20-, 50-, 100-, 200-, and 400 ms. The ratio of the 2nd stimulus-evoked current over the first gives the paired pulse ratio (PPR) summarized *(right)*. Summary plot of PPR data. *(B)* Cumulative probability plot of mEPSC frequency from D1(+) MSNs. *(C)* Summary plot of mEPSC frequency from D1(+) MSNs (WT n/N = 12/5; TLR4.KO n/N = 9/4). *(D-F)* Representative traces, summary plots, and cumulative probability plot for PPR (WT = green, n/N = 14/5 ; TLR4.KO = black, n/N = 7/4) and mEPSC frequency (WT n/N = 8/5; TLR4.KO n/N = 10/4) from D1(-) MSNs. Scale bars: 100 pA, 50 ms. *P < 0.05, NS = not significant, 2-way ANOVA with Sidak post hoc test for PPR, unpaired t test for mEPSC frequency.

observations suggest that TLR4.KO MSNs exhibit altered NMDAR stoichiometry without alterations in AMPAR transmission or presynaptic release properties.

The specific GluN2 subunit greatly influences NMDAR decay kinetics. GluN2A subunits exhibit the fastest decay kinetics with the widest abundance in the adult synapse while GluN2D has the slowest with GluN2B and 2C in the middle (Paoletti *et al*, 2013). To determine the functional NMDAR profile we applied the GluN2B antagonist

Ifenprodil (3 μM) (Schwartz *et al*, 2014) to pharmacologically isolated NMDAR currents from NAc MSNs. This was followed by D-APV (50 μM) to confirm recorded currents were from NMDARs. If TLR4.KO MSNs exhibit increased GluN2B function, then Ifenprodil will cause a greater depression of NDMAR transmission in these cells. Ifenprodil caused a significant decrease in NMDAR currents from WT D1(+) MSNs (Figure 6A-B). However, we found TLR4.KO D1(+) MSNs to be insensitive to Ifenprodil (Figure 6A-B).

Though less common, increased NMDAR decay kinetics may also be caused by GluN2C or GluN2D subunits (Paoletti *et al*, 2013). To test for this, we assessed the effect of the GluN2C/D positive allosteric modulator CIQ (30 μM) (Joffe and Grueter, 2016) on isolated NMDAR currents from D1(+) MSNs. Whereas CIQ did not cause a significant difference from baseline in WT MSNs, it caused a significant potentiation in TLR4.KO cells (Figure 6C-D). The Ifenprodil and CIQ experiments were also repeated on D1(-) MSNs. Ifenprodil did not cause any significant difference from baseline in either WT or TLR4.KO D1(-) MSNs (Figure 6E-F). CIQ did not significantly alter NMDAR transmission from WT D1(-) MSNs, however, the compound caused a modest yet significant increase in NMDAR currents in TLR4.KO D1(-) MSNs (Figure 6E-H). Taken together, we provide evidence for decreased GluN2B function on TLR4.KO D1(+) MSNs along with increased GluN2C/D function in both D1(+) and D1(-) cells. Our finding that TLR4.KO animals express altered NMDAR properties on both subtypes of MSNs compared to WTs suggests a shared mechanism through which TLR4 affects synaptic physiology. Altered NMDARs in NAc MSNs are associated with behavioral adaptations

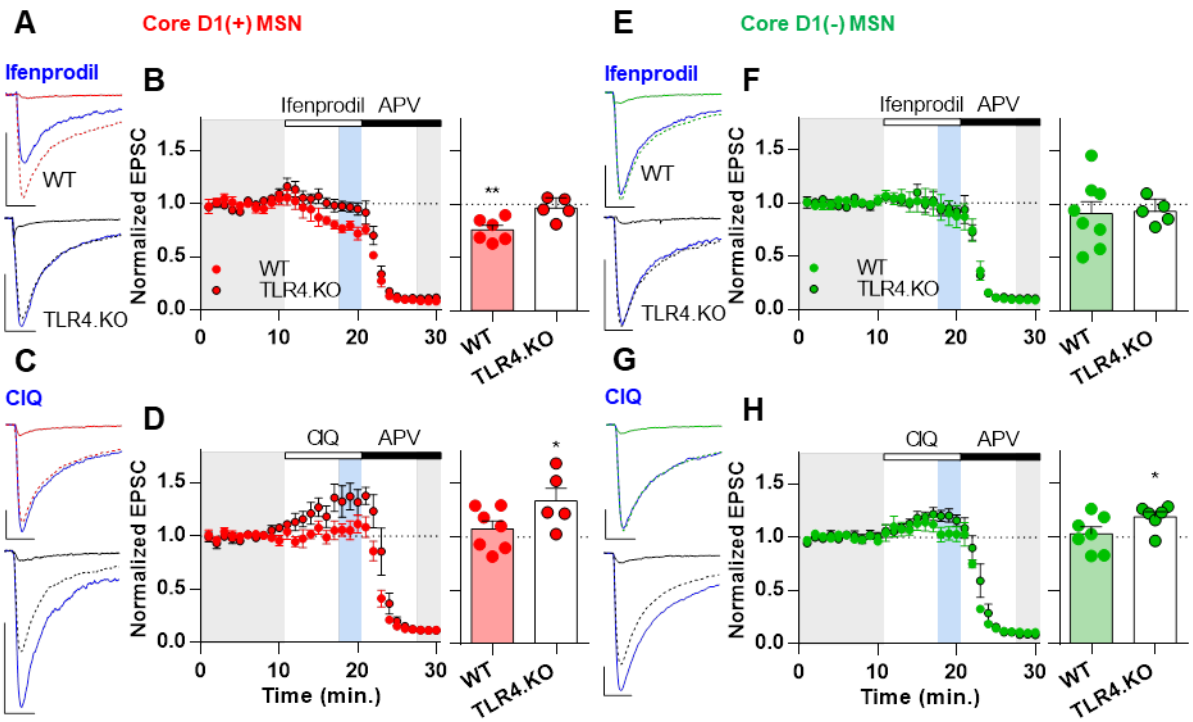

Figure 6. Altered NAc core NMDAR pharmacological profile in TLR4.KO mice. *(A)* Representative D1(+) MSN NMDAR traces from WT (red) and TLR4.KO (black) animals overlaid with traces following Ifenprodil (3 μM; blue) and APV (50 μM; solid non-blue) application. *(B) (left)* Summary plot of D1(+) Ifenprodil experiments. *(right)* Quantification of Ifenprodil response on the normalized EPSCs (WT n/N = 6/3; TLR4.KO n/N = 5/4). *(C)* Representative D1(+) MSN NMDAR traces from WT and TLR4.KO animals overlaid with traces following CIQ (30 μM; blue) and APV (solid non-blue) application. *(D) (left)* Summary plot of D1(+) CIQ experiments. *(right)* Quantification of CIQ response on normalized EPSCs (WT n/N = 7/4; TLR4.KO n/N = 5/4). *(E-H)* Representative traces, summary plots, and quantification of D1(-) MSNs for Ifenprodil (WT = green, n/N = 8/5; TLR4.KO = black, 5/4) and CIQ experiments (WT n/N = 7/4; TLR4.KO n/N = 6/4). All experiments performed holding the cell at -50 mV using a low-Mg^{2+} solution with picrotoxin (50 μM) and NBQX (10 μM). Scale bars: 100 pA; 50 ms. *P < 0.05, **P < 0.01, one-sample t test vs baseline value of 1.0.

affecting motivation including chronic social defeat (Jiang *et al*, 2013), chronic pain (Schwartz *et al*, 2014), cocaine experience and withdrawal (Joffe and Grueter, 2016), aversion-resistant ethanol intake (Seif *et al*, 2013), and chronic intermittent ethanol exposure (Renteria *et al*, 2017). A basal difference in NMDAR transmission on both

MSN types raises the possibility that TLR4.KO animals may exhibit altered learning mechanisms related to NAc core-dependent motivational and reward behavior.

The anatomy and physiology of the NAc shell is distinct from the NAc core with different hippocampal (Groenewegen *et al*, 1987), prefrontal cortical- (Ma *et al*, 2014), and midbrain dopaminergic inputs (Everitt and Robbins, 2005; Sesack and Grace, 2010). Thus, it is not surprising that experience-dependent changes in MSN synaptic physiology differ between the subregions (Grueter *et al*, 2013; Ma *et al*, 2014; Martin *et al*, 2006). To determine whether synaptic differences seen in TLR4.KO animals are specific to the NAc core, we also performed cell-type specific voltage clamp recordings from NAc shell MSNs. Unlike the core subregion, we observed no post-synaptic differences between TLR4.KO and WT D1(+) and D1(-) NAc shell MSNs as assessed through A/N ratios and $1/CV^2_{N:A}$ (Figure 7, $P > 0.05$ for all comparisons, unpaired t test). We did however observe a reduction of PPR in TLR4.KO D1(-) MSNs (Figure 7; genotype effect $F(1,13) = 6.632$, $P = 0.0231$, 2-way repeated measures ANOVA) suggesting altered presynaptic release probability. Taken together, we conclude that TLR4.KO animals exhibit an alteration in post-synaptic properties in both MSNs subtypes of the NAc core but not shell subregions.

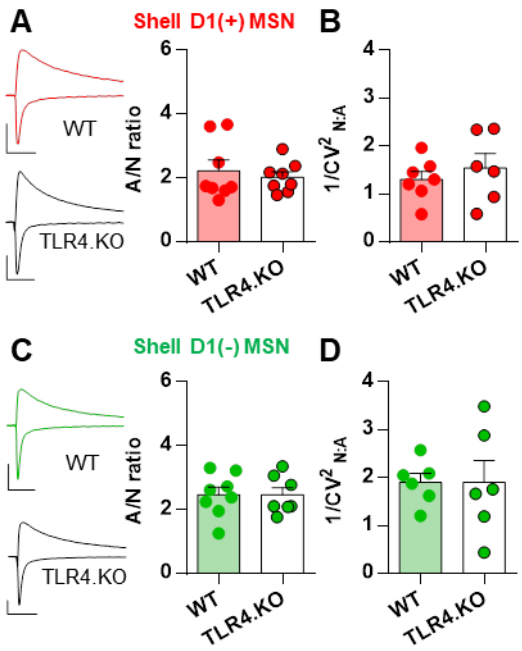

Figure 7. Lack of post-synaptic differences in the NAc shell.
(A) (left) Representative evoked current traces recorded from -70 mV and +40 mV from D1(+) MSNs of WT (red) and TLR4.KO (black) animals. *(right)* Summary plot of A/N ratios (WT n/N = 8/5, TLR4.KO n/N = 8/4). *(B)* Summary plot of $1/CV^2_{N:A}$ in D1(+) MSNs (WT n/N = 7/4; TLR4.KO n/N = 6/4). *(C-D)* Representative traces and summary plots of D1(-) MSNs for A/N ratio (WT = green, n/N = 8/5; TLR4.KO = black, n/N = 7/4), $1/CV^2_{N:A}$ (WT n/N = 6/5; TLR4.KO n/N = 6/4), All recordings taken in the presence of picrotoxin (50 μM). Scale bars: 100 pA; 50 ms. $P > 0.05$ for all comparisons, unpaired t test.

II-2b. TLR4.KO mice exhibit LTD deficits in the NAc core

Synaptic plasticity is a substrate for learning and memory. Within the NAc, perturbations in plasticity mechanisms are associated with alterations in reward and motivation-related behaviors (Grueter *et al*, 2010; Lim *et al*, 2012; Schwartz *et al*, 2014). In addition, behavioral experiences related to stress (Lim *et al*, 2012), pain (Schwartz *et al*, 2014), social reward (Dölen *et al*, 2013), and drugs of abuse (Graziane *et al*, 2016; Joffe and Grueter, 2016; Lee *et al*, 2013; Ma *et al*, 2014; Thomas *et al*, 2001) alter plasticity mechanisms in the NAc. With evidence for altered NMDAR transmission on NAc core

MSNs, we thought that TLR4.KO animals might exhibit deficits in NMDAR-dependent synaptic long-term depression. To test this hypothesis, we performed extracellular field potential recordings from the NAc core. Using a well-established NMDAR-dependent LFS stimulation protocol (3 x 3 min., 5 Hz stimulation of NAc afferents with 5 min. between each LFS train) (Lim *et al*, 2012; Mary Brown *et al*, 2015; Thomas *et al*, 2001), we assessed LTD in WT and TLR4.KO mice. In support of our hypothesis, this stimulation protocol induced a depression of evoked field potential responses in slices from WT animals but not TLR4.KO animals (Figure 8A-C). To confirm that this lack of LFS-LTD is not due to general lack of plasticity mechanisms in TLR4.KO animals, we assessed LTD dependent on Group II mGluR. Application of the Group II mGluR agonist LY 379268 (200 nM; 10 min) (Kasanetz *et al*, 2010) caused a significant reduction in field potentials in both WT and TLR4.KO animals (Figure 8D-F). Thus, the NAc core of TLR4.KO animals exhibit impairments in NMDAR-dependent LTD without deficits in Group II mGluR-dependent LTD. In combination with our whole-cell electrophysiology results showing differences in NMDAR subunit composition, these data suggest the lack of LTD in TLR4.KO is due to impairments in induction mechanisms. Loss of TLR4 function therefore hinders the ability of LFS to reduce synaptic strength onto NAc core MSNs.

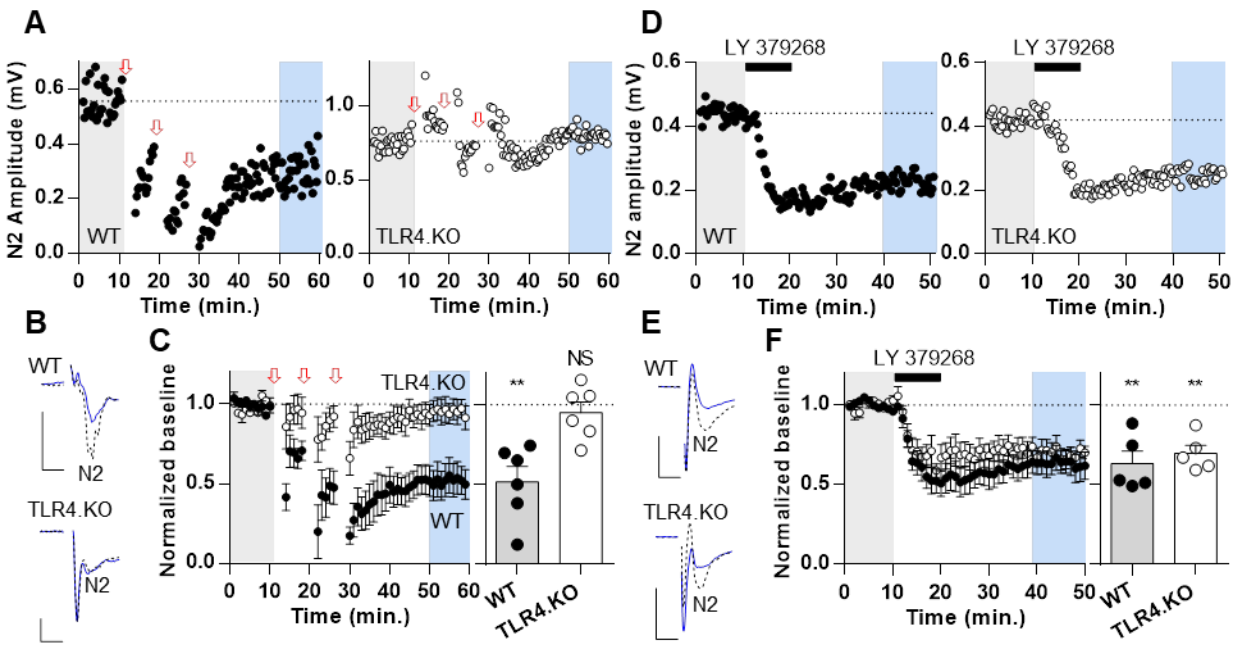

Figure 8. TLR4.KO mice exhibit impaired NMDAR-dependent long-term depression (LTD) but maintain Group II mGluR LTD in the NAc core.
(A) (left) Representative NMDAR LTD field potential experiment from WT NAc core slice. Following 10 min. of stable N2 amplitudes, a low-frequency stimulation (LFS) protocol is induced (3 x 3 min., 5 Hz with 5min. between each bout). N2 amplitudes were monitored for 30 min. following LFS. Arrows denote LFS. *(right)* Representative experiment from TLR4.KO NAc core slice. *(B)* Representative traces from baseline (dashed black) and post LFS (blue) of the experiment from WT and TLR4.KO animals. (C) *(left)* Summary plot and *(right)* quantification of LTD experiments (WT $n_{experiments}$/N = 6/5; TLR4.KO n/N = 6/4). A one-sample t test was performed to compare the normalized EPSCs from the last 10 min. of post-LFS responses to a value of 1.0. *(D-F)* Representative experiments, summary plot, and quantification of Group II mGluR LTD field potential experiments. Following 10 min. of stable baseline N2 amplitudes, the Group II mGluR agonist LY 379268 (200 nM) was bath applied for 10 min. Responses were monitored for an additional 30 min. following drug removal (WT n/N = 5/4; TLR4.KO n/N = 5/3). All recordings taken in the presence of picrotoxin (50 μM). Scale bars: 0.4 mV; 4 ms. NS = not significant, **P < 0.01, one-sample t test vs. baseline value of 1.0.

II-2c. TLR4.KO mice exhibit deficits in drug reward learning

The NAc core is a nexus for drug seeking and motivational behavior (Sesack and Grace, 2010), therefore the inability to regulate synaptic strength in this region has implications in associated learning. Deficits in NMDAR-dependent synaptic plasticity mechanisms are associated with altered drug reward behavior (Kasanetz *et al*, 2010; Kauer and Malenka, 2007; Martin *et al*, 2006). With evidence for a deficit in NAc core NMDAR-dependent LTD, we hypothesized that TLR4.KO animals exhibit altered drug reward learning. To test this, we performed a cocaine place conditioning (conditioned place preference, or CPP) assay as previously described (Joffe *et al*, 2017) (Figure 9A). In this assay, all mice were given 3 injections of cocaine and 3 injections of saline (1 of each/context pairing day; Figure 9A). We found that TLR4.KO mice have a significant attenuation in preference following conditioning with a 5 mg/kg dose (Figure 9B). To test whether this result signifies an impairment or a complete loss of cocaine reward learning, we examined two higher doses of cocaine (10 and 15 mg/kg). In support of TLR4.KO animals having decreased cocaine reward learning, we found no significant differences in CPP between WT and TLR4.KO animals for 10- and 15 mg/kg cocaine (Figure 9B).

Additionally, TLR4.KO animals did not maintain a change in preference for 10 mg/kg cocaine when assessed 14 days later (Figure 9C), suggesting TLR4.KO animals may have a decreased persistence of drug reward learning. Furthermore, TLR4.KO animals display less cocaine-induced hyperactivity than WT mice (Figure 9D). The differences between genotypes are most evident at the highest cocaine dose tested.

