Expanding the Xenobiotic Hypothesis of Drug Reward and Reinforcement: Implications of TLR4 Dependent Cocaine and Methamphetamine Induced Neuroinflammation on Reward, Relapse, Priming, and Neurotoxicity

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Expanding the Xenobiotic Hypothesis of Drug Reward and Reinforcement: Implications of TLR4 Dependent Cocaine and Methamphetamine Induced Neuroinflammation on Reward, Relapse, Priming, and Neurotoxicity

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Defended: March 31, 2015

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Abstract

Conventionally, cocaine is thought to exert its rewarding and reinforcing effects on the mesolimbic dopamine pathway, which is commonly referred to as the reward circuitry of the brain. Cocaine increases dopamine levels within the NAc by blocking dopamine reuptake by antagonizing dopamine transporters (DATs) on the VTA dopaminergic axon terminals. Traditionally, it is believed that with repeated cocaine use, this neuronal action causes neuroadaptations that underlie addiction. However, developments within the last two decades indicate that glial cells within the central nervous system’s immune system act as non-neuronal modulators of drug reward. It was recently found that cocaine interacts with the TLR4/MD-2 complex to produce a proinflammatory response, and that this interaction is necessary for cocaine reward. The present investigation proceeds to demonstrate that cocaine can directly activate microglia cells through TLR4, causing them to upregulate mRNA of the proinflammatory cytokine, IL-1β, within the VTA. Furthermore, cocaine-induced dopamine increases in the NAc depend on cocaine-induced IL-1β signaling within the VTA. TLR4 and IL-1β are also important aspects underlying cocaine-primed reinstatement to drug seeking in a self-administration paradigm. In this self-administration model, repeated exposure to cocaine appeared to induce glial priming, and led to the expression of additional proinflammatory markers throughout the mesolimbic dopamine pathway, providing new targets for further investigation into cocaine reinforcement. The investigation was expanded to include another commonly abused psychostimulant, methamphetamine (meth), which has also been recently shown to interact with the TLR4/MD-2 complex. Here, it is shown that the meth-induced neuroinflammation revealed within the mesolimbic dopamine pathway is TLR4 dependent; however, unlike cocaine, meth reward and reinforcement may be driven by mechanisms other than IL-1β signaling. Comparisons between cocaine-induced and meth-induced neuroinflammation suggest that IL-6 may also be an important molecule in mediating mesolimbic dopamine pathway functionality. The present studies provide evidence that the rewarding and reinforcing effects of psychostimulants arise from synergistic mechanisms involving both direct neuronal actions and neuroimmune interactions.
Acknowledgements

If I were to look back, years from now, at my experience as an undergraduate research assistant in the Maier/Watkins lab, I would undoubtedly consider it to be what confirmed and fortified my passion for scientific research. I must first thank my primary thesis advisor Dr. Linda Watkins for giving me the opportunity to work in her lab; an opportunity that has enabled me to become a true scientist which presents countless doors leading to my future endeavors. Being able to work with such a prominent member of the scientific community, from whom I learned new ways to ask meaningful questions to enhance research, has not only been an honor, but a true inspiration.

Working closely with my mentor and postdoctoral fellow Alexis Northcutt has been invaluable to my growth as a scientist. Interacting often with her strengthened my ability to think critically when analyzing data or designing experiments. From Alexis I learned to perform numerous important lab techniques that I used extensively in the work behind this thesis. This allowed me to become a confident researcher, comfortable carrying out tasks both with others and independently which will prove quite useful in my future research career. Furthermore, the passion, positivity, and resiliency she has displayed throughout this project was highly motivational. Without her I would not have been able to take part in a study that became so significant to me.

Additionally, this thesis would not have been possible if it were not for the collaboration of Dr. Ryan Bachtell and his lab, for which I am extremely grateful. I would also like to thank former PRA Tom Cochran, whose many hours dedicated to teaching me lab techniques and sharpening my skills have enabled me to be an effective research assistant. Together, Alexis and Tom have both contributed to much of the work presented in this thesis. The knowledge I now hold thanks to them is the key to opening the doors I am presented.

Next, I would like to thank the neuroscience, psychology, and MCDB professors that I had the pleasure of interacting with throughout my undergraduate career. Serge Campeau, Tor Wager, Ravinder Singh, Dick Olson, and Jerry Rudy all helped me discover where my real interests within neuroscience lie. In their own ways these professors, along with other MW lab postdoctoral fellows like Mike Baratta and A.J. Kwilasz, provided guidance to me in pursuing my interests. Now as I encounter various doors leading to different research career paths, I will know which ones to walk through in order to best contribute to the scientific community and to society.

Finally, I must thank my family and friends who supported me and helped me both stay focused and unwind while working on this project. The friends I made while working in this lab, especially PRA Meagan Kitt, provided ceaseless advice and help that I could not have gone without. These colleagues facilitated my emergence into a career in science by making me feel like a part of the Maier/Watkins lab family.
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Introduction

Cocaine use is a prevalent societal issue throughout the world. Globally, between 14.25 and 20.52 million people used cocaine in 2009 (UNODC 2011). An alarming 5.69 million of those users were in the United States, where 36% of the world’s cocaine supply was consumed (UNODC 2011). Fortunately, the number of Americans who reported using cocaine within the previous month decreased from 2.1 to 1.7 million from 2007 to 2012 (NIDA). Despite the decline, high rates of use among young adults, emergency room visits, and relapse make cocaine a predominant social, economic, and public health issue in the US. Many of those who use cocaine are at risk of addiction, jeopardizing their own health and overwhelming the health care system, as evidenced by the more than 420,000 cocaine related emergency room visits in 2009 (SAMSHA, 2009). Arguably the most debilitating aspect of cocaine addiction is the high rate of relapse to cocaine use after recovery. One study in 2004 estimated the rate of relapse for cocaine addicts to be 61.9% (TEDS).

Successful treatment of cocaine addiction typically requires a variety of factors: a safe environment not associated with former drug use, healthy living conditions, proper social support, and some form of psychotherapy such as cognitive behavioral therapy (NIDA). Safe, healthy environments and social interaction are thought to act as natural rewards that stimulate the reward circuitry making drug-induced reward less appealing (Puhl, et. al. 2012). Social support and therapy is aimed at providing both the motivation to recover and the tools to resist the intense cravings and impulses that come with cocaine withdrawal (Winstanley et al., 2009). Unfortunately, not only do many afflicted individuals not have access to these aids, but even in ideal circumstances this treatment alone is largely ineffective. In fact, during withdrawal cocaine addicts often suffer from hindered motivation, self-control, and decision making that may lead to relapse before therapy can take effect (Goodman, 2008).

Changes brought on by chronic cocaine use drive these drug-seeking, impulsive behaviors characteristic of withdrawal due to the drug’s effect on the mesolimbic dopamine pathway, which serves as the reward circuitry of the brain. Any process that is essential to survival, such as eating and reproducing, results in an increase in extracellular dopamine in the nucleus accumbens (NAc) that instills a rewarding, euphoric affect to reinforce these behaviors (Ikemoto et al., 1999). The NAc receives dopaminergic projections from the ventral tegmental area (VTA), which also projects to the prefrontal cortex (PFC) (Wise, 1996 &1998). Behaviors that result in stimulation of this pathway are perceived as pleasurable, effectively creating a type of conditioned context and reward associative learning within this system (Hyman et al., 2006). Drugs of abuse, like cocaine, co-opt this system by manipulating dopamine signaling, thus leading to reward, or the euphoria that reinforces drug-taking behaviors. When studying drug addiction, measures of reward relate to the pleasure received from a drug while measures of reinforcement relate to tendency to repeat drug taking behaviors which is indicative of addiction.

Dopamine signaling in the NAc is regulated by a variety of mechanisms including GABAergic inhibitory connections in the VTA, dopamine metabolism in the synapse by
monoamine oxidase (MAO), and dopamine reuptake by dopamine transports (DAT) on VTA axon terminals (Spanagel & Weiss, 1999). These processes maintain a constant basal level of extracellular dopamine in the NAc. Cocaine’s primary mechanism of action is the direct antagonism of the dopamine transporters in the NAc (Ritz et al., 1987; Volkow et al., 1997). This causes extracellular dopamine levels to rise in the NAc (Chiara, 1988), which is associated with generating euphoria that, when paired to cocaine, produces the rewarding effect of the drug. As cocaine use persists, the elevated levels of dopamine in the NAc trigger neural adaptations that lower dopamine activity in an attempt to maintain homeostasis. One such adaptation involves the down-regulation of postsynaptic dopamine receptor expression (Volkow et al., 1990), resulting in decreased baseline dopamine signaling in the reward circuitry. This