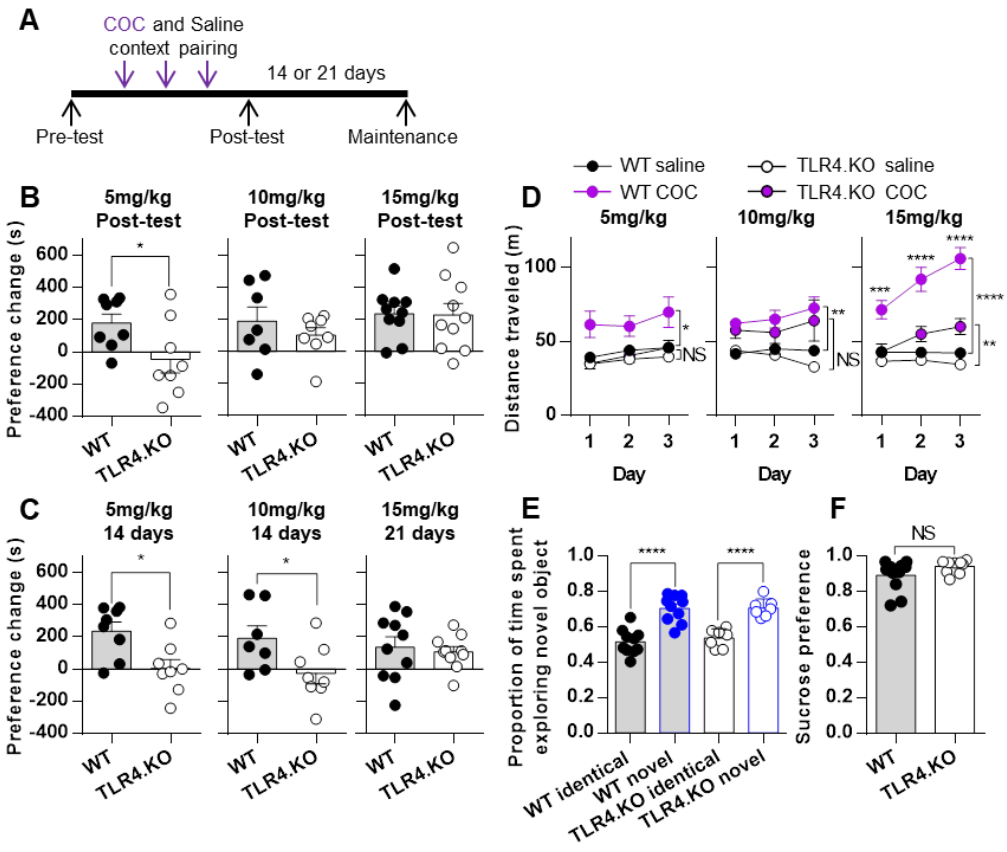

Figure 9. TLR4.KO mice exhibit attenuated drug reward learning without deficits in episodic memory or expression of anhedonia.
(A) Timeline of cocaine (COC) conditioned place preference (CPP) at three doses of COC (5, 10, and 15 mg/kg). *(B)* Preference changes assessed at the post-test time point compared to pre-test. *P < 0.05, unpaired t-test. *(C)* Preference changes at the maintenance time point as compared to pre-test. *P < 0.05, unpaired t-test. *(D)* Locomotor response to saline and COC during context pairing days. 5 mg/kg WT saline vs COC $F(1,14) = 5.851$, $P = 0.0298$; 5 mg/kg TLR4.KO saline vs COC $F(1,14) = 0.4099$, $P = 0.5324$; 10 mg/kg WT saline vs COC $F(1,12) = 14.55$, $P = 0.0025$; 10 mg/kg TLR4.KO saline vs COC $F(1,14) = 4.178$, $P = 0.0602$; 15 mg/kg WT saline vs COC $F(1,18) = 92.46$, $P < 0.0001$; 15 mg/kg TLR4.KO saline vs COC $F(1,18) = 13.83$, $P = 0.0016$, 2-way repeated measures ANOVA. ***P < 0.001, ****P < 0.0001, Sidak post hoc test for TLR4.KO COC vs WT COC. *(E)* Proportion of time(s) spent exploring objects in novel object recognition task. Genotype effect $F(1,15) = 0.1981$, $P = 0.6626$; Object effect $F(1,15) = 87.32$, $P < 0.0001$; WT identical vs novel, $t = 7.658$, $P < 0.0001$; TLR4.KO identical vs novel, $t = 5.777$, $P < 0.0001$, 2-way repeated measures ANOVA. ****P < 0.0001, Sidak post hoc test. (F) Summary of 18 h 2-bottle choice sucrose preference test. NS = not significant, unpaired t test. N = 7-11 animals/group for all experiments.

Importantly, these reductions in preference are not due to differences in episodic memory as TLR4.KO do not significantly differ in preference change from WT mice at 15 mg/kg cocaine (Figure 9B) and show no deficits in novel object recognition, a hippocampus-associated task (Figure 9E). In addition, anhedonia is not a likely cause for impaired drug reward learning as we found no differences between WT and TLR4.KO animals for the sucrose preference test (Figure 9F). Finally, to control for basal behavioral states, we assessed open field locomotor activity and center time. No differences were observed for distance traveled. However, there was a trend towards decreased center time in the TLR4.KO animals (WT = 843.5 ± 98.52 s; TLR4.KO = 615.3 ± 79.72 s; P = 0.0923, unpaired t test) (Figure 10A-B). These results support our hypothesis that TLR4.KO animals exhibit specific deficits in drug reward learning.

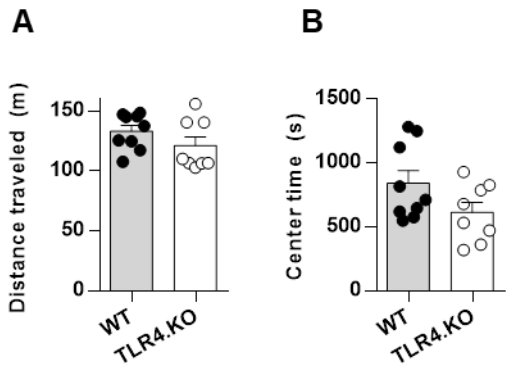

Figure 10. No significant differences seen in distance traveled or center time as assessed by 60 min. open field test.
(A) Locomotor activity of WT and TLR4.KO animals. *(B)* Total center time of same assessed in same test. WT N = 9; TLR4.KO N = 8. P > 0.05 for both measurements, unpaired t test.

II-2d. TLR4 in NAc core expressed primarily on microglia

To determine where *Tlr4* was expressed within the NAc core, we performed multiplex fluorescent in-situ hybridization from frozen NAc core sections taken from naïve WT mice. Prior studies looking into *Tlr4* expression in the NAc used fluorescence activated cell sorting followed by qPCR but did not differentiate between the core and shell subregions (Schwarz *et al*, 2013). Consistent with results from the NAc as a whole (Schwarz *et al*, 2013), we found that the majority (~80%) of TLR4 expressing cells in the NAc core could be classified as microglia (Figure 11; *Tlr4*+, *Iba1*+, *Gfap*-; $n_{(cells)}$ = 114/143; $N_{(animals)}$ = 4). The rest included astrocytes (*Tlr4* +, *Gfap*+, *Iba1*-; n = 9/143), cells expressing both astrocytic and microglial markers (*Tlr4*+, *Iba1*+, *Gfap*+; n = 13/143) and some other cell population (*Tlr4*+, *Iba1*-, *Gfap*-; n = 7/143). These results suggest the possibility of an interaction between NAc core MSNs and microglia mediating the synaptic and behavioral effects observed in TLR4.KO animals.

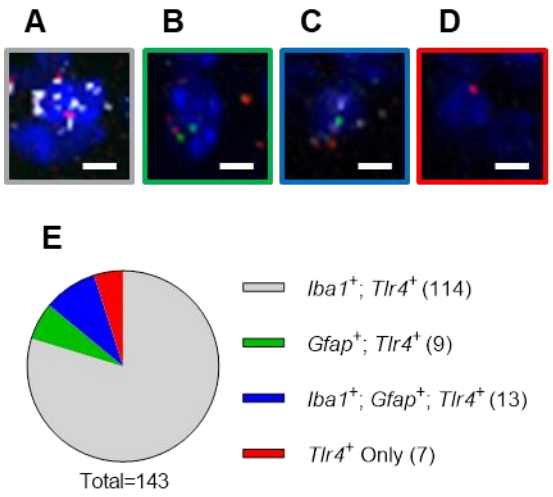

Figure 11. NAc core *Tlr4* expression is primarily on microglia.
Multiplex fluorescent in-situ hybridization was performed using RNAscope to detect mRNA transcripts for *Tlr4* (red), *Iba1* (white), and *Gfap* (green) on a background of DAPI (blue). *(A)* Representative *Iba1*+, *Tlr4*+ cell. *(B)* Representative *Gfap*+, *Tlr4*+ cell. *(C)* Representative *Iba1*+, *Gfap*+, *Tlr4*+ cell. *(D)* Representative *Tlr4*+ cell. *(E)* Summary of cell counts (n/N = 143/4). 79.72% of quantified cells were *Iba1*+, *Tlr4*+. Scale bars: 5 μm.

II-3. Discussion

In the present study, we utilized TLR4.KO and cell-type specific reporter mice to investigate the interaction between the innate immune system and key elements of the reward circuit. We provide evidence that TLR4 significantly influences NAc core NMDAR synaptic transmission, synaptic plasticity, and cocaine-induced behavioral plasticity. Whereas we observed altered NMDAR transmission and plasticity in the NAc core, we found no differences between WT and TLR4.KO animals in the neighboring NAc shell. Furthermore, we found these mice exhibit blunted behavioral responding to cocaine and that NAc core *Tlr4* is primarily expressed on microglia. These results suggest TLR4, likely expressed on microglia, is a novel molecular regulator in the NAc associated with reward learning.

II-3a. TLR4 and drug reward behavior

Though numerous neuron-centric studies have revealed important insights into how drugs of abuse alter behavior and NAc physiology, far less is known about the role of the innate immune system in this sequela. One intriguing molecular target is the pattern recognition molecule TLR4. Along with a storied history in innate immunity and pathogen detection (O'Neill *et al*, 2013), recent research suggests TLR4 may play a role in reward behaviors associated with alcohol (June *et al*, 2015), morphine (Hutchinson *et al*, 2012), and cocaine (Northcutt *et al*, 2015). Northcutt et al. (Northcutt *et al*, 2015) demonstrate that TLR4 antagonists diminish cocaine self-administration in rats through an effect mediated by the VTA. These investigators also found cocaine reward behavior diminished in the C3H/HeJ mouse line. Though the C3H/HeJ line is deficient in TLR4, these mice are also homozygous for an inversion spanning 20% of chromosome 6 (The Jackson Laboratory, n.d.) making it difficult to draw specific conclusions. A later study also found that the pharmacologic reduction in cocaine reward in rats may be due to non-specific effects as the same doses TLR4 antagonists also caused decreased food reward behavior (Tanda *et al*, 2016). Additionally, TLR4's role in alcohol reward has also been disputed (Harris *et al*, 2017). These discrepancies prompt continued investigation of the nature of TLR4's involvement in drug reward.

II-3b. TLR4 and NAc synaptic physiology

The NAc is a brain region that integrates information on motivation and reward to initiate goal-directed behavior (Sesack and Grace, 2010). Virtually every drug of abuse causes changes within the NAc (Grueter *et al*, 2012; Joffe *et al*, 2014), and reversal of synaptic

changes leads to reversal of drug reward learning (Lee *et al*, 2013; Ma *et al*, 2014; Pascoli *et al*, 2014). These changes occur in a subregion (Ma *et al*, 2014; Martin *et al*, 2006) and cell-type (Graziane *et al*, 2016; Grueter *et al*, 2010; Hearing *et al*, 2016) specific manner.

Given the importance of NAc MSN synaptic physiology on drug reward, we carried out electrophysiology experiments to test TLR4's involvement NAc core and shell D1(+) and D1(-) MSNs. We found that naïve TLR4.KO mice had decreased A/N ratios in both D1(+) and D1(-) MSNs in the NAc core but not shell. Decreased A/N ratios may broadly result from decreased AMPAR transmission, altered NMDAR transmission, or some combination of both. Reduced AMPAR transmission may be a result of altered AMPAR stoichiometry or reduced AMPAR density. No observed differences in AMPAR I-V or rectification argues against differences in AMPAR stoichiometry. A lack of difference in spontaneous and evoked quantal events in the form of mEPSC, sEPSC, and asEPSC amplitudes argues against reduced AMPAR density. This leaves altered NMDAR transmission as the likely cause for decreased A/N ratios.

Our results in TLR4.KO animals showed significantly slower isolated NMDAR decay kinetics in putative D2 MSNs and a trend towards slower dual component (AMPAR and NMDAR) kinetics in D1 MSNs. As increased NMDAR decay kinetics are commonly associated with upregulation of GluN2B subunits (Schwartz *et al*, 2014) but could also be due to greater functional expression of GluN2C-D, we hypothesized an upregulation of their function TLR4.KO MSNs. We found blunted Ifenprodil sensitivity of TLR4.KO D1 MSNs along with a significant potentiation of NMDAR currents with CIQ in D1 and putative D2 MSNs. These results suggest *decreased* GluN2B function in D1

MSNs from TLR4.KOs along with *increased* GluN2C/D function in both MSN populations. As GluN2C and GluN2D NMDAR subunits exhibit decay kinetics even slower than that of GluN2B (Wyllie *et al*, 2013), their upregulation in TLR4.KO MSNs provides an explanation for decreased A/N ratios.

A second possible explanation for decreased A/N ratios is an increase in NMDAR-only containing silent synapses. In the neighboring NAc shell, silent synapses generated in the context of cocaine exposure are enriched with the GluN2B NMDAR subunit (Graziane *et al*, 2016). We found no significant differences in $1/CV^2_{N:A}$, an estimation of the number of silent synapses (Grueter *et al*, 2013), and reduced GluN2B function on D1(+) MSNs from TLR4.KOs. Taken together, these results argue against increased NMDAR transmission due to increased silent synapse number. Although we did not test for synaptic NMDAR density, our results point towards altered NMDAR subunit stoichiometry contributing to the differences observed for A/N ratios. Alterations of NAc NMDAR GluN2 subunits are associated with behavioral paradigms known to affect motivation and reward. In the NAc core, this includes increased GluN2B function in D1(-) MSNs following chronic pain (Schwartz *et al*, 2014), increased GluN2C/D function of thalamic inputs onto D1(+) MSNs following cocaine exposure and withdrawal (Joffe and Grueter, 2016), and aversion-resistant ethanol intake requiring GluN2C function on prefrontal-cortical and insular inputs (Seif *et al*, 2013). In addition, studies performed examining the neighboring shell subregion or the NAc as a whole provide additional evidence for the importance of GluN2 subunits on motivation/reward behaviors (Graziane *et al*, 2016; Jiang *et al*, 2013; Renteria *et al*, 2017).

We showed that TLR4.KO mice exhibit a deficit in LTD associated with NMDARs. This idea was supported through extracellular field potential recordings revealing a lack of LFS-LTD but intact Group II mGluR-dependent LTD from TLR4.KO mice. Much controversy remains on the role of the specific NMDAR subunits in scaling synaptic plasticity from LTP to LTD where some suggest LTD is dependent on GluN2B whereas GluN2A is important for LTP (Paoletti *et al*, 2013). An intriguing possibility is that TLR4 regulates the NMDAR-dependent threshold for inducing LTP vs LTD. A reduced sensitivity to the GluN2B antagonist Ifenprodil, along with greater GluN2C/D sensitivity in TLR4.KO mice may shift the propensity of synaptic plasticity rather than prevention. Further experiments are necessary to test this possibility. In combination, our experiments suggest TLR4.KO animals exhibit a NAc core-specific alteration of NMDAR transmission and a deficit in NMDAR-dependent synaptic plasticity. Thus, TLR4 may play a role in the developmental profile of NMDARs at NAc core synapses.

A lack of LFS- LTD in the NAc core is associated with a range of behavioral manipulations affecting motivation and reward including cocaine experience (Kasanetz *et al*, 2010; Martin *et al*, 2006), exposure to palatable foods (Mary Brown *et al*, 2015), chronic social defeat (Jiang *et al*, 2013), and chronic restraint stress (Lim *et al*, 2012). However, many of these plasticity assays were only examined following behavioral experiments and distinctions between LTD induction and expression were not always clarified. Psychostimulants such as cocaine can depress excitatory synapses in the NAc core (Kauer and Malenka, 2007) and blocking NMDAR-dependent LTD expression through inhibiting AMPAR endocytosis prevents locomotor sensitization (Brebner *et al*, 2005). On the other hand, loss of NMDAR-dependent LTD is associated with cocaine-

exposed mice exhibiting signs of “addiction” (drug seeking, motivation, and continued use despite negative consequences) compared to cocaine-exposed “non-addicted” mice (Kasanetz *et al*, 2010). Given the range of behavioral adaptations associated with NMDAR-dependent plasticity, it is difficult to make a specific prediction for the behavioral effect of a presumed change NMDAR-dependent LTD induction mechanism in naïve TLR4.KO animals. We found that TLR4.KO mice express attenuations in cocaine CPP and associated locomotor sensitization, both non-contingent drug reward learning processes.

To confirm the cell type(s) expressing TLR4 in the NAc, we performed fluorescent in-situ hybridization on brain sections. We found that the majority of *Tlr4*-expressing cells in the NAc core are microglia, echoing fluorescent-assorted cell sorting/qPCR results from the NAc as a whole (Schwarz *et al*, 2013). Given the many differences seen in WT mice when examining NAc synaptic physiology in an input specific manner, it will be interesting to see whether TLR4 and/or microglia influence any part of this.

II-3c. Microglia, immune signaling, and drug reward

Throughout the brain, microglia and their associated cytokines play important roles in modulation of synapses via multiple mechanisms. These include complement-mediated pruning of spines during development of the reticulogeniculate system (Schafer *et al*, 2012), regulation of synaptogenesis/elimination in the motor cortex through microglial BDNF (Parkhurst *et al*, 2013), and the homeostatic scaling up of synapses in the hippocampus through TNFα (Stellwagen and Malenka, 2006). Microglia also exhibit

brain-region specific differences in cellular aging and transcriptional profiles (Grabert *et al*, 2016) which may underlie observed synaptic differences between the NAc core and shell. Importantly, microglia have also been implicated in drug addiction. Methamphetamine induce activation of microglia in humans (Sekine *et al*, 2008). Additionally, rodent models suggest microglial changes in protein expression following cocaine administration (Wang *et al*, 2017).

In the NAc core, microglial TNFα scales down synaptic strength on D1(+) MSNs in response to cocaine and that lacking this cytokine exacerbates cocaine locomotor sensitization (Lewitus *et al*, 2016). This suggests TNFα combats drug-induced increases in synaptic strength. In the peripheral organs, TNFα is one of several known cytokines released in response to ligands binding TLR4 (Bohannon *et al*, 2013). Cytokines originating from the periphery can also directly influence synaptic connections. TNFα from peripheral monocytes also influence motor learning and cortical dendritic spine dynamics independent of microglia (Garre *et al*, 2017). We showed that TLR4.KO animals express a basal difference NAc NMDARs with an attenuation of cocaine reward learning and associated motor changes. This suggests a mechanism independent of TNFα underlying the associated findings. Given the importance of microglia in shaping synaptic physiology and behavior along with TLR4's role in detecting factors such as free fatty acids (Shi *et al*, 2006) and damaged tissue signals (O'Neill, 2008) in addition to pathogens (O'Neill *et al*, 2013), it is tempting to think about loss of a constitutively active signaling cascade or altered gut-brain communication (Fung *et al*, 2017) perturbing microglia to cause a basal change in NAc physiology.

In summary, we show that TLR4 influences NAc synaptic function and cocaine behavior. These results expand upon the spectrum of neurobiological adaptations underlying drug-induced behavioral learning. These findings also contribute to the implication of TLR4 in a range of medical issues beyond infection. These include stress (Cheng *et al*, 2016b), visceral pain-linked depression (Tramullas *et al*, 2014, 2016), opioid reward (Hutchinson *et al*, 2012), and insulin resistance (Shi *et al*, 2006). With many of these conditions linked to alterations in motivation and reward, it is tantalizing to imagine the NAc as a nexus for such TLR4-associated pathologies.

CHAPTER III

TLR4 AND NAc SYNAPTIC PHYSIOLOGY WITH COCAINE EXPOSURE

III-1. Introduction

Cocaine and other drugs of abuse cause synaptic changes in the NAc core that are important for expression of drug-reward behavior (Kauer and Malenka, 2007). Acting as a central hub for reward, the NAc core integrates glutamatergic signals from multiple brain areas signifying different aspects of the drug experience. These inputs drive the activity of otherwise quiescent MSNs—the principle cells making up 90-95% of the NAc's neuronal population (Sesack and Grace, 2010). MSNs are further-divided into 2 populations: those expressing the D1 dopamine receptor and those expressing the D2 dopamine receptor (Sesack and Grace, 2010). MSN subtypes differentially regulate drug-reward behavior (Lobo *et al*, 2010).

Exposure to drugs of abuse such as cocaine cause NAc core synaptic changes occurring in input, cell-type, and region-specific manner (Jedynak *et al*, 2016; Joffe and Grueter, 2016; Ma *et al*, 2014). Such changes are thought important for drug-reward as restoration of synaptic properties modulate drug-reward behavior (Loweth *et al*, 2014; Ma *et al*, 2014; Moussawi *et al*, 2009; Scheyer *et al*, 2016). Over the past 3 decades, research on the NAc primarily focused on the nature of pre- and postsynaptic proteins and adaptations occurring in response to drug experience. Such studies gave important insights into neuronal factors underlying learning, memory, and behavior. More recent studies implicated the importance of non-neuronal factors influencing synaptic

physiology and behavior. The innate immune system—particularly astrocytes, microglia, and associated cytokines such as TNFα—have been demonstrated to sculpt NAc synaptic physiology and influence drug-reward behavior (Knackstedt *et al*, 2010; Lewitus *et al*, 2016). More recently, it was demonstrated loss of the pattern-recognition molecule TLR4 results in changes in basal NAc synaptic physiology as well as cocaine-reward behavior (Kashima and Grueter, 2017). However, its role in cocaine-induced synaptic changes is unknown. In addition, drug-reward behavioral assessment of males vs. females in the context of neuro-immune influences is lacking. To address these gaps in knowledge, we performed cocaine locomotor sensitization on male and female WT and TLR4.KO mice. We further characterized NAc core MSN synaptic physiology from male WT and TLR4.KO mice in a cell-type specific manner at 3 time points following cocaine exposure. We provide evidence for sex-based differences in cocaine locomotor sensitization following withdrawal as well as cell-type specific alterations in NAc MSNs associated with acquisition but not expression of cocaine locomotor sensitization.

III-2. Results

III-2a. TLR4.KO animals exhibit impaired cocaine locomotor response

TLR4 has previously been linked to drug-reward behaviors associated with morphine (Hutchinson *et al*, 2012), alcohol (June *et al*, 2015), and cocaine (Kashima and Grueter, 2017; Northcutt *et al*, 2015). Locomotor-sensitization to cocaine has not been reported. In addition, published behavioral studies examining TLR4 and drug-reward largely ignored females. Therefore, we performed cocaine locomotor sensitization in WT and

TLR4.KO mice in a sex-specific manner (Figure 12). We found that the locomotor response to cocaine was attenuated in both male and female TLR4.KO mice for the first 5 days of injections (Figure 12B-C). However, while TLR4.KO males maintained a reduced locomotor response compared to WT following abstinence and a challenge dose of cocaine, we found that the response to cocaine in TLR4.KO females increased with abstinence and was no different from WT with a challenge dose (Day 21, Figure 12B-C). Though this points to a gender-difference in TLR4.KO animals, there is also a commonality for initial reduction in cocaine-induced hyperlocomotion.

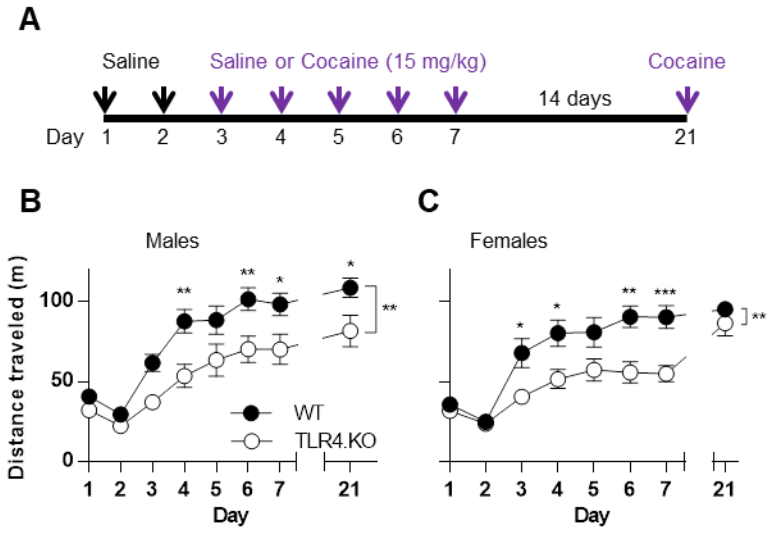

Figure 12. TLR4 knockout (TLR4.KO) animals exhibit reduced locomotor response to cocaine.
(A) Experimental timeline. Following 2 days of saline injections, mice were injected with cocaine (15 mg/kg) for 5 days. Following a 14 day withdrawal/abstinence period, a challenge dose of cocaine was given. Locomotor activity was assessed for 15 min. following each injection in an open field chamber. (B) Cocaine locomotor response in male WT and TLR4.KO mice (WT N = 12; TLR4.KO N = 10). (C) Locomotor response in female mice (N = 11; TLR4.KO N = 9). *P < 0.05; **P < 0.01; ***P < 0.001, 2-way repeated measures ANOVA with Sidak's post-hoc test.

III-2b. Cocaine exposure differentially affects NAc core post-synaptic properties in TLR4.KO animals

Cocaine experience causes cell-type specific synaptic changes on NAc core MSNs (Grueter *et al*, 2012; Kauer and Malenka, 2007). To study MSNs in a cell-type specific manner, we used WT and TLR4.KO mice carrying a bacterial artificial chromosome expressing the tdTomato fluorophore driven by the D1 dopamine receptor promotor as previously described (Grueter *et al*, 2013). Whole-cell voltage-clamp recordings were made in D1(+) and D1(-) MSNs corresponding to D1 and D2 MSNs, respectively (Kashima and Grueter, 2017). We used male mice to perform voltage-clamp recordings following acquisition of cocaine locomotor sensitization (Figure 2A), a time point associated with behavioral attenuations in both genders of TLR4.KO animals.

Non-contingent cocaine experience is associated with the generation of synapses containing only NMDAR on D1 MSNs (Graziane *et al*, 2016). These are termed “silent” synapses and play an important role in meta-plasticity (Graziane *et al*, 2016). We hypothesized that cocaine exposure causes differential changes in NAc core MSN synaptic properties in WT compared to TLR4.KO animals. To this end, we assessed a series of pre- and post-synaptic properties the day following 5 days of saline or cocaine (15 mg/kg) injections (Figure 13A). We began by assessing the ratio of $1/CV^2_{N:A}$, a measure of silent synapses (Grueter *et al*, 2013). In D1(+) MSNs, we found that cocaine significantly increased $1/CV^2_{N:A}$ in both WT and TLR4.KOs suggesting an increase in the proportion of silent synapses (Figure 13B-C, L-M). We also examined PPR, A/N ratio, dual-component decay kinetics, and sEPSC amplitude and frequency. We found that cocaine experience did not alter PPR, an inverse measurement of

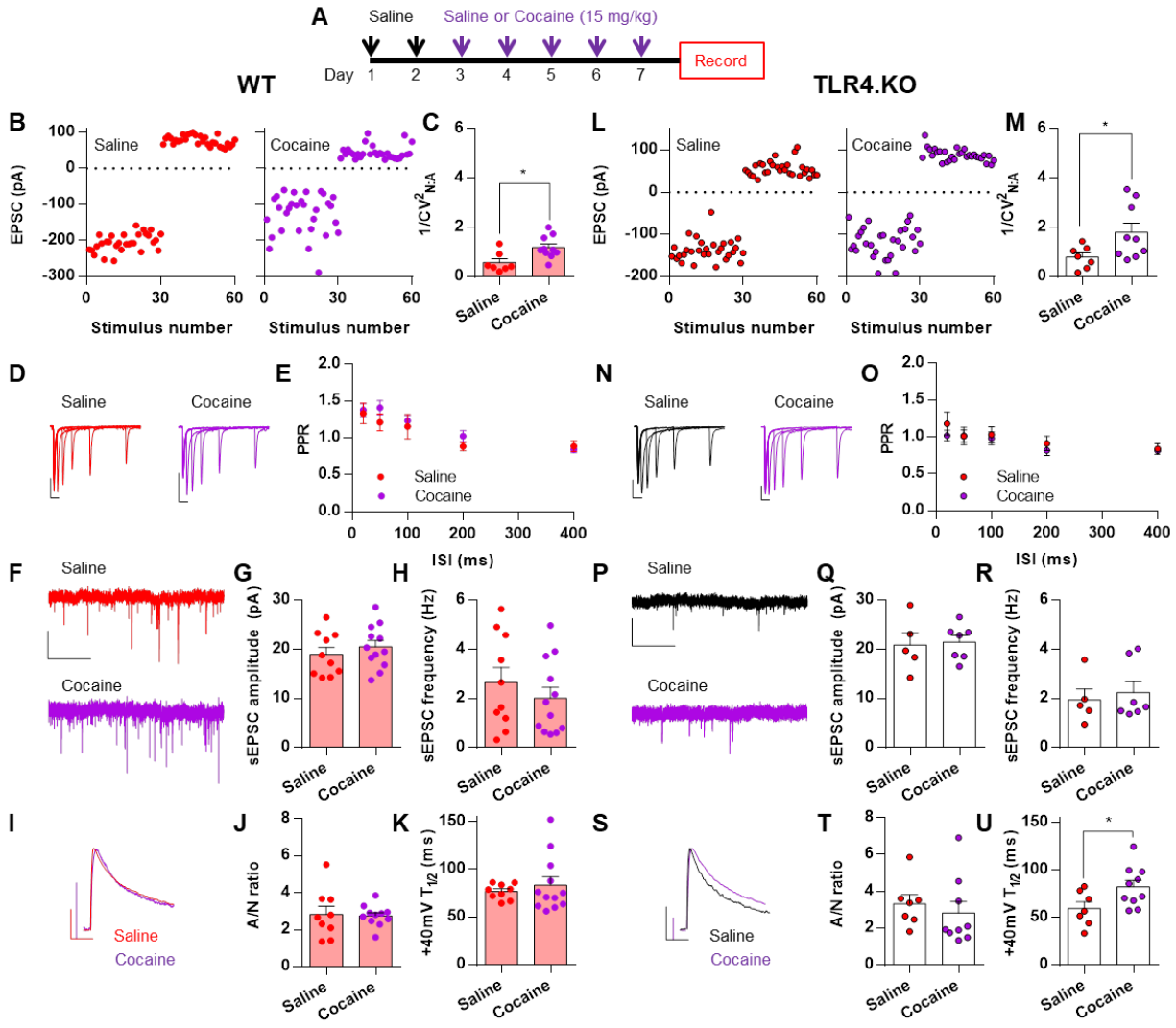

Figure 13. Cocaine alters synaptic properties in TLR4.KO D1(+) MSNs.
(A) Experimental schematic. Recordings were made 24 h following 2 days saline habituation and 5 days of saline or cocaine injections. Similar to behavioral experiments, each injection was followed by 15 min. exposure to an open field chamber. (B) Representative plot of WT coefficient of variance (CV) experiments. AMPAR (-70 mV) and NMDAR EPSCs (+40 mV) from D1(+) MSNs taken from saline (red) and cocaine (purple)-treated mice. (C) Summary ratio of $1/CV^2_{N:A}$ from WT D1(+) MSNs. (D) Representative traces from PPR experiments. (E) Summary plot of WT D1(+) PPR experiments. (F) Representative sEPSC traces. (G) Summary plot of sEPSC amplitude and (H) frequency. (I) Representative evoked current traces taken at +40 mV. (J) Summary plot of A/N ratio and (K) Time to half-peak ($T_{1/2}$) of dual-component currents at +40 mV. (L-U) Example traces, experiments, and summary plots for $1/CV^2_{N:A}$, PPR, sEPSC amplitude, sEPSC frequency, A/N ratio, and dual component $T_{1/2}$ from D1(+) MSNs from TLR4.KO mice treated with either saline (black) or cocaine (purple). All recordings taken in the presence of picrotoxin (50 µM). n/N = 5-12 cells from 3-5 animals per group. Error bars denote SEM. *P < 0.05, unpaired t test.

presynaptic release probability, in either WT or TLR4.KO D1(+) MSNs (Figure 13D-E, N-O). We also found no differences with cocaine in the A/N ratio and sEPSC amplitude; measures of post-synaptic strength, or sEPSC frequency (Figure 13F-J). Interestingly, we found that cocaine exposure increased the time to half-peak ($T_{1/2}$) of AMPAR and NMDAR dual component responses in D1(+) TLR4.KO but not WT MSNs (Figure 13K), suggestive of a change in NMDAR function.

In contrast to D1(+) MSNs, $1/CV^2_{N:A}$ was unchanged in D1(-) cells from cocaine-treated WT similar to what is seen in the NAc shell (Graziane *et al*, 2016) (Figure 14B-C). However, cocaine significantly increased $1/CV^2_{N:A}$, decreased sEPSC frequency, and increased dual-component $T_{1/2}$ in TLR4.KO animals (Figure 14L-M, R, U). In both WT and TLR4.KO D1(-) MSNs following cocaine, we observed no changes to PPR, A/N ratio, or sEPSC amplitude (Figure 14D-K, N-Q, S-T). The decrease in sEPSC frequency observed in TLR4.KO D1(-) MSNs suggests one of: decreased presynaptic release probability, decreased number of synapses sampled from, or decreased network activity. Cocaine's lack of effect on PPR argues against altered presynaptic release probability. In combination with the observed increase in $1/CV^2_{N:A}$, the most parsimonious explanation for these findings is cocaine "silencing" previously active synapses on D1(-) MSNs. In addition, an increase in the dual-component $T_{1/2}$ is consistent with decreased current flow through the AMPARs although it may also be due to a change in NMDAR function (Kashima and Grueter, 2017). Though we cannot rule out the possibility of *de novo* silent synapse generation paired with a reduction in network activity, this seems less likely given no differences in sEPSC frequencies were

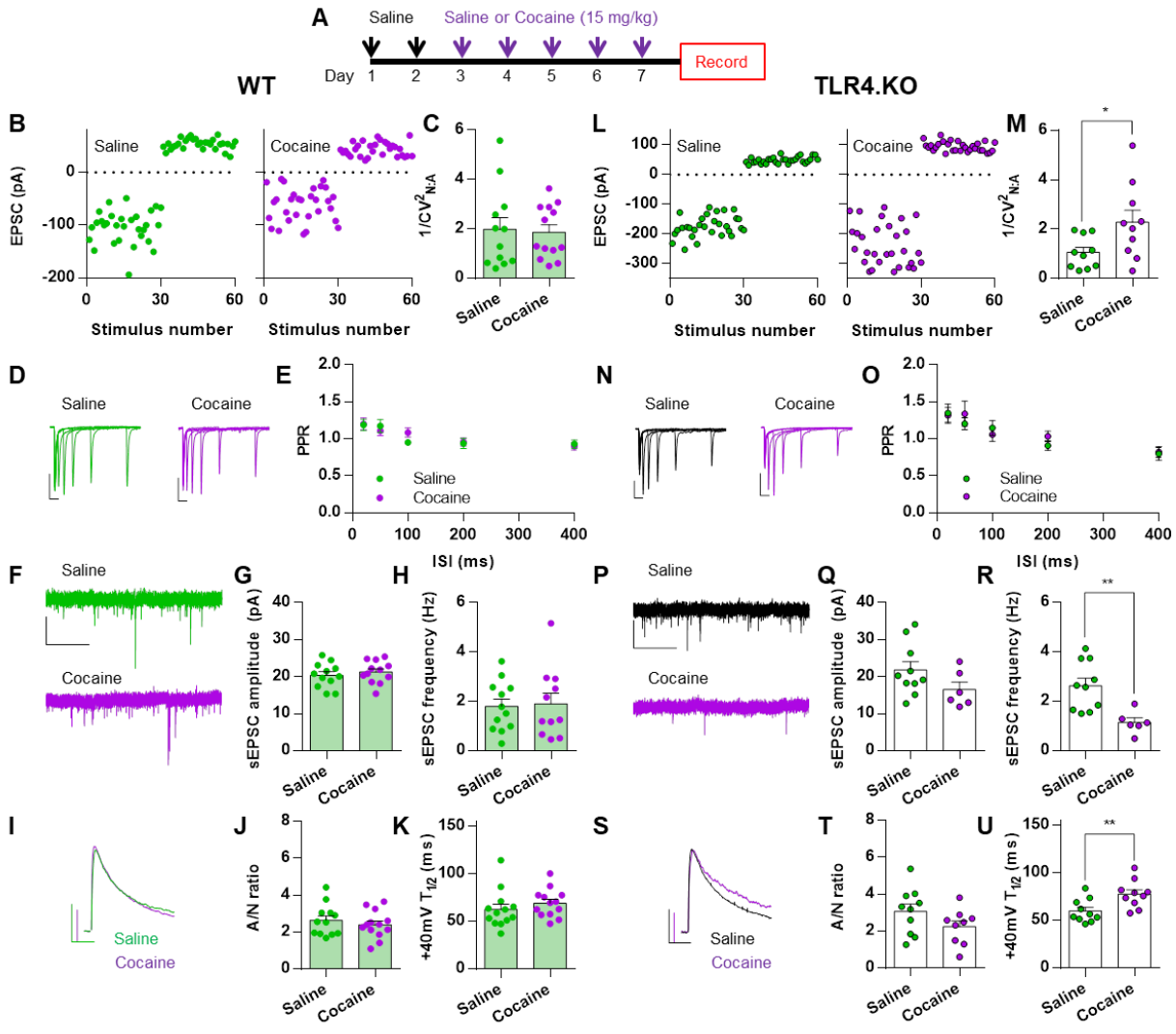

Figure 14. Cocaine alters synaptic properties in TLR4.KO D1(-) MSNs.
(A) Experimental schematic. (B) Representative plot of WT coefficient of variance (CV) experiments taken from saline (green) and cocaine (purple)-treated mice. (C) Summary of $1/CV^2_{N:A}$ from WT D1(-) MSNs. (D) Representative traces from PPR experiments. (E) Summary plot of WT D1(-) PPR experiments. (F) Representative sEPSC traces. (G) Summary plot of sEPSC amplitude and (H) frequency. (I) Representative evoked current traces taken at +40 mV. (J) Summary plot of A/N ratio and (K) $T_{1/2}$ of dual-component currents at +40 mV. (L-U) Example traces, experiments, and summary plots for $1/CV^2_{N:A}$, PPR, sEPSC amplitude, sEPSC frequency, A/N ratio, and dual component $T_{1/2}$ from D1(-) MSNs from TLR4.KO mice treated with either saline (black) or cocaine (purple). All recordings taken in the presence of picrotoxin (50 µM). n/N = 6-13 cells from 4-6 animals per group. Error bars denote SEM. *P < 0.05; **P < 0.01, unpaired t test.

seen in D1(+) MSNs. Regardless, we provide evidence for cocaine exposure differentially affecting WT and TLR4.KO MSNs in a cell-type specific manner.

III-2c. Few differences observed in synaptic properties following withdrawal from cocaine

Withdrawal from cocaine exposure causes a distinct set of synaptic changes across the NAc core (Jedynak *et al*, 2016; Joffe and Grueter, 2016; Loweth *et al*, 2014; Moussawi *et al*, 2009). To determine whether TLR4.KO and WT animals differed in their synaptic remodeling during withdrawal, we assessed synaptic properties of NAc core D1(+) and (-) MSNs 10-14 days after cocaine exposure (Figure 15A). For both D1(+) and D1(-) MSNs from WT and TLR4.KO animals, we found no differences between saline and cocaine treatments for $1/CV^2_{N:A}$, PPR, dual-component $T_{1/2}$, sEPSC amplitude, or sEPSC frequency (Figure 15B-K, M). Interestingly, we observed an increase in the A/N ratio of WT D1(-) MSNs following cocaine exposure (Figure 15L). With no differences seen in $1/CV^2_{N:A}$, sEPSC amplitude or dual component decay kinetics, this raises the possibility for decreased NMDAR content on synapses.

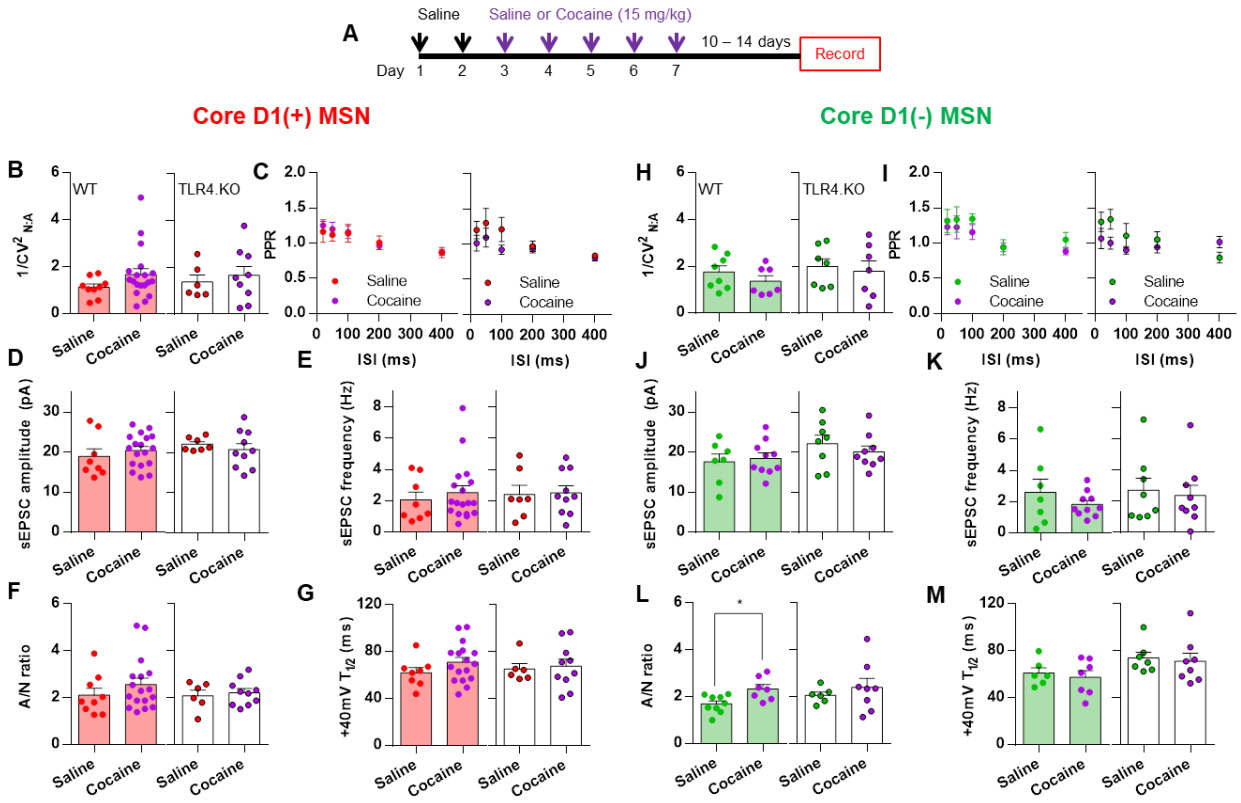

Figure 15. Normalization MSN synaptic properties following withdrawal from cocaine. (A) Experimental schematic. Recordings were made following 10-14 of abstinence from saline or cocaine (15 mg/kg) injections. (B) (left) Summary ratio $1/CV^2_{N:A}$ from D1(+) MSNs from saline/cocaine-exposed WT and (right) TLR4.KO mice. (C) Summary plot of PPR experiments from WT (left) and TLR4.KO (right) D1(+) MSNs. (D) Summary plot of sEPSC amplitudes from D1(+) MSNs of WT and TLR4.KO animals. (E) Summary plot of sEPSC frequency in D1(+) MSNs from WT and TLR4.KO animals. (F) Summary plot of A/N ratio in WT and TLR4.KO D1(+) MSNs. (G) Summary plot of dual component $T_{1/2}$ from WT and TLR4.KO D1(+) MSNs. (H-M) Summary plots of $1/CV^2_{N:A}$, PPR, sEPSC amplitude, sEPSC frequency, A/N ratio, and dual component $T_{1/2}$ from D1(-) MSNs from cocaine/saline withdrawn WT and TLR4.KO mice. All recordings taken in the presence of picrotoxin (50 µM). WT D1(+) saline n/N = 8-9 cells/5 animals; cocaine n/N = 16-19 cells/8 animals; TLR4.KO D1(+) saline n/N = 6-7 cells/3 animals; cocaine n/N = 9-10 cells/5 animals; WT D1(-) saline n/N = 6-7 cells/4 animals; cocaine = 7-10 cells/5 animals; TLR4.KO D1(-) saline n/N = 6-8 cells/4 animals; cocaine n/N = 7-10 cells/5 animals. Error bars denote SEM. *P < 0.05, unpaired t test.

III-2d. Differences in dual-component decay times following a challenge dose of cocaine

A challenge dose of cocaine following withdrawal produces additional changes in NAc synaptic physiology (Jedynak *et al*, 2016). To test whether cocaine history differentially affects WT and TLR4.KO mice at this time point, we recorded from these animals following a challenge dose given after 10-14 days withdrawal from 5 days of cocaine or saline injections similar to previously described (Thomas *et al*, 2001) (Figure 16A). At this time point, we found no differences 24 h following cocaine injections between animals with prior saline or cocaine history for $1/CV^2_{N:A}$, A/N ratio, or PPR (Figure 16B-D, F-H), though we noted a trend towards prior cocaine exposure decreasing $1/CV^2_{N:A}$ in TLR4.KO D1(-) MSNs (Figure 16F). Interestingly, we found that a history of cocaine exposure caused a significant decrease in the dual-component $T_{1/2}$ of WT D1(+) MSNs compared to animals experiencing cocaine for the first time (Figure 16E). This suggests that cocaine history affects the synaptic response to acute drug administration and is an effect not observed in TLR4.KO D1(+) MSNs (Figure 16E). This may point to genotype-based differences in metaplasticity *to* cocaine. Whether the difference in $T_{1/2}$ of is driven by changes to AMPAR or NMDAR function remains to be determined.

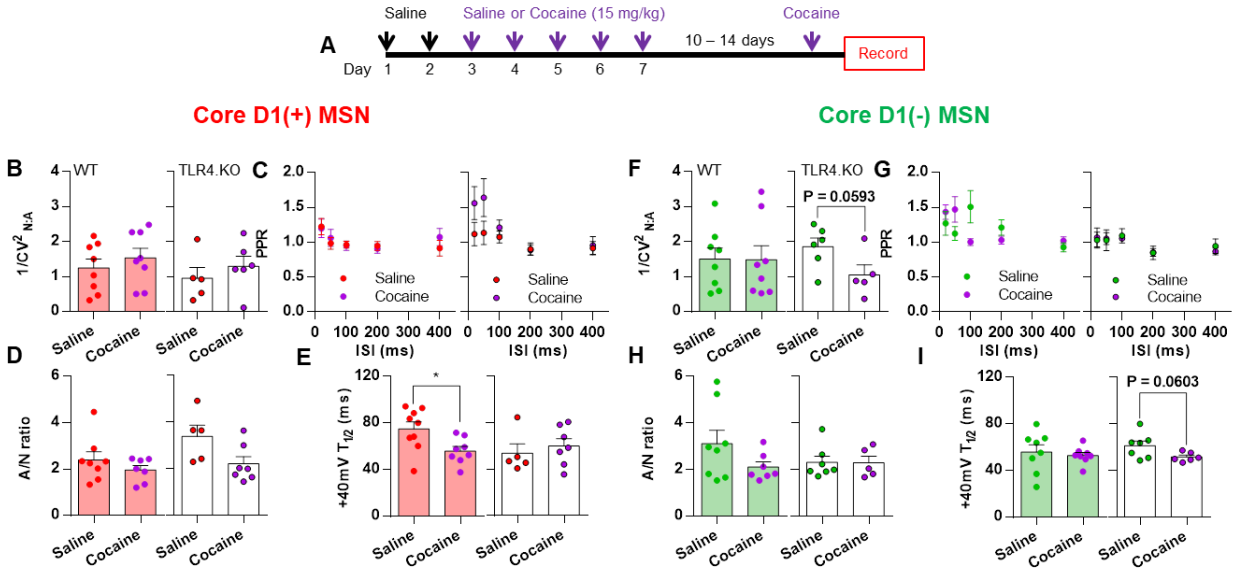

Figure 16. Synaptic properties following challenge dose of cocaine.
(A) Experimental schematic. Recordings were made 24 hours following a challenge dose of cocaine (15 mg/kg) in mice withdrawn from saline or cocaine. (B) (left) Summary ratio $1/CV^2_{N:A}$ from D1(+) MSNs from withdrawn and cocaine-challenged WT and (right) TLR4.KO mice. (C) Summary plot of PPR experiments from WT (left) and TLR4.KO (right) D1(+) MSNs. (D) Summary plot of A/N ratio in WT and TLR4.KO D1(+) MSNs. (E) Summary plot of dual component $T_{1/2}$ from WT and TLR4.KO D1(+) MSNs. (F-I) Summary plots of $1/CV^2_{N:A}$, PPR, A/N ratio, and dual component $T_{1/2}$ from D1(-) MSNs from withdrawn and cocaine-challenged WT and TLR4.KO mice. All recordings taken in the presence of picrotoxin (50 µM). WT D1(+) saline n/N = 6-9 cells/4 animals; cocaine n/N = 7-8 cells/4 animals; TLR4.KO D1(+) saline n/N = 5 cells/3 animals; cocaine n/N = 6-7 cells/3 animals; WT D1(-) saline n/N = 7-8 cells/4 animals; cocaine = 6-8 cells/4 animals; TLR4.KO D1(-) saline n/N = 6-7 cells/3 animals; cocaine n/N = 4-6 cells/2 animals. Error bars denote SEM. *P < 0.05, unpaired t test.

III-3. Discussion

TLR4 is a pattern recognition molecule of the innate immune system associated with drug-reward behavior (Hutchinson *et al*, 2012; Kashima and Grueter, 2017; Northcutt *et al*, 2015). In the NAc core, TLR4 is found primarily on microglia (Kashima and Grueter, 2017)—an innate immune cell which modulates synaptic function through several mechanisms including synaptic pruning, synaptic stripping, and cytokine release (Beggs and Salter, 2016; Lewitus *et al*, 2016). A global deficit in TLR4 is associated with basal alterations in NMDAR subunit composition and plasticity (Kashima and Grueter, 2017). This is linked to an attenuation in CPP without affecting non-drug behaviors including locomotor activity, episodic memory, or anhedonia (Kashima and Grueter, 2017). Less understood is the role of TLR4 on NAc synaptic changes that occur with drug experience. In addition, there is a paucity of research examining gender-specific effects of TLR4 on cocaine behavior. To address these issues, the present study characterized cocaine locomotor sensitization in both WT and TLR4.KO mice for males and females. Male cell-type-specific reporter mice were further used to examine synaptic properties of NAc core MSNs at several time-points following cocaine experience.

III-3a. Sex-specific differences in cocaine response

We report sex-specific differences for cocaine locomotor response in TLR4.KO mice. Although both male and female TLR4.KO mice exhibit reductions in the initial locomotor response to cocaine injections, we found that females further sensitize during a withdrawal period to match WT locomotion after a cocaine challenge. Males did not. This suggests at a sexually dimorphic role of TLR4 in the reward system. Sex-specific

differences are well-documented in humans and rodents for cocaine behavior (Lynch *et al*, 2002) as well as neuro-immune responses such as that seen with microglia in chronic pain (Brings and Zylka, 2015). For drug-reward, female rats acquire CPP at lower cocaine doses compared to males (Russo *et al*, 2003). On the other hand, male mice extinguish from low-dose cocaine CPP slower than females (Hilderbrand and Lasek, 2014) suggesting sex-specific differences to different aspects of drug-experience or species-specific differences. In line with behavioral differences, other studies found sex-specific biochemical changes such as an upregulation of the *Auts2* gene in male but not female mice with cocaine exposure (Engmann *et al*, 2017) or the protein p11 found dispensable for female but not male acquisition of cocaine CPP (Thanos *et al*, 2016b). For neuro-immune interactions, sex-specific differences in microglia and T cells have been shown to underlie behavioral and therapeutic responses to treatments of chronic pain (Sorge *et al*, 2015). Our results add to this line of work by implicating TLR4 as important in the initial behavioral response to cocaine in both sexes but dispensable in females for further adaptations occurring during withdrawal.

III-3b. Differential remodeling of synaptic properties following cocaine exposure

Prior studies established associations between microglia in the NAc and exposure to drugs of abuse including cocaine, methamphetamine, and morphine. In response to cocaine, microglia upregulate the cytokine TNFα in the NAc core of mice to depress synaptic strength on D1(+) MSNs (Lewitus *et al*, 2016). As loss of TNFα leads to an exaggerated behavioral response to cocaine, upregulation of this cytokine is thought to be a homeostatic response to cocaine-induced changes in NAc. In rats, morphine

administration resulted in chemokine/cytokine production from microglia (Schwarz *et al*, 2013) and methamphetamine administration led to increased NAc microglial activation as assessed through [3H]PK 11195 autoradiography (Thanos *et al*, 2016a).

We found that 5 days of non-contingent cocaine injections increases $1/CV^2_{N:A}$, suggesting an increase in the proportion of silent synapses on D1(+) but not D1(-) MSNs in the NAc core of WT mice. This result echoes findings seen in the neighboring shell subregion following a similar drug administration paradigm (Graziane *et al*, 2016). With TLR4.KO animals, our data suggests cocaine upregulates silent synapses on both MSN subtypes. On D1(+) MSNs, this was associated with an increase in dual-component (AMPAR + NMDAR) current decay time. Given a lack of change in AMPAR-mediated sEPSC amplitudes, this change in dual-component decay-time points to either a larger increase in NMDAR number compared to WT or a change in NDMAR subunit composition. On TLR4.KO D1(-) MSNs, the upregulation in silent synapses was associated with a decrease in sEPSC frequency, increase in dual component decay-time, and no change in PPR. This suggests “silencing” of previously active synapses. Interestingly, this phenomenon is observed in the NAc shell following morphine administration and is associated with reward-learning (Graziane *et al*, 2016). This discrepancy with our results associating this change with a reduction in drug-locomotor response magnitude may be due to differences between the NAc shell and core subregions for silent synapse modulation in reward valence such as that observed for prefrontal cortical inputs to the NAc (Ma *et al*, 2014) or due to differences in input-specific changes. Future studies may tease this apart.

III-3c. Withdrawal from cocaine exposure

Withdrawal from cocaine exposure causes synaptic remodeling throughout the NAc. In the shell subregion, both contingent and non-contingent cocaine exposure generates silent synapses made up of GluN2B-containing NMDAR *de novo* (Huang *et al*, 2009; Ma *et al*, 2014). These changes occur on inputs from the IfL as well as BLA (Lee *et al*, 2013; Ma *et al*, 2014; Pascoli *et al*, 2012). Following prolonged withdrawal, these connections are strengthened through “unsilencing” of synapses through increased surface expression of calcium-permeable AMPARs (Lee *et al*, 2013; Ma *et al*, 2014). Such changes are thought to underlie drug-reward behavior as optogenetic depotentiation of these inputs reverses drug seeking and locomotor response (Lee *et al*, 2013; Ma *et al*, 2014; Pascoli *et al*, 2012).

The NAc core receives input from the PrL portion of mPFC (Sesack and Grace, 2010). In rats, cocaine self-administration increases the proportion of silent synapses on these inputs (Ma *et al*, 2014). With prolonged withdrawal, the proportion of silent synapses normalizes to that of saline treated animals. However, the input becomes sensitive to LFS-induced synaptic depotentiation associated with an increase in silent synapses *ex vivo* and an increase in drug-seeking behavior *in vivo* (Ma *et al*, 2014). The parsimonious interpretation of these findings is the generation of silent synapses *de novo* into which calcium-impermeable AMPARs are inserted.

We characterized NAc core synaptic properties in WT and TLR4.KO mice following a 10-14 day abstinence from cocaine or saline exposure. We noted a lack of effect with cocaine in both genotypes. In WT mice, a lack of difference in AMPAR transmission following withdrawal is consistent with previously published observations

(Dobi *et al*, 2011; Joffe and Grueter, 2016). This may signify a restoration of baseline synaptic properties in the NAc core during abstinence.

A lack of difference in synaptic properties in cocaine and saline-treated WT mice following withdrawal—particularly sEPSC amplitude and frequency—suggests against global insertion of AMPARs into silent synapses generated during the acquisition phase. The observed differences with Ma et al., may be due to one of several biological or technical reasons: input specificity, animal species, or drug administration paradigm. Ma et al. (2014) used optogenetics to examine a specific input into the NAc core whereas our study sampled inputs indiscriminately. Input specificity has been shown to uncover synaptic differences otherwise hidden with electrical stimulation in cocaine administration paradigms (Joffe and Grueter, 2016). In addition, Ma et al. (2014) used rats self-administering cocaine (contingent behavior) whereas our study employed mice receiving cocaine in a non-contingent manner. Further study taking these variables into account will be useful.

In assessing synaptic properties following a challenge dose of cocaine in mice with a prior history of saline or cocaine exposure, we found a no differences in the A/N ratio or PPR similar to previously published results (Thomas *et al*, 2001). However, we observed a difference in dual-component decay times for D1(+) MSNs from WT but not TLR4.KO animals. Given the lack of differences seen during drug-withdrawal, this result may be suggestive of a change in extra-synaptic “metaplasticity” wherein there is an alteration in the ability to modulate synaptic properties in response to future experiences. A prior study found that withdrawal from cocaine self-administration causes a general loss of inducible synaptic long-term potentiation and long-term

depression in the NAc core associated with drug-seeking (Moussawi *et al*, 2009). Our results raise the possibility of altered metaplasticity for the cocaine experience itself in TLR4.KOs. Given the importance of glial cells in metaplasticity (Kalivas, 2009) and the association of TLR4 with glial cells (Kashima and Grueter, 2017), future studies may focus on connecting these observations.

III-3d. Cocaine and the immune system

A growing list of studies implicates the importance of immunologic factors sculpting synaptic transmission and behavior (Beggs and Salter, 2016; Liddelow and Barres, 2015). TLR4 is a pattern-recognition molecule that detects gram-negative bacteria, as well as a range of endogenous signals such including the “danger” signals HMG-B1 and heat shock proteins, free-fatty acids, and many others (O’Neill, 2008; Shi *et al*, 2006; Trotta *et al*, 2014). In the NAc and brain as a whole, TLR4 is associated with microglia (Schwarz *et al*, 2013). This protein is also linked to drug-reward behavior through direct binding to both alcohol and morphine (Hutchinson *et al*, 2012; June *et al*, 2015). For cocaine, the mechanism of interaction with TLR4 is comparatively nebulous. On one hand, there is biophysical evidence showing cocaine can to bind MD-2, a co-receptor of TLR4 (Northcutt *et al*, 2015). On the other hand, cocaine’s ability to bind TLR4 has not been shown beyond computational modeling while behavioral studies using pharmacologic antagonists have been muddled by off-target effects (Tanda *et al*, 2016). Separate studies using cultured rat microglial cells as well as isolated microglia from cocaine exposed mice demonstrated that cocaine downregulates MicroRNA-124 (MiR-124) through promoter methylation (Guo *et al*, 2016). MiR-124 regulates multiple

pathways associated with TLR4 and its overexpression attenuates cocaine-induced locomotor activity and microglial activation (Periyasamy *et al*, 2017). These studies all demonstrate the importance of TLR4 in cocaine reward behavior. However, the question of what molecular factor mediates TLR4's activation with drugs of abuse remains unanswered. Future studies may focus on the endogenous TLR4 ligands in order to better understand the biology and develop novel therapies for addiction. Regardless, our study adds to this growing body of work by linking TLR4 to cocaine-induced changes in the NAc synaptic physiology as well as gender-specific behaviors.

CHAPTER IV

PHYSIOLOGY OF SICKNESS

IV-1. Introduction

Alterations in motivational state underlie a variety of clinically diagnosed psychiatric conditions including major depressive disorder (MDD). Treatments continue to lack due in part to an incomplete understanding of how the brain's reward circuits are altered in such pathologies. One brain region implicated in MDD is the NAc (Francis and Lobo, 2016). Acting as a center for motivation and reward, this heterogeneous structure is part of the ventral striatum with 90-95% of the neurons consisting of medium spiny neurons (MSN) (Sesack and Grace, 2010). These cells are divided into roughly 2 equal populations: those expressing the D1 dopamine receptor and those expressing the D2 dopamine receptor. The NAc is further divided into the shell and core subregions with distinct afferent and efferent projections (Sesack and Grace, 2010). In the context of drug-reward, NAc D1 and D2 MSNs broadly represent pro- and anti-reward signaling, respectively (Lobo *et al*, 2010). In rodent models of depression, the distinction is less clear. Animal models of depression are frequently studied in the context of repeated stressors to alter motivational state. Such stressors include restraint, social defeat, and pain which are associated with differential changes in NAc synaptic physiology (Francis and Lobo, 2016; Lim *et al*, 2012; Schwartz *et al*, 2014).

In addition to stressors, activation of the immune system alters motivational state. Infections or injection of LPS, a component of gram-negative bacterial cell walls which

binds TLR4, causes anhedonia, hypolocomotion, and despair (Dantzer *et al*, 2007). This is termed “sickness behavior” and is thought to depend on cytokines and prostaglandins signaling in the brain (Dantzer *et al*, 2007). The behavioral and immunologic changes seen in infections are well characterized. Less-understood are neuronal responses underlying this process. Given behavioral manifestations of anhedonia and despair, sickness may alter the brain’s reward system. Thus, we hypothesize that sickness behavior alters NAc synaptic physiology. To test this idea, we performed whole-cell voltage-clamp recordings from the NAc of mice injected with either saline or LPS to induce sickness. These mice express fluorescent cell markers permitting distinction of D1 [D1(+)] and D2 [D1(-)] MSNs. We report that sickness behavior is associated with NAc subregion and cell-type-specific changes in MSN synaptic physiology.

IV-2. Results

IV-2a. LPS injections cause behavioral alterations as part of sickness behavior

We began by confirming behavioral effects of LPS injections in mice. Compared to saline injections, LPS (2.5 mg/kg) administration followed by a 24 hour wait resulted in significant changes in behavior consistent with “sickness behavior.” This included a reduction in locomotor activity, increased anxiety as assessed through open field chamber center time, and trends towards increased despair as assessed through forced-swim tests when compared to saline-treated controls (Figure 17). However, we did not observe any treatment effects for the tail-suspension test (Figure 17). This work in progress suggests LPS injections induce behavioral alterations as part of sickness behavior.

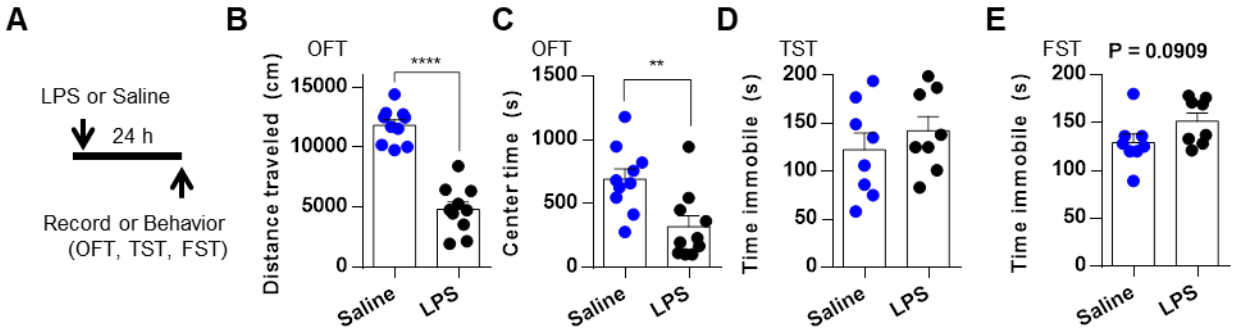

Figure 17. LPS injections alter behavior assessed 24 hours later.
(A) Experimental schematic. LPS (2.5 mg/kg) or saline were injected 24 hours prior to behavior or electrophysiology experiments. Behaviors tested include open field test (OFT), tail-suspension test (TST), and forced-swim test (FST). (B) Distance traveled in 60 min. OFT. (C) Center time in 60 min. OFT. (D) Time spent immobile in 6 min. TST. (E) Time spent immobile in 6 min. FST. Error bars denote SEM. $N_{animals}$ = 8-10 animals/group. **P < 0.01; ****P < 0.0001, unpaired t test.

IV-2b. Sickness behavior is associated with alterations in NAc core synaptic physiology

Simultaneous to determining if LPS induces behavioral despair, we assessed whether this treatment causes synaptic changes in NAc MSNs. To this end, we used whole-cell voltage-clamp to test a variety of pre- and post-synaptic properties in a cell-type specific manner. Specifically, we assessed A/N ratio, dual-component decay kinetics1/$CV^2_{N:A}$, sEPSC amplitude and frequency, and PPR. In the NAc core D1(+) MSNs, we found that LPS injections followed by a 24 hour wait results in a significant reduction in sEPSC frequency without difference for A/N ratio, 1/$CV^2_{N:A}$, sEPSC amplitude, or PPR compared to saline treated controls (Figure 18). With D1(-) MSNs, we found no significant difference between LPS and saline-treated animals for all measurements (Figure 19).

Finally, we performed simultaneous paired-recordings of adjacent D1(+) and D1(-) MSNs to test for differences in the “D1/D2” ratio. This measurement assesses the

relative excitatory tone onto the 2 populations of MSNs. Changes in this ratio are associated with alterations in reward signaling such as an increase seen in morphine or cocaine exposure (Graziane *et al*, 2016). We found that LPS did not change this ratio in the NAc core (Figure 20).

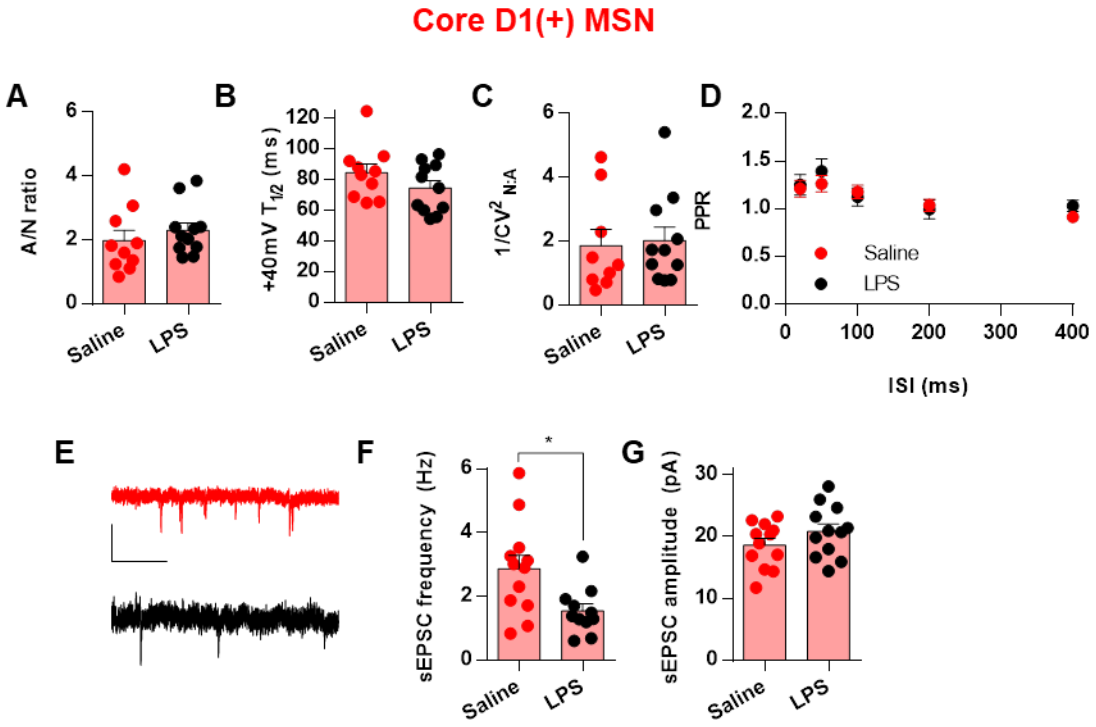

Figure 18. LPS injections alter excitatory transmission onto D1(+) MSNs of the NAc core.
(A) Summary of A/N ratio. Saline $n_{cells}/N_{animals}$ = 10/5; LPS n/N = 11/7. (B) Summary of dual-component decay kinetics. Saline n/N = 10/5; LPS n/N = 11/7. (C) Summary of $1/CV^2_{N:A}$. Saline n/N = 9/5; LPS n/N = 11/7. (D) Summary of PPR. Saline n/N = 9/5; LPS n/N = 9/7. (E) Representative traces from sEPSC experiments. (F) Summary of sEPSC frequency and (G) amplitudes. Saline n/N = 12/5; LPS n/N = 12/7. Error bars denote SEM. *P < 0.05, unpaired t test.

Decreased sEPSC frequency in D1(+) MSNs suggests one of 3 things: 1) decreased presynaptic release probability, 2) decreased number of sampled synapses, or 3) decreased network activity. As PPR is inversely related to presynaptic release

probability, a lack of change in this measure argues against altered decreased release probability underlying the change in sEPSC frequency. Additionally, if there is either a reduction in presynaptic release probability or a reduction in the number of sampled synapses on D1(+) MSNs, then the D1/D2 ratio (made up of evoked responses) will decrease. With no observed difference in the D1/D2 ratio, decreased network activity on D1(+) MSNs may underlie the reduction in sEPSC frequency. mEPSC experiments will provide more strength to this idea.

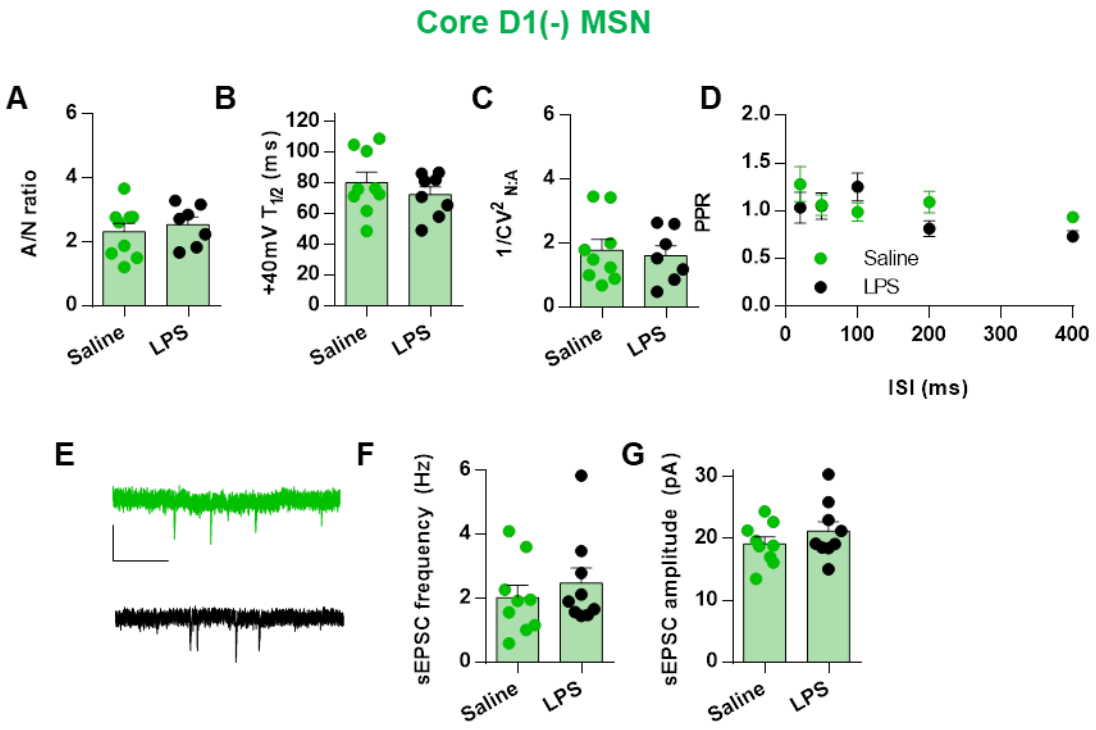

Figure 19. LPS effects on D1(-) MSNs of the NAc core.
(A) Summary of A/N ratio. Saline n/N = 9/5; LPS n/N = 8/7. (B) Summary of dual-component decay kinetics. Saline n/N = 9/5; LPS n/N = 8/7. (C) Summary of $1/CV^2_{N:A}$. Saline n/N = 9/5; LPS n/N = 7/7. (D) Summary of PPR. Saline n/N = 9/5; LPS n/N = 10/7. (E) Representative traces from sEPSC experiments. (F) Summary of sEPSC frequency and (G) amplitudes. Saline n/N = 9/5; LPS n/N = 9/7. Error bars denote SEM. P > 0.05 for all comparisons, unpaired t test.

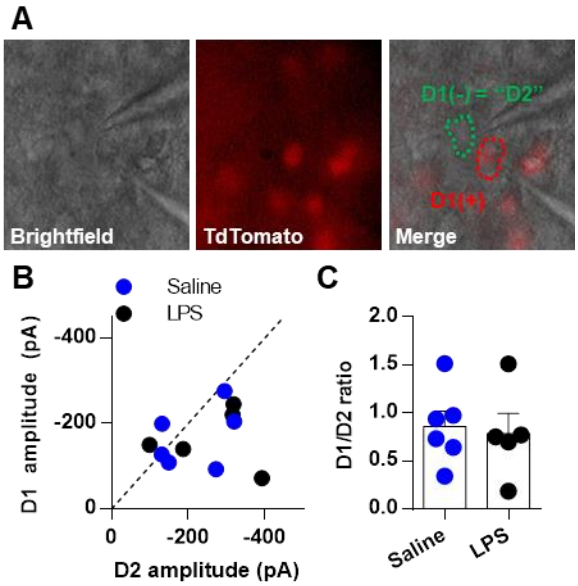

Figure 20. LPS does not bias excitatory inputs towards either MSN subtype.
(A) Representative images illustrating paired recordings from adjacent D1(+) and D1(-) [putative D2] MSNs. (B) Plot of EPSCs from paired D1 and D2 cells. (C) Summary plot of D1/D2 EPSC ratio. Saline $n_{pairs}/N_{animals}$ = 6/4; LPS n/N = 5/4. Error bars denote SEM. P > 0.05, unpaired t test.

IV-2c. Differential changes in the NAc shell

Though anatomically and functionally related, the NAc shell and core subregions subtly differ in anatomic inputs, projections, and experience-dependent synaptic changes (Jedynak *et al*, 2016; Ma *et al*, 2014). Thus, we also performed voltage-clamp recordings from MSNs from this region. In NAc shell D1(+) MSNs, we found that LPS significantly decreases the PPR in LPS treated animals (Figure 21). This was associated with no significant differences between saline and LPS treated mice for the A/N ratio, dual-component decay kinetics, $1/CV^2_{N:A}$, sEPSC amplitude, or sEPSC frequency (Figure 21). This suggests that LPS may increase the presynaptic release probability onto D1(+) MSNs of the NAc shell.

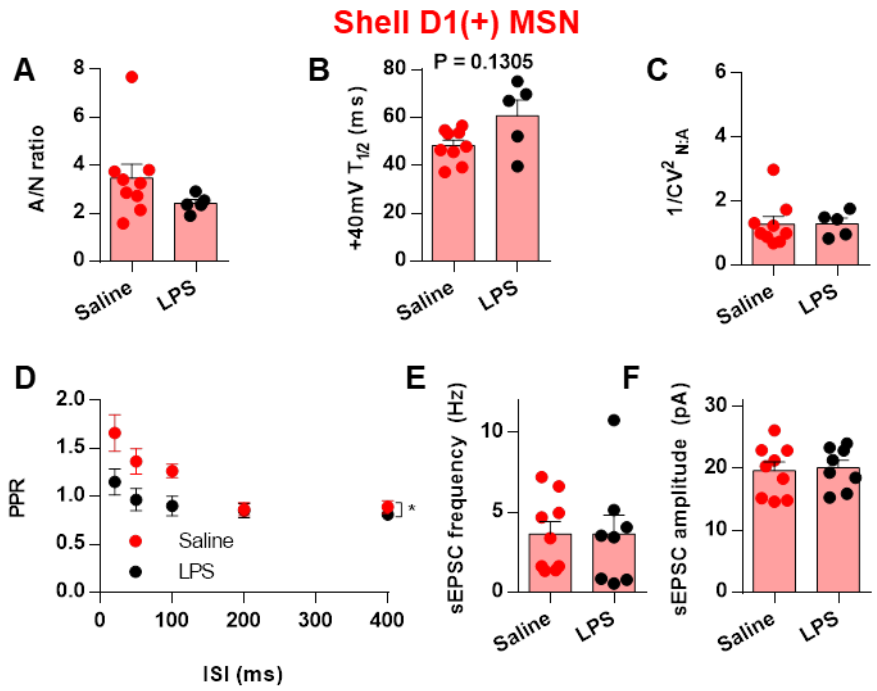

Figure 21. Synaptic properties of D1(+) NAc shell MSNs following LPS administration. (A) Summary of A/N ratio. Saline $n_{cells}/N_{animals}$ = 9/5; LPS n/N = 5/3. (B) Summary of dual-component decay kinetics. Saline n/N = 9/5; LPS n/N = 5/3. (C) Summary of $1/CV^2_{N:A}$. Saline n/N = 9/5; LPS n/N = 5/3. (D) Summary of PPR. Saline n/N = 7/5; LPS n/N = 9/5. (E) Summary of sEPSC frequency and (F) amplitudes. Saline n/N = 9/5; LPS n/N = 8/4. Error bars denote SEM. *P < 0.05, 2-way ANOVA.

In D1(-) MSNs, however, we found that LPS significantly decreased the amplitude of sEPSCs (Figure 22). No differences were observed for the A/N ratio, dual-component decay kinetics, $1/CV^2_{N:A}$, PPR, and sEPSC frequency (Figure 22). These results indicate that LPS may decrease the synaptic strength onto D1(-) MSNs of the NAc shell.

Similar to the NAc core, we also performed simultaneous paired-recordings of adjacent D1(+) and D1(-) MSNs in the NAc shell. With results indicating a decreased presynaptic release probability onto D1(+) MSNs and decreased synaptic strength on to D1(-) MSNs, we predicted that LPS increases the D1/D2 ratio in the NAc shell.

Paradoxically, we found a significant *decrease* in this ratio following LPS administration (Figure 23). Further studies need to be performed to determine the mechanism underlying the altered D1/D2 ratio.

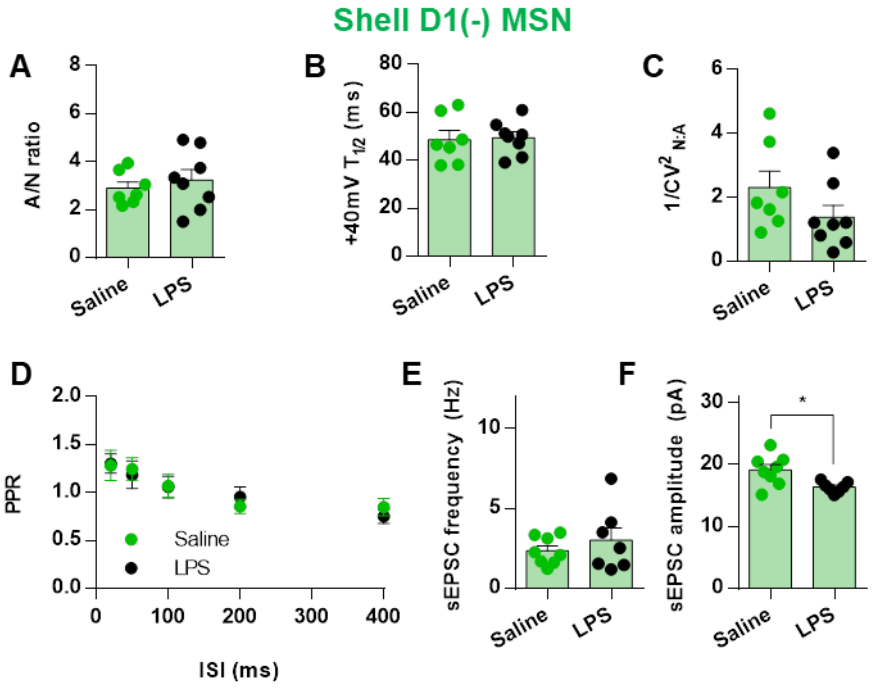

Figure 22. Synaptic properties of D1(-) NAc shell MSNs following LPS administration. (A) Summary of A/N ratio. Saline $n_{cells}/N_{animals}$ = 7/5; LPS n/N = 8/5. (B) Summary of dual-component decay kinetics. Saline n/N = 7/5; LPS n/N = 8/5. (C) Summary of $1/CV^2_{N:A}$. Saline n/N = 7/5; LPS n/N = 8/5. (D) Summary of PPR. Saline n/N = 7/5; LPS n/N = 9/5. (E) Summary of sEPSC frequency and (F) amplitudes. Saline n/N = 8/5; LPS n/N = 7/5. Error bars denote SEM. *P < 0.05, unpaired t test.

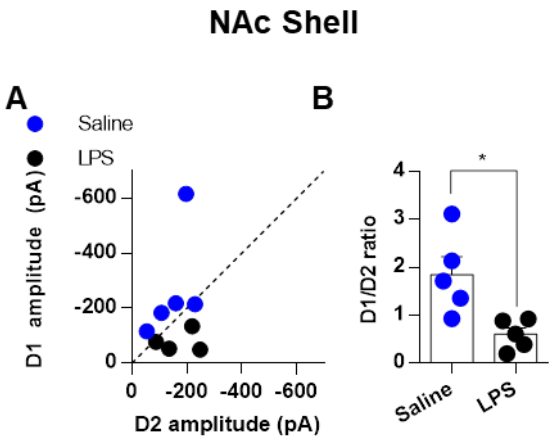

Figure 23. LPS biases excitatory inputs towards D1 MSNs in the NAc shell.
(A) Plot of EPSCs from paired D1 and D2 cells. (C) Summary plot of D1/D2 EPSC ratio. Saline $n_{pairs}/N_{animals}$ = 5/4; LPS n/N = 5/3. Error bars denote SEM. *P < 0.05, unpaired t test.

IV-3. Discussion

IV-3a. Infections, sickness, and depression

Infections are associated with a host of immunologic and behavioral changes falling under the umbrella term of “sickness behavior.” This includes despair, anhedonia, and hypolocomotion—characteristics also associated with MDD. Though medical conditions including sickness and infections preclude the diagnosis of MDD (American Psychiatric Association, 2013), it is worth noting that many suffering from MDD exhibit altered immunologic profiles (Dantzer *et al*, 2007). Furthermore, administration of cytokines alone is sufficient to recapitulate many of the mood changes seen in sickness and depression (Dantzer *et al*, 2007). Studies performed on human male subjects found that immune activation using a gram-negative bacterial mimetic (*Salmonella typhi* vaccine) was associated with an upregulation of peripheral IL-6 and mood changes shared with depression (Harrison *et al*, 2009). Functional imaging of these subjects revealed a

concurrent decrease in functional connectivity between the subgenual anterior cingulate cortex and NAc. Importantly, reported changes in mood occurred independently of other "illness" symptoms including fever, cortisol levels, nausea, and joint aches pointing towards a primary effect of immune activation on mood (Harrison *et al*, 2009).

A greater understanding of the neuronal physiology underlying changes in motivation/reward caused by sickness may shed insight into other pathologies associated with altered motivational states. To this end, we characterized behavioral and synaptic manifestations in the NAc following LPS administration. LPS is a component of gram-negative bacterial cell walls and is detected by TLR4. LPS binding to TLR4 leads to signaling through MyD88 and TRIF pathways resulting in upregulation of pro-inflammatory cytokines (Bohannon *et al*, 2013). These include IL-6, CCL2, and CXCL10 in the periphery and CCL2 and CXCL10 in the brain of mice when assessed 24 hours after injection of LPS (Davis *et al*, 2017). Measured concentrations of each of these cytokines in their respective compartments correlated with hypolocomotion (Davis *et al*, 2017).

We add to this body of work through examination of changes in synaptic physiology following in vivo LPS administration. Following behavioral verification of LPS-induced behavioral changes, we found modest cell-type and NAc-subregion-specific changes in MSNs. In the NAc core, decreased sEPSC frequency on D1(+) MSNs without change in PPR, D1(-) MSN properties, or D1/D2 ratio leaves the possibility of decreased network activity onto D1(+) MSNs. In the NAc shell, our data points to differences in both MSN subtypes with LPS causing a decreased PPR on D1(+) MSNs and decreased sEPSC amplitude on D1(-) cells. Though these changes

would each be expected to bias the excitatory drive towards D1 MSNs, we found a paradoxical *decrease* in the evoked D1/D2 ratio. A decrease in this ratio is in line with the simplified idea of D1 and D2 MSNs mediating pro- and anti-motivation/reinforcement, respectively (Francis and Lobo, 2016). Future experiments such as examining evoked quantal events via asEPCs or corroborating the decrease in NAc shell D1(+) MSN PPRs with mEPSCs resolve the apparent discrepancy between the paired recordings and cell-type properties. Regardless, these results suggest that similar to other behavioral experiences affecting the reward system, differential and specific synaptic adaptations occur as a response to systemic immunologic activation.

IV-3b. The NAc and emotional valence

Experiences that affect motivational states cause cell-type-specific synaptic alterations in the NAc MSNs. Rewarding experiences which elicit motivational behavior such as exposure to drugs of abuse are well-studied and lead to the biasing of excitatory inputs towards D1 MSNs (Graziane *et al*, 2016; Pascoli *et al*, 2014). However, comparatively less is known about NAc synaptic adaptations associated with negative experiences leading to decreased motivation/reward (Francis and Lobo, 2016). Several studies suggest that certain stressors including chronic social defeat or restraint are associated with NAc synaptic adaptations opposite to that seen in drug abuse. Mice susceptible to expression of “depressed” symptoms following chronic social defeat exhibit decreased mEPSC frequency on D1 MSNs and increased mEPSC frequency on D2 MSNs (Francis *et al*, 2015). Correlation of MSN electrophysiological properties with behavior showed that D2 (but not D1) MSN mEPSC frequency inversely correlated with social-

defeat-induced behaviors (Francis and Lobo, 2016). A separate study found that chronic restraint stress reduces excitatory inputs on D1 MSNs through endocytosis of AMPAR without affecting the D2 population (Lim *et al*, 2012). Prevention of AMPAR endocytosis protected against the decrease in behavioral despair associated with restraint stress (Lim *et al*, 2012). These studies provide evidence for stress biasing NAc excitatory inputs towards D2 MSNs. However, this synaptic adaptation is not universal for negative experiences decreasing motivation/reward. Beyond stressors, chronic pain is another condition associated with depression (Turk *et al*, 2010). In rodent models, chronic inflammatory pain via injection of Complete Freund's Adjuvant or neuropathic pain via spinal nerve injury results in loss of motivation (Schwartz *et al*, 2014). In contrast to stress, both models of chronic pain led to reduction of excitatory tone onto NAc D2 MSNs (Schwartz *et al*, 2014). These results paint a complicated picture signifying the need to further investigate of the relation of D1 and D2 MSN afferent signaling to reward valence.

Certain experiences also confer subregion-specific adaptations in the NAc. In the context of cocaine exposure, experience-dependent changes in MSN synaptic physiology differ between the core and shell subregions (Grueter *et al*, 2013; Jedynak *et al*, 2016; Ma *et al*, 2014). This may be in part due to each region's distinct anatomy and basal physiology. Each region receives differential hippocampal (Britt *et al*, 2012; Groenewegen *et al*, 1987), prefrontal cortical- (Ma *et al*, 2014), and midbrain dopaminergic inputs (Everitt and Robbins, 2005; Sesack and Grace, 2010). In addition, optogenetic reversal of drug-induced changes of cortical inputs onto the NAc core vs. shell cause opposing effects on drug seeking behavior (Ma *et al*, 2014). Stress-induced

alterations in the NAc have also been associated with subregion-specific changes (Francis and Lobo, 2016). Our results showing differential alteration of NAc core vs. shell synaptic properties furthers our understanding of the physiology of sickness and despair.

Unlike addiction or depression, sickness and infections are typically temporary (Yirmiya, 1996). Thus, examination into NAc MSN synaptic properties through the time course between onset and end of sickness behavior provides a unique opportunity to study endogenous changes in physiology associated with recovery from depression-like motivational states. Future studies may also utilize optogenetics to examine input-specific changes in NAc synaptic physiology underlying sickness behavior. Such insights may have implications in other conditions associated with neuro-inflammation and altered motivation including MDD, schizophrenia, and autism.

CHAPTER V

DISCUSSION

V-1. Introduction to the end

Substance use disorders as well as major depressive disorders remain as highly prevalent psychiatric disease states associated with a significant healthcare and economic burden. Treatments continue to lack in part due to an incomplete understanding of the underlying disease pathophysiology. Though clinically separate conditions, both depression and substance use disorders involve alterations of motivation and reward (American Psychiatric Association, 2013). The NAc is an integrator of such information and is altered in both disease states (Francis and Lobo, 2016; Kauer and Malenka, 2007). In rodents, exposure to drugs of abuse as well as stress/pain behavioral manipulations associated with depression cause cell-type specific changes in NAc MSNs. The nature of these synaptic changes are well-characterized (Francis *et al*, 2015; Graziane *et al*, 2016; Pascoli *et al*, 2014; Schwartz *et al*, 2014). Less understood are synaptic adaptations occurring in the context of alterations in the immune system.

Alterations in the immune system including an increase in inflammatory markers are a hallmark of many neuropsychiatric diseases including depression, bipolar, anxiety disorders, schizophrenia, and substance use disorders (Miller *et al*, 2017; Schleifer, 2007). In addition, exogenous administration of cytokines can modulate mood/reward (Harrison *et al*, 2009) suggesting direct interplay between

the nervous and immune systems. One immune-associated protein implicated in reward behavior is TLR4. Though inhibition of this protein has been implicated in drug-reward behavior (Hutchinson *et al*, 2012; Northcutt *et al*, 2015) with some controversy (Tanda *et al*, 2016), its involvement in synaptic physiology remained relatively unexplored. On the flip side, activation of TLR4 through mimetics of a gram-negative bacterial infection causes a well-characterized release of inflammatory cytokines resulting in behavioral anhedonia and despair (Sekio *et al*, 2015; Yirmiya, 1996). Similarly, the synaptic consequence of this manipulation is less known. The work presented in this dissertation addressed these gaps in knowledge through examination of cell-type and region-specific NAc MSN synaptic physiology as it relates to behavioral manipulations. These results provide a key step in developing more effective treatments for neuropsychiatric conditions through bettering our understanding of immunologic factors influence disease states affecting motivation and reward.

V-2. A NAc for crack: TLR4.KOs and drug-reward

In order to elucidate the contribution of TLR4 to drug-reward behavior and synaptic physiology, we utilized a KO approach. We examined the effects of lacking TLR4 on NAc synaptic physiology in a cell-type and region-specific manner, we bred TLR4.KO mice to fluorescent reporter mice so as to express the tdTomato fluorophore on D1(+) MSNs. With these mice along with WT animals also expressing the reporter protein, we performed *ex vivo* electrophysiological recordings from acute slices from the anatomically distinct

NAc core and shell subregions. Using whole-cell voltage clamp recordings, we assessed AMPAR and NMDAR properties. With extracellular field-potential recordings, we examined plasticity mechanisms.

Without prior studies examining NAc synaptic physiology and TLR4, we initially characterized naïve synaptic properties in TLR4.KO animals. In the NAc core, we found no significant differences in basal AMPAR transmission. However, when assessing NMDAR properties, we found evidence for TLR4.KO animals expressing cell-type specific differences in NMDAR subunit composition (Figure 24). This was further associated with an attenuation in both cocaine-reward learning as well as LFS-LTD when assessed through extracellular field potentials. Importantly, LTD mediated by a Group II mGluR agonist remained intact and no synaptic differences were seen in the NAc shell. These points argue against both a non-specific loss of plasticity mechanisms and a global change in synaptic physiology. Behaviorally, we found that TLR4.KO animals did not differ from WT animals for non-drug behavior tests including open field locomotion, novel object recognition, and sucrose preference tests. This supports a drug-reward specific behavioral phenotype. We further showed that *Tlr4* mRNA in the NAc core was primarily found on microglia suggestive of a neuro-immune interaction mediating the synaptic and behavioral effects.

Following characterization of naïve animals, we assessed changes in NAc synaptic physiology following drug exposure. Such changes are important as they underlie drug-reward learning (Kauer and Malenka, 2007) and their

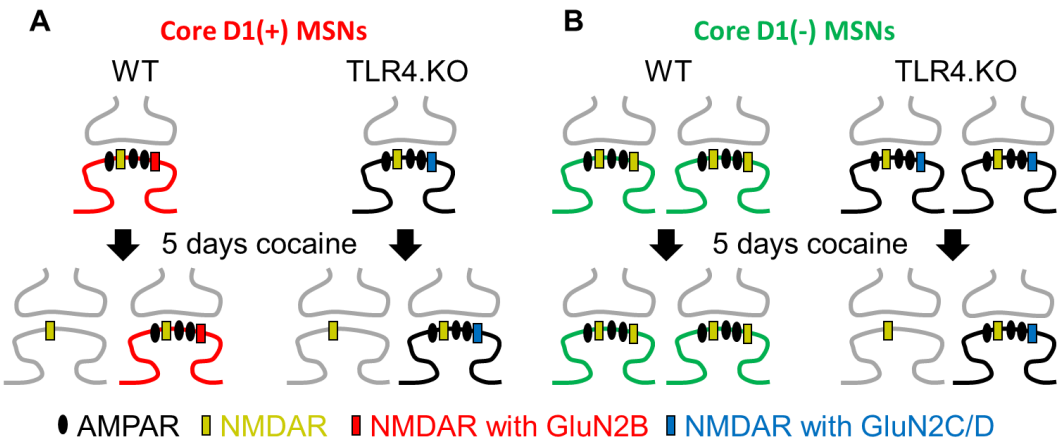

Figure 24. Synaptic summary.
(A) In WT NAc core D1(+) MSNs, NMDARs with GluN2B subunits are present in the synapse under naïve conditions. Exposure to 5 days of cocaine generates silent synapses *de novo*. In TLR4.KO D1(+) MSNs, there is decreased function of NMDARs with GluN2B but increased function of GluN2C/D subunits. (B) In WT D1(-) MSNs, there is a lack of Ifenprodil sensitivity suggesting little/no GluN2B function. Exposure to 5 days of cocaine does not cause synaptic change. In TLR4.KO D1(-) MSNs, there is an upregulation of GluN2C/D function. Exposure to 5 days of cocaine may silence active synapses. These synaptic properties may underlie the observed behavioral differences.

restoration modulates drug-seeking behavior (Ma *et al*, 2014; Pascoli *et al*, 2012, 2014). To this end, we exposed WT and TLR4.KO mice to a locomotor sensitization assay (Joffe and Grueter, 2016; Pascoli *et al*, 2012). With this paradigm, we recorded from D1(+) and D1(-) MSNs from the NAc core at 3 separate time-points. In this set of experiments, we focused on the NAc core as that was the site of a synaptic phenotype seen in naïve animals (Kashima and Grueter, 2017). Locomotor activity in response to cocaine-exposure was also assessed and revealed sex-specific differences. Specifically, male TLR4.KO mice exhibit attenuations in locomotor activity throughout initial exposure and challenge from cocaine whereas females only exhibit a decreased locomotor

response during initial exposure. This behavioral result suggests sex-specific modulation of drug-reward by TLR4 and warrants further investigation. In WT female mice, oesterus is associated with increased neuronal activity in the VTA and a heightened effect of cocaine in blocking dopamine reuptake (Calipari *et al*, 2017). Examination into a possible interaction between TLR4, the oesterus cycle, and drug-reward may reveal important insights into sex-specific differences in addiction biology. Through assessment of NAc core synaptic properties in male mice, we found genotype and cell-type specific differences following the initial 5 days of cocaine or saline as well as following a cocaine challenge. Of note, the day 5 time-point is associated with attenuated drug-response in both male and female TLR4.KO mice.

In WT mice, we found evidence for cocaine increasing the proportion of silent synapses on D1(+) MSNs through their generation *de novo*—an observation in line with that of the neighboring NA shell (Graziane *et al*, 2016). In TLR4.KO animals, our results suggest that cocaine increases the proportion of silent synapses on both D1(+) and D1(-) MSNs. However, the change in D1(-) may be due to the “silencing” of previously active synapses (Figure 24). Importantly, these conclusions about changes in the proportion of silent synapses were based on analysis of $1/CV^2_{N:A}$. While this measure has been used in past studies (Grueter *et al*, 2013; Huang *et al*, 2009) and has the advantage of allowing simultaneous assessment of A/N ratio, silent synapse measurements are typically confirmed with a minimal-stimulation assay (Graziane *et al*, 2016; Huang *et al*, 2009). In addition, our interpretation of changes on TLR4.KO D1(-)

MSNs relied on a combination of increased $1/CV^2_{N:A}$, a lack of change in PPR, and decreased sEPSC frequency to argue for a silencing of synapses. While this is the most parsimonious explanation, it does not rule out the possibility of silent synapses generated *de novo* combined with a profound decrease in network activity. In order to rule this possibility out, future experiments should also include recordings of mEPSCs. As mEPSCs are recorded in the presence of tetrodotoxin to block action potentials, differences observed between saline and cocaine treated animals will be due to factors independent of network activity. If the increase in the proportion of silent synapses in cocaine-treated TLR4.KO D1(-) MSNs is due to silencing of previously active synapses, then the mEPSC frequency will also be decreased in cocaine-treated compared to saline treated animals. Beyond electrophysiology experiments, these results may be corroborated with morphologic analysis of dendritic arbors in dye-filled MSNs (Graziane *et al*, 2016; Grueter *et al*, 2013). If cocaine increases $1/CV^2_{N:A}$, in D1(+) MSNs of both WT and TLR4.KO animals through generation of silent synapses *de novo*, then dye filling experiments will show an increase in the number/density of dendritic spines on D1(+) MSNs. On the other hand, if cocaine increases $1/CV^2_{N:A}$, in TLR4.KO D1(-) MSNs through the silencing of previously active synapses, then there will be no difference in the number/density of dendritic spines on D1(-) MSNs. Should the conclusion of synapse silencing on TLR4.KO D1(-) MSNs be substantiated, it leaves a puzzling phenomenon where decreased drug-associated behavior is associated with a decrease in excitatory tone onto D1(-) MSNs. This is at odds with the view of D1(+) and D1(-) MSNs

representing “pro” and “anti” drug/reward, respectively (Graziane *et al*, 2016; Lobo *et al*, 2010). One possible explanation for this discrepancy is an input-specific change in synaptic properties. This has been observed in NAc shell D1(+) MSNs, where optogenetic experiments revealed that cocaine causes projection-specific synaptic changes associated with drug-experience (MacAskill *et al*, 2014; Pascoli *et al*, 2014). In particular, MacAskill et al. showed in the NAc shell that 5 days of non-contingent cocaine exposure results in the biasing of BLA inputs towards D1 MSNs while biasing vHipp inputs towards D2 MSNs (MacAskill *et al*, 2014). In the NAc core, vHipp inputs onto D2 MSNs are unique in that the synapses are located further from the soma compared to other inputs such as the mPFC and thalamus (MacAskill *et al*, 2012). A preferential silencing of vHipp synapses on TLR4.KO D1(-) MSNs would oppose the normal cocaine-induced changes at this input and provides a plausible explanation for a reduction in drug-locomotor behavior. In order to assess whether the increase in silent synapses on TLR4.KO D1(-) NAc core MSNs occurs in such a projection-specific manner, future experiments may use viral-mediated expression of channelrhodopsin into NAc core inputs. While changes in vHipp inputs are the hypothesized locus of change, examination of the inputs from the mPFC, BLA, and mThal may yield additional insights (Joffe and Grueter, 2016; Ma *et al*, 2014; MacAskill *et al*, 2012). If the change in silent synapse numbers is driven by the vHipp input, then cocaine will cause $1/CV^2_{N:A}$ to be increased in this input but not others.

A second possible explanation for the association between silencing of D1(-) synapses and attenuated cocaine locomotor behavior is physiologic/anatomic differences between the core and shell subregions. Studies relating cell-type specific physiology to *in vivo* optogenetic behavioral manipulations of drug-reward largely focused on the NAc shell to argue D1 and D2 MSNs represent pro- and anti-reward, respectively (Lobo *et al*, 2010; Pascoli *et al*, 2014). Strengthening of inputs onto D1 MSNs via increased AMPAR transmission has been repeatedly demonstrated in the NAc shell following non-contingent cocaine exposure (Graziane *et al*, 2016; Pascoli *et al*, 2012, 2014). However, cell-type specific effects in the NAc core are less understood. Lewitus et al. demonstrated that 5 days of non-contingent cocaine decreases the A/N ratio of NAc core D1(+) but not D1(-) MSNs (Lewitus *et al*, 2016). Whether this was due to a decrease in AMPAR transmission or an increase in NMDAR transmission was not clarified. Separately, Joffe et al. found that 5 days of non-contingent cocaine exposure followed by 10-14 days of withdrawal results in no differences between saline or cocaine-treated animals for D1(+) and D1(-) cells when assessed through electrical stimulation (Joffe and Grueter, 2016). However, input-specific optogenetic experiments uncovered changes in NMDAR subunits in thalamic- but not prefrontal-cortical inputs onto NAc core D1(+) MSNs (Joffe and Grueter, 2016). These subregion-specific differences in cocaine-associated physiology may also underlie our observed results for drug-associated physiology in TLR4.KO animals. Future studies may examine cell-type specific effects of cocaine exposure in the NAc shell.

Interestingly, synaptic differences between saline and cocaine-treated animals were largely absent for both WT and TLR4.KO when examined following 10-14 days of withdrawal. Highlighting a likely regional difference within the NAc core, this data from WT animals is consistent with previously published data from the our lab (Joffe and Grueter, 2016) but not with another study recording from a more lateral portion (Jedynak *et al*, 2016). Perplexingly, we also found that cocaine or saline history affects synaptic responses to a cocaine challenge in WT but not TLR4.KO animals. This difference may indicate a difference in metaplasticity to cocaine. Future experiments may examine this possibility.

V-3. Activation: LPS and MPLA

V-3a. LPS and despair

Whereas drugs of abuse promote rewarding sensations and motivated behavior, sickness does the opposite. While we demonstrated that a deficiency in TLR4 attenuates drug-reward behavior, there remains the question of behavioral and synaptic effects following its activation. LPS is a canonical activator of TLR4 that causes upregulation of pro-inflammatory cytokines via signaling through MyD88 and TRIF pathways (Bohannon *et al*, 2013). When LPS is administered in the periphery, the subsequent release of cytokines such as IL-6 cause a decrease in motivational and reward sensations/behaviors associated with “sickness.” Of note, such clinical symptoms overlap with many of those seen in depression (Table 2) (American Psychiatric Association, 2013; Dantzer *et al*, 2007). With the NAc acting as a hub for motivation and reward-related behaviors, we sought to

characterize synaptic changes occurring as a result of sickness. Following confirmation of LPS-induced behavioral effects, we found that this was associated with cell-type and region-specific changes in NAc synaptic physiology. It will be interesting to see whether these changes reverse along the time-course of behavioral recovery.

Major depressive episode (5+ of the following for at least 2 weeks)
1) Individual has depressed mood most of the day, nearly every day
2) Individual loses interest or pleasure in most or all activities, nearly every day
3) Indidivdual has insomnia or hypersomnia nearly every day
4) Individual has significant weight loss/weight gain OR decreased/increased in appetite nearly every day
5) Individual exhibits psychomotor retardation or agitation nearly every day that is observable by others
6) Individual has fatigue or low energy, nearly every day
7) Individual has decreased ability to concentrate, think, or make decisions, nearly every day
8) Individual has thoughts of worthlessness or excessive or inappropriate guilt, nearly every day
9) Individual has recurrent thoughts of death or suicidal ideation, or has attempted suicide

Table 2. Diagnostic criteria for major depressive episode.
At least one symptom must be either depressed mood or loss of interest/pleasure. Major depressive disorder (MDD) involves a history of one or more major depressive episodes without history of mania or hypomania. Modified from DSM-V (American Psychiatric Association, 2013).

V-3b. What else can TLR4 signaling be used for? A foray into ischemia.

In addition to their use in mapping out immune function and associated behaviors, activators of immune signaling also have clinical utility as vaccine adjuvants. Such compounds are combined with vaccine antigens to boost antibody responses (Mata-Haro *et al*, 2007). One immune signaling pathway targeted for the development of adjuvants is TLR4. TLR4 is unique among toll-like receptors in that it signals through both MyD88 and TRIF pathways en route

to inducing the cytokine response (Watters *et al*, 2007; Yamamoto *et al*, 2003). While LPS may seem like an obvious candidate for TLR4 stimulation, its utility beyond pre-clinical research is limited due to its high toxicity in humans (Bohannon *et al*, 2013). LPS signals through both MyD88 and TRIF pathways. However, a molecular derivative known as monophosphoryl lipid A (MPLA) was later developed which acts as a TLR4 biased-agonist with preferential signaling through TRIF (Mata-Haro *et al*, 2007). This compound has 1/1000 the toxicity of LPS in humans and is now used as an adjuvant in papilloma and hepatitis virus vaccines (Bohannon *et al*, 2013). Interestingly, exposure to LPS or MPLA alone has differential effects dependent on dose. Low/moderate doses cause endotoxin "tolerance" where the host is hyporesponsive to subsequent LPS exposure whereas ultra-low doses prime the host to mount a more exaggerated response (Bohannon *et al*, 2013). With demonstrated clinical utility in boosting immune function, a follow-up question is whether MPLA can improve outcomes for neuronal pathologies associated with inflammation. One such condition with currently limited therapeutic options is stroke.

Every year, stroke affects ~795,000 Americans and accounts for 1 in 18 deaths in the United States (Roger *et al*, 2012). Beyond mortality, stroke is associated with significant disability (Regenhardt *et al*, 2013). Despite this disease burden, few options exist to limit the damage and long-term disability brought by stroke (Regenhardt *et al*, 2013). Stroke causes injury to the brain parenchyma and is associated with an upregulation of inflammatory cytokines (McColl *et al*, 2007). We wondered whether modulation of the innate immune

system using MPLA influences functional and/or physiologic adaptation following infarct. To this end, a colleague performed a middle cerebellar artery occlusion (MCAO) in mice and I performed extracellular field potential recordings from the contralateral dorsal striatum to assess physiologic changes. The dorsal striatum is part of the extrapyramidal motor circuit involved in motor learning and procedural planning (Kreitzer and Malenka, 2008). The contralateral hemisphere was assessed for physiologic changes as strokes preclude stable/viable recordings from affected regions (Brad Grueter, personal communication). With this paradigm, we assessed synaptic strength through input/output (i/o) relationships as well as presynaptic release probability via PPR. Compared to naïve animals, we found that MCAO caused a significant reduction of PPR without altering i/o relationships for the fiber volley or population spike response (Figure 25). This suggests that MCAO causes an increase in the presynaptic release probability of glutamatergic afferents onto the contralateral dorsal striatum. Interestingly, we found that 24 hour pre-treatment with MPLA mitigates the decrease in PPR (Figure 25B).

Though these electrophysiological results raise the possibility of MPLA protecting against changes in synaptic function due to a contralateral stroke, a control experiment assessing the effects of MPLA alone is still lacking. Future studies need to include this control experiment as well as corroborate these findings with immunohistochemical tests to assess tissue damage/death as well as functional tests such as open field tests to examine locomotor behavior.

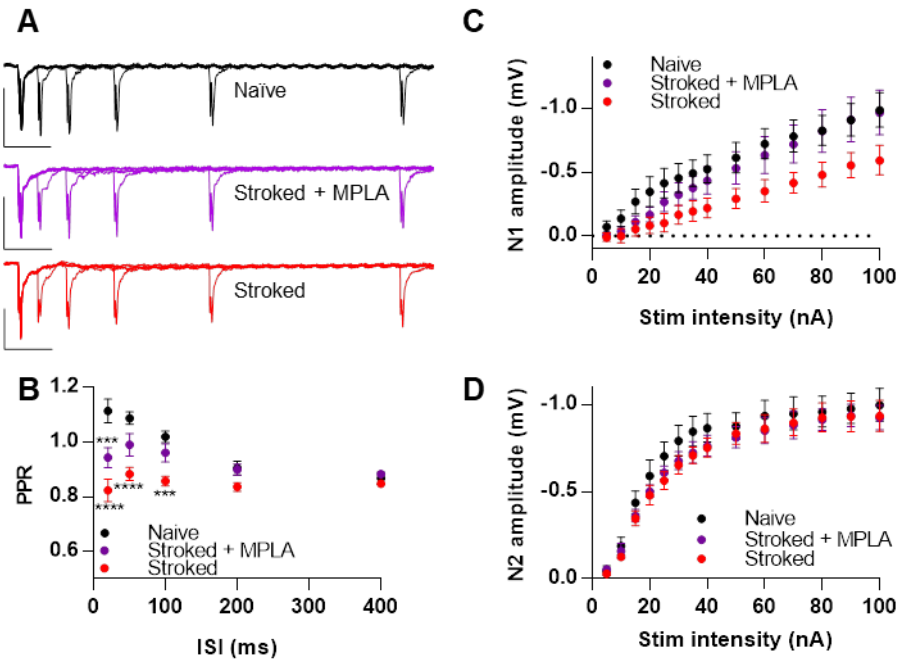

Figure 25. Mono-phosphoryl lipid A (MPLA) administration alters physiologic synaptic adaptations to contralateral stroke.
Mice were pre-treated with MPLA 24 h before middle cerebral artery occlusion (MCAO). Field potential recordings were performed 24 h after MCAO in the dorsal striatum. (A) Representative traces from paired pulse ratio (PPR) experiments. (B) Summary of PPR experiments. Significant effects by treatment were observed (Naïve vs Stroke $F(1,18) = 32.52$, $P < 0.0001$; Stroke vs Stroke + MPLA $F(1,16) = 7.818$, $P = 0.0129$; Naïve vs Stroke + MPLA $F(1,16) = 4.424$, $P = 0.0516$, 2-way repeated measures ANOVA). (C) Summary data of fiber volley (N1) amplitudes as a relation of stimulation (stim) intensity. (D) Summary data for population spike response (N2) as a relation of stim intensity. ***$P < 0.001$; ****$P < 0.0001$, 2-way repeated measures ANOVA with Sidak's post-hoc test. Comparisons made against naïve responses for a given interstimulus interval (ISI). Naïve n/N = 10/4; Stroked + MPLA n/N = 8/3; Stroked n/N = 10/4. All error bars denote SEM. Scale bars: 50 ms, 0.5 mV.

V-4. Study Limitations

It is important to acknowledge limitations of the findings within this dissertation for at least 3 reasons: 1) it provides an appropriate context for the findings, 2) it permits interpretation of the results' validity, and 3) is necessary for proper assessment of whether the conclusions are warranted (Ioannidis, 2007). Perhaps the biggest limitation to these studies is use of the mouse to model neurologic/psychiatric disease processes. Though murine behavioral assays may test for specific aspects of disease features such as anhedonia, despair, and drug-reward learning, there remains the inability to ask mice about the subjective nature of their condition. Mice are not humans and there will always be a question of how accurately they model human conditions. Nonetheless, mice remain powerful as model organisms in neuroscience as they are mammals which learn complex behaviors, are permissive for genetic manipulations, and allows for *ex vivo* physiologic assessment of transcriptionally distinct neurons in the context of a circuit. "Less advanced" organisms do not have a sufficiently developed CNS to study physiology in the context of neural circuits whereas "more advanced" species are less amenable to genetic manipulations and have longer gestation/maturation periods requiring significantly more time and resources to perform enough experiments needed for statistical power. Put another way, we could not have performed this set of experiments on another organism.

A major limitation for our drug-reward experiments (Chapters 2 and 3) is the use of germline whole-body knockouts of TLR4. An issue with studying

germline knockouts is the inability to rule out developmental effects of the gene. There is some evidence suggesting that TLR4 plays a role in hippocampal development (Okun *et al*, 2012; Rolls *et al*, 2007). In addition, our results did not demonstrate sufficiency of microglial TLR4 causing the behavioral/physiologic phenotype. We attempted address these issues through breeding a mouse expressing a tamoxifen-inducible cre on microglia and peripheral monocytes ($Cx3Cr1^{CreER}$) to a floxed-TLR4 line. Due to differential turnover rates of microglia and the periphery (Parkhurst *et al*, 2013), tamoxifen administration followed by a 28 day wait results in mice lacking TLR4 exclusively in microglia. Unfortunately, we found that tamoxifen administration alone has an effect on cocaine locomotor activity (Figure 26).

An alternate approach to address the developmental issue is through the use of pharmacologic antagonists of TLR4. Northcutt et al. (2015) found that administration of (+)-naloxone/(+)-naltrexone—enantiomers that act on TLR4 but not μ-opioid receptors—decreases cocaine self-administration. However, the doses used were later demonstrated to cause non-specific effects making it difficult to draw specific conclusions (Tanda *et al*, 2016). Future studies may approach this issue through use of another TLR4 antagonist such as Tak-242/CLI-095 (Hua *et al*, 2015). Alternatively, different administration time courses and administration routes for tamoxifen may be tested. Should these fail, genetic models using a tetracycline-on/off-dependent cre mouse bred to floxed TLR4 lines could be used as a way to genetically excise TLR4 after development.

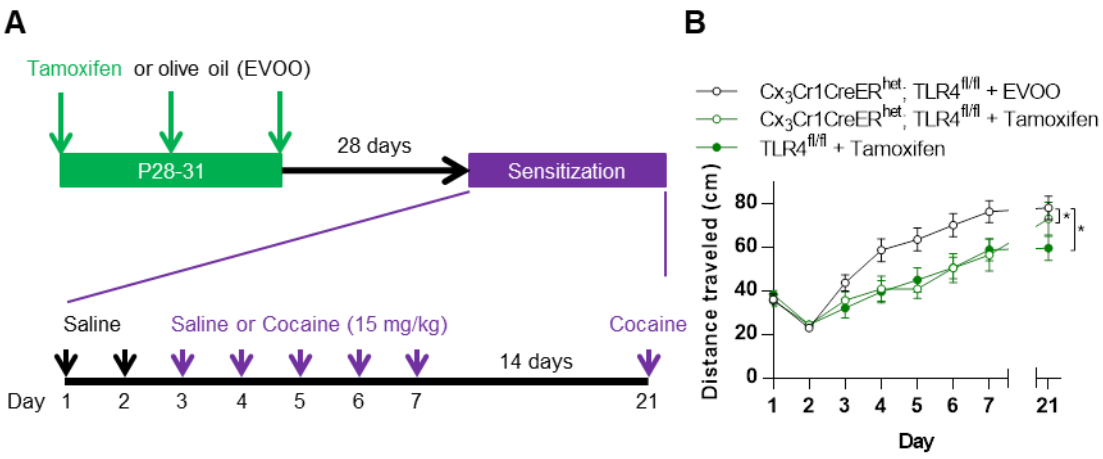

Figure 26. Tamoxifen administration alone attenuates cocaine locomotor response.

(A) Experimental timeline for tamoxifen administration and behavioral experiments. From P28 – P31, mice were injected 3 times with either extra-virgin olive oil (EVOO) or tamoxifen (2 mg dissolved in EVOO) subcutaneously. Following a 28 day wait, cocaine locomotor sensitization was performed. This was performed on $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ and $TLR4^{flox/flox}$ mice. $Cx_3CR1\text{-}Cre^{ER}$ is a tamoxifen-inducible cre on a promoter expressed on microglia and a subset of peripheral myeloid cells. When crossed to have $TLR4^{flox/flox}$, Tamoxifen administration ablates *Tlr4* from both populations. Due to differences in cell turnover, a subsequent 28-day wait results in animals lacking *Tlr4* exclusively on microglia (Parkhurst *et al*, 2013). (B) Results from sensitization experiments. Tamoxifen administration alone depresses cocaine locomotor response (Effect of treatment $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ + EVOO vs $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ + Tamoxifen $F(1,28) = 5.066$, $P = 0.0324$, $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ + EVOO vs $TLR4^{flox/flox}$ + Tamoxifen $F(1,33) = 6.842$, $P = 0.0133$, $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ + Tamoxifen vs $TLR4^{flox/flox}$ + Tamoxifen $F(1,29) = 0.04752$, $P = 0.8290$, 2-way repeated measures ANOVA). $N_{animals}$ = 17 $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ + EVOO, 13 $Cx_3CR1\text{-}Cre^{ER}$; $TLR4^{flox/flox}$ + Tamoxifen, 18 $TLR4^{flox/flox}$ + Tamoxifen. All error bars denote SEM.

V-5. Future Directions of TLR4 function in the NAc

Historically, neuroscience and immunology represented distinct fields of study with little overlap ("Neuroimmune communication," 2017). This notion has gradually changed over the past decade as studies showed bi-directional communication between the nervous and immune systems. Broadly, the nervous system influences immune function in the context of inflammation or pathogen clearance (Ben-Shaanan *et al*, 2016; Pavlov and Tracey, 2017) while immunologic cells and factors affect synaptic function, learning, and memory (Klein *et al*, 2017; Prinz and Priller, 2017). The work presented in this dissertation focused on TLR4, a pattern recognition molecule of the innate immune system, on glutamatergic synaptic physiology and behavior. Our demonstration of this protein's importance in NAc synaptic physiology, cocaine-reward behavior, and sickness, brings up a multitude of interesting questions worth pursuing in future studies.

V-5a. TLR4-dependent signaling involved in drug-reward learning

The most substantial question is that of mechanism in drug-reward. We demonstrated the synaptic and drug-behavioral consequence of lacking TLR4. Others have studied behavioral effects following pharmacologic antagonism (Hutchinson *et al*, 2012; Northcutt *et al*, 2015; Tanda *et al*, 2016). However, insight into factors upstream- and downstream of TLR4 mediating drug-reward behavior is lacking. Downstream, TLR4 signals through both MyD88 and TRIF-dependent pathways to upregulate inflammatory cytokines and type I interferons

(Bohannon *et al*, 2013; Watters *et al*, 2007; Yamamoto *et al*, 2003). MyD88 has been implicated in opioid-reward (Hutchinson *et al*, 2012). Whether these pathways are also necessary for cocaine-reward may be tested using commercially available MyD88 and TRIF knockout mice. Beyond MyD88 and TRIF, there is also a question of what signaling factors downstream of TLR4 mediate the synaptic and behavioral phenotype. Identification of factors upstream to TLR4 will be more challenging. Some investigators posit that cocaine directly binds to microglial TLR4 resulting in cytokine responses (Bachtell *et al*, 2015; Northcutt *et al*, 2015). However, direct activation of microglia by cocaine is disputed (Lewitus *et al*, 2016). This leaves the possibility of an endogenous ligand signaling through TLR4 for drug-reward. Unfortunately, the identification of such a ligand is difficult because TLR4 binds a wide variety of endogenous factors including heat-shock proteins, saturated fatty acids, myeloid-related proteins, fibrinogen, fibronectin, and many others (Trotta *et al*, 2014). Future studies may take an exploratory approach using RNAseq to identify which known TLR4 ligands are upregulated with cocaine exposure. Subsequent behavioral and synaptic characterization of knockout mice will be telling. Similar approaches may be taken to understand which inflammatory factors associated with sickness behavior are necessary for the observed synaptic changes.

V-5b. Other substances: Morphine, alcohol, and beyond

Although virtually every drug of abuse alters the NAc (Nestler, 2005), the specific nature of the synaptic changes depend on the drug identity (Graziane *et al*, 2016;

Hearing *et al*, 2016; Jedynak *et al*, 2016). The drug-reward behavior and physiology examined in this dissertation centered on cocaine in the NAc core. An obvious next step is examination of morphine on TLR4 and synaptic physiology. Morphine acts on the μ-opioid receptor and causes a variety of effects including analgesia, euphoria, and sedation (Al-Hasani and Bruchas, 2011).

With common and widespread prescription of morphine and other opiates, the US faces an "opioid crisis" (National Institute on Drug Abuse, 2017b). In WT mice, non-contingent exposure to morphine initially causes the "silencing" of synapses on D2 MSNs in the NAc shell (Graziane *et al*, 2016). Over the course of several weeks of withdrawal, synaptic strength increases on D1 MSNs and decreases on D2 MSNs (Hearing *et al*, 2016). This contrasts to cocaine where experience and withdrawal preferentially affects D1 MSNs (Graziane *et al*, 2016; Lewitus *et al*, 2016; Pascoli *et al*, 2014). In addition, there is evidence for neuro-immune involvement associated with morphine experience. TLR4 deficiency decreases morphine reward-learning and there is *in silico* evidence for morphine interacting directly with TLR4 (Hutchinson *et al*, 2012).

Along with morphine and cocaine, TLR4 plays a role in some of the physiologic changes associated with alcohol intake. Mice lacking TLR4 are protected from alcohol-induced glial activation, inflammation, and cell death (Alfonso-Loeches *et al*, 2010). In alcohol-preferring P rats, TLR4 is upregulated in VTA dopaminergic neurons and their knockdown results in reduced impulsivity (Aurelian *et al*, 2016). However, experiments performed using TLR4.KO rats and viral-mediated knockout of TLR4 in the NAc of mice suggest that TLR4 does not

affect alcohol intake (Harris *et al*, 2017). This series of observations begs the question of how morphine, alcohol, and other drugs of abuse affect NAc synaptic physiology in the presence/absence of TLR4.

V-5c. Intersection of neuro-immune system, input-specificity, and drugs of abuse

With the development and refinement of optogenetic tools, the past decade saw an explosion of research dissecting synaptic properties in a circuit/pathway-specific manner (Aston-Jones and Deisseroth, 2013). Cocaine and morphine alter NAc synaptic properties in an input-specific manner (Joffe and Grueter, 2016; Lee *et al*, 2013; Ma *et al*, 2014; MacAskill *et al*, 2014; Pascoli *et al*, 2014; Zhu *et al*, 2016). Such changes may not be detected when sampling inputs in a non-specific manner (Joffe and Grueter, 2016; Pascoli *et al*, 2014). Importantly, different inputs modulate distinct aspects of the drug experience (Lee *et al*, 2013; Ma *et al*, 2014; Pascoli *et al*, 2014; Zhu *et al*, 2016).

In other brain regions, there is some evidence for neuro-immune-mediated synaptic properties occurring in a pathway-specific manner. One example is activity-dependent, input-specific pruning of synapses in the visual system dependent on the compliment cascade (Schafer *et al*, 2012). Despite these insights, little is known about neuro-immune effects on input-specific synaptic physiology. Future work would do well to address this using optogenetics to interrogate input-specific properties. Beyond the experiments proposed above to study of input-specific cocaine-induced changes in TLR4.KO NAc core MSNs,

input-specific changes may also be studied in the context of other drugs of abuse.

V-5d. Further characterization of TLR4.KO animals

Beyond examination of the signaling pathways tying together sickness, drug-reward behavior and the immune system, the work presented in this dissertation leaves open further characterization of TLR4.KO animals.

One observation we noted was a trend towards decreased center time in the open field test suggesting increased basal anxiety in TLR4.KO animals. Future experiments may expand on this characterization through more explicit tests of anxiety through the elevated-plus/elevated-zero maze (McCall *et al*, 2015) or novelty-induced hypophagia (Bluett *et al*, 2017). Stress and anxiety are modulated in part by the eCB system (Bluett *et al*, 2017). eCB function may be assessed in the short-term through depolarization-induced suppression of excitation (DSE) (Shonesy *et al*, 2013). This measures retrograde eCB signaling dependent on calcium and diacylglyceral-lipase-α resulting in a temporary reduction in presynaptic release probability (Ohno-Shosaku *et al*, 2012) previously demonstrated in the NAc (Renteria *et al*, 2014). Long-term eCB effects may be assessed through LFS (10-13 Hz) plasticity protocols (Grueter *et al*, 2010; Robbe *et al*, 2002). Preliminary data examining the NAc core shows a trend towards reduced DSE in TLR4.KO animals compared to WTs (Figure 27). Future experiments examining NAc DSE as well as eCB-dependent LFS plasticity in relation to stress/anxiety.

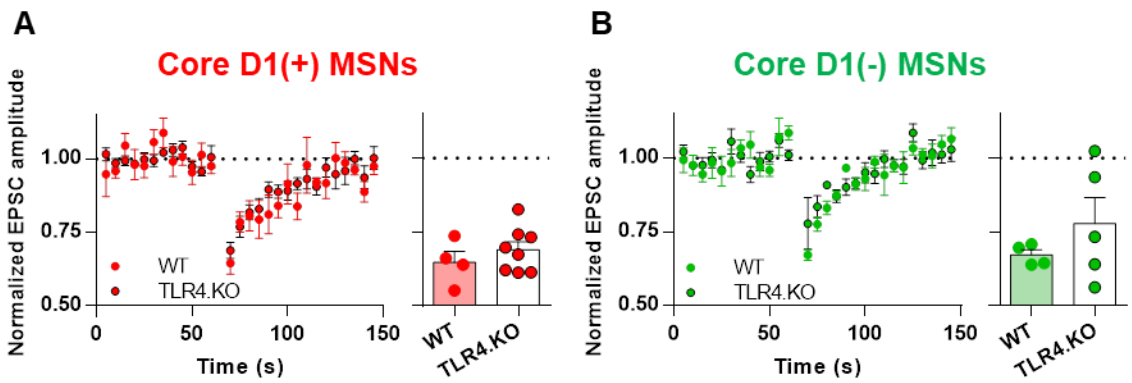

Figure 27. Trends for altered depolarization-induced suppression of excitation (DSE) in TLR4.KO animals.
(A) Summary plot and quantification of DSE from NAc core D1(+) MSNs. WT n/N = 4/2; TLR4.KO n/N = 8/4 (B) Summary plot and quantification of DSE from NAc core D1(-) MSNs. WT n/N = 4/2; TLR.KO n/N = 5/3. P > 0.05, unpaired t test.

Finally, the physiology results presented in this dissertation focused entirely on excitatory synaptic transmission. Future experiments may also examine basal- and experience-dependent changes in NAc inhibitory transmission in relation to TLR4. Inhibitory neurotransmission plays an important role in sculpting circuit responses and behavior (Tepper *et al*, 2010). The role and synaptic properties of NAc inhibitory interneurons are just beginning to be elucidated (Wright *et al*, 2016). It will be interesting to see how TLR4 and the neuroimmune system affect this component of the NAc circuit.

V-6. Conclusion

The ability to dynamically alter the strength of synaptic connections underlies learning, memory, and behavior. Improper regulation or maintenance of this process is a feature or defining hallmark shared by virtually every neurologic and psychiatric disease state. This list includes but is not limited to: substance use disorders, depression, schizophrenia, autism, Alzheimer's disease, Huntington's Disease, ischemia, and epilepsy. Better-understanding of the factors regulating synapses will provide insights into many of these conditions and may uncover novel therapeutic targets.

Starting with Eric Kandel's seminal work in sea slugs implicating synaptic plasticity as a substrate for learning, research over the past half-century revealed many neuron-centric mechanisms of synaptic regulation. Less focus was placed on extra-neuronal factors such as glia and the associated innate immune system. Originally thought to act as nothing more than support cells, glia have since been shown to play an active role in processes underlying development, plasticity, and behavior. Dysregulation of the neuro-immune system is also a feature found in most neuro/psychiatric diseases.

The work presented in this dissertation focused on NAc synaptic physiology and behaviors in relation to either loss or activation of the pattern recognition molecule TLR4. Our findings advanced the growing field of neuro-immunology by showing NAc region- and cell-type specific differences associated with drug-reward and sickness behaviors. These results demonstrate the specificity of neuro-immune interactions beyond that of homeostatic scaling.

More broadly, this body of work highlights the importance of the innate immune system in synaptic physiology and associated behaviors. Beyond increasing our basic understanding of the brain, these insights may prove useful in developing better treatments to help those suffering from neuropsychiatric diseases

CHAPTER VI

METHODS

VI-1. Animals

All mice were used in accordance with policies approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Male mice aged 6-12 weeks were used. Animals were separated by gender and housed together in groups of 2-5/cage on a 12/12-hour light/dark cycle. All experiments were performed during the light cycle. WT C57/B6 mice used in behavior were purchased from Jax Labs (Bar Harbor/ME). TLR4.KO mice on a C57/B6 background were generously donated by Luc Van Kaer and maintained in our colony. For all electrophysiology experiments, these mouse lines were also bred to carry a bacterial artificial chromosome carrying the tdTomato fluorophore under control of the *Drd1a* promoter (Joffe and Grueter, 2016). All mice are on a C57Bl/6 background.

VI-2. Histology

RNAscope fluorescent in situ hybridization was performed on fresh-frozen tissue similar to previously described (Li and Kim, 2015). Mice were anesthetized, decapitated, and the brains were rapidly extracted into an ice-cold sucrose solution (in mM: Sucrose 182.6, NaCl 19.8, KCl 0.5, $MgCl_2$ 1.0, $CaCl_2$ 2.0, NaH_2PO_4 1.2, $NaHCO_3$ 25.9, Glucose 10.0). Brains were blocked, covered with

O.C.T. (Sakura Finetek, Torranace, CA), and rapidly frozen with Super Friendly Freeze-It (Fisher Scientific, Waltham, MA). 16µm coronal sections were made using a HM 505N cryostat (Microm International, Walldorf, Germany).

Multiplex RNAscope probe binding, amplification, and mounting were performed per written instructions. Probes against the following mRNA were used: *Aif1* (*Iba1*), *Gfap*, and *Tlr4*. Confocal microscopy was performed using a Fluoview FV-1000 (Olympus, Center Valley, PA).

VI-3. Electrophysiology

VI-3a. Slice Preparation

Whole cell voltage clamp recordings were performed from the NAc core and shell similar to previously described (Grueter *et al*, 2010; Joffe and Grueter, 2016). Briefly, parasagittal slices (250 µm) were prepared from mouse brains using a Leica VT1200 vibratome submerged in an oxygenated (95% O_2; 5%CO_2) ice-cold sucrose solution (in mM: Sucrose 182.6, NaCl 19.8, KCl 0.5, $MgCl_2$ 1.0, $CaCl_2$ 2.0, NaH_2PO_4 1.2, $NaHCO_3$ 25.9, Glucose 10.0). Slices then sat undisturbed for >1 hour in an oxygenated artificial cerebral spinal fluid (ACSF) solution (NaCl 118.93, KCl 2.49, $MgCl_2$ 1.30, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 12.21, Glucose 23.57) in a holding chamber (~25° C) prior to recording.

We performed whole cell voltage clamp recordings from NAc core and shell MSNs using IR-DIC video microscopy. The NAc core and shell were identified using the anterior commissure as a landmark. D1-tdTomato-positive (D1+) MSNs were identified through the presence of the tdTomato fluorophore.

Cells lacking the tdTomato fluorophore (D1(-)) are defined putative D2 MSNs as 90-95% of neurons in the NAc are MSNs roughly divided into those expressing D1 or D2 dopamine receptors (Sesack and Grace, 2010). Whole-cell configuration was achieved using electrodes (3.0-7.0 MΩ) filled with a cesium-based internal solution (in mM: $CsMeSO_3$ 120, CsCl 15, NaCl 8, HEPES 10, EGTA 0.2, TEA-Cl 10, MgATP 4, NaGTP, Spermine 0.1, QX-314 Bromide 5). Oxygenated ACSF was continuously perfused into the recording chamber at a rate of ~2 ml/min. Picrotoxin (50 μM) was added to block current through $GABA_A$-receptors. Afferent fibers were stimulated with a bipolar nichrome wire electrode placed near the border between the NAc and cortex. Recordings were performed using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz, and digitized at 10 kHz. EPSCs of 100-500 pA were evoked at a frequency of 0.05 – 0.2 Hz. Data acquisition and analysis were performed using ClampX (Molecular Devices).

VI-3b. Voltage-clamp basal properties

A/N ratios for a given cell were calculated as its EPSC magnitude while held at -70 mV divided by the EPSC magnitude 50 ms after the start of current efflux while held at +40 mV. For each cell's A/N ratio, a minimum of 10 consecutive responses at -70 mV and +40 mV were averaged together. CV was calculated by dividing the standard deviation of 30-60 consecutive EPSCs by the mean. $1/CV^2_{N:A}$ was calculated as $(1/CV_{2NMDAR}) / (1/CV^2_{AMPAR})$ as described previously (Grueter *et al*, 2013).

AMPAR current-voltage plots were performed in the presence of AP5 (10 μM). EPSC magnitudes were acquired at holding potentials of: -70, -40, 0, +20, and +40 mV. A minimum of 3 responses were averaged for each holding potential and normalized to the EPSCs at -70 mV. Rectification index (RI) was calculated as the current magnitude at +40 mV / current magnitude at -70 mV.

NMDAR current-voltage plots were performed in the presence of NBQX (10 μM). EPSC magnitudes were acquired at holding potentials of: -80, -40, -20, 0, +20, and +40 mV. A minimum of 3 responses were averaged for each holding potential. All responses were normalized to the EPSC magnitude at +40 mV. Time to half-peak was calculated using traces recorded at +40 mV.

mEPSCs were recorded in the presence of tetrodotoxin (1μM) and analyzed using a template search. sEPSCs and asEPSCs were recorded in an ACSF solution where Sr^{2+} (2.5 mM $SrCl_2$) replaced Ca^{2+}. 500 ms leading up to each afferent stimulation was used for sEPSC analysis. The time period of 200 ms following each initial electrically-evoked release event was used for asEPSC analysis. sEPSC and asEPSCs were analyzed using a template search.

PPRs were acquired through application of two successive afferent stimuli of equal intensity. The interstimulus intervals (ISI) examined were 20, 50, 100, 200, and 400 ms. The PPR was calculated as EPSC2/EPSC1. For each ISI of each cell, the ratios of 6 consecutive responses were averaged.

VI-3c. NMDAR pharmacology experiments

Ifenprodil and CIQ wash-on experiments were performed in a low-Mg^{2+} ACSF solution with NBQX and picrotoxin to block AMPAR and $GABA_A$ transmission (Joffe and Grueter, 2016). For these experiments, cells were clamped to -50mV and NMDAR EPSCs were recorded. After acquisition of a 10 min. stable baseline, Ifenprodil (3 μM) or CIQ (30 μM) was washed-on for 10 min. Responses from the final 3 min. of drug wash-on were averaged and compared to baseline. This was followed by D-APV to confirm NMDAR currents.

VI-3d. Depolarization induced suppression of excitation

DSE was performed using a cesium-free internal solution similar to previously described (Shonesy *et al*, 2013). MSNs were held at -70 mV and afferents were electrically stimulated with a 5 s inter-sweep interval. Following 60 s of a stable baseline, a post-synaptic depolarization (+30 mV; 10 s) was induced. This was followed by 90 s of evoked responses at -70 mV. This protocol was repeated 3 times per cell. The initial evoked response following depolarization was compared to baseline.

VI-3e. Field potential recordings

Extracellular field potentials from the NAc core were performed similar to previously described (Xu *et al*, 2013) using slices prepared similar to that of whole cell experiments. Recordings were performed using lower impedance electrodes (1.0-2.0 MΩ) filled with ACSF. Afferents were stimulated as described

above at 0.05 Hz. Fiber volley (N1) and population spike (N2) were assessed. For plasticity experiments, a 10 min. stable N2 baseline was acquired prior to either low frequency stimulation (LFS; 3 x 3 min., 5 Hz stimulation with 5 min. between each bout) or 10 min. wash-on of the Group II mGluR agonist LY 379268 (200nM). Responses were recorded for 30 min. after LFS induction or drug removal. Experiments with unstable N1 were discarded.

VI-4. Behavior

VI-4a. Cocaine conditioned place preference (CPP)

A biased CPP assay was performed as previously described (Joffe *et al*, 2017). Each session (pre-test, conditioning, post-test) was 20 min. in duration. Overhead video recordings of mouse activity was tracked and analyzed with automated software (EthoVision XT; Noldus, Leesburg, VA). During the pre-test, animals were placed in an open field chamber (ENV-510; Med Associates, Georgia, VT) equally divided to two sections with contextually distinct wall patterns and floor textures for 20 min. The amount of time spent on each side was recorded. Conditioning sessions were performed daily for the following 3 days, separated by 4 h. All mice received an i.p. injection of cocaine (5, 10, or 15 mg/kg) prior to exposure to the initially less-preferred pattern/texture combination. The same day, all mice received an i.p. injection of saline prior to exposure to the context where more time was initially spent. Treatment order was varied for each day. Finally, on day 5, a post-test was performed where the animal is given 20 min. with access both pattern/texture combinations. A change

in the amount of time spent on the initially less-preferred context was assessed. A second post-test was performed either 14 or 21 days later to assess the persistence of the learned preference. No animals were discarded based on an exclusion criteria of >80% initial preference for either context during the prestest.

VI-4b. Novel object recognition

Novel object recognition was performed similar to previously described (Leger *et al*, 2013). We performed a 5 min. habituation session an empty test chamber prior to the assay. During the familiarization session, animals were given 10 min. to explore 2 identical objects. Following a 1 h intersession interval, a test session was performed. Here, the animals were given 10 min. to explore the same chamber with one of the identical objects replaced with a novel one. Familiarization and test sessions were recorded with an overhead camera. We analyzed the first 20 s of total object exploration. Proportion of time spent exploring the novel object (test session) and the identical object it replaced (familiarization session) were quantified over the 20 s total exploration. Animals were excluded if there was >70% preference for either identical object. Videos were manually analyzed blinded to genotype.

VI-4c. Sucrose preference test

A 2-bottle choice sucrose preference test was performed modified from previously described methods (Christoffel *et al*, 2012). A solution of 2% sucrose dissolved in water or drinking water alone was placed in 50mL conical tubes fit

with a rubber stopper and sipper tube (Fisher, Waltham, MA). Conical tube assemblies were weighed before and after acclimation and test sessions. Mice were initially acclimatized to two-bottle choice conditions for 18 h with water alone. The less-preferred bottle/side was then filled with the sucrose solution before the mouse was placed back in the cage for an 18 h test. Sucrose preference was calculated as the proportion of sucrose solution consumed during the test session [change in mass of sucrose tube / (change in mass of sucrose tube + water tube)]. All mice explored/drank from both water tubes during acclimation. One mouse from each genotype was discarded due to leaky stoppers.

VI-4d. Open field test

Open field tests were performed similar to previously described (Joffe *et al*, 2017). Mice were placed in an open field activity chamber (ENV-510; Med Associates, Georgia, VT) for 60 min. Overhead video recordings were analyzed for locomotor activity and center time using automated software (Ethovision XT; Noldus, Leesburg, VA).

VI-4e. Tail-suspension and forced swim tests

Tail-suspension tests (TST) and forced-swim tests (FST) were performed similar to previously described (Joffe *et al*, 2017). For the TST, the caudal 1-2 cm of each mouse's tail was attached to a metal pole using tape. This was used to suspend the mouse ~20 cm above a surface. For FST, mice were placed in 2 L

beakers filled with ~1700mL room temperature water (changed between each session). All trials were 6 min. in length and video-taped. Scoring of immobility was performed by a blinded observer.

VI-4f. Tamoxifen administration

Tamoxifen was dissolved at 20 mg/ml in extra-virgin olive oil (EVOO) similar to previously described (Heffner, 2011). From P28 – P31, mice were injected once per day for 3 days with either EVOO or tamoxifen (100 µL) subcutaneously. The solution was heated to 37° C before injection. Following a 28 day wait, cocaine locomotor sensitization was performed.

VI-5. Stroke and the dorsal striatum

VI-5a. Middle cerebral artery occlusion (MCAO)

MCAO was performed by a collaborator. Under an operating microscope, the left common carotid artery was carefully separated and isolated from the vagus nerve. An 11 mm, silicon-coated suture was routed into the left internal carotid artery and advanced until it occluded the middle cerebral artery. A suture was tightened around the filament and left in place for 30 min. MCAO was considered to be technically adequate at ≥80% reduction in cerebral blood flow was observed immediately following placement of the occluding catheter. Animal temperature was carefully controlled using a water heated circulating pad using a rectal probe. The suture was removed for reperfusion after 30 min. and the incision closed. MPLA (20 µg) or vehicle were given 24 h prior to MCAO.

VI-5b. Dorsal striatum field recordings

Field potential recordings from the dorsal striatum were performed in a similar manner to that of the NAc core. 24 h after MCAO, Coronal tissue slices (250 µm) were prepared using sucrose and ACSF solutions similar to that used for the NAc core. Recordings were performed in the dorsal-lateral portion of the striatum. Afferents were stimulated at 0.05 Hz. i/o relationships were assessed for both the N1 and N2 by acquiring these values for a range of stimulation intensities (5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, and 100 nA). Each stimulation was repeated 3 times. PPRs were examined for ISIs of 20, 50, 100, 200, and 400 ms. The PPR was calculated as $N2_{second}/N2_{first}$. For each ISI of each cell, the ratios of 6 consecutive responses were averaged. Recordings were performed while blinded to treatment.

VI-6. Drugs

Cocaine-HCl, picrotoxin, and tamoxifen were purchased from Sigma-Aldrich Corp. (St. Louis, MO). NBQX, D-APV, Ifenprodil, CIQ, tetrodotoxin, and LY 379268 were purchased from Tocris Bioscience (Bristol, United Kingdom).

VI-7. Data analysis/Statistics

All data are presented as a mean ± SEM. Individual data points represent individual cells for whole-cell physiology/histology, slices for field potentials, and animals for behavioral assays. Sample sizes are presented as *n/N* where *n* is the number of cells (whole cell or histology) or slices (field potentials) and *N* is the

number of mice. Statistical significance was tested using one-sample t tests, unpaired t tests, two-way, and repeated measures ANOVA with further comparisons made using Sidak post hoc tests. Representative traces have had stimulus artifacts removed.

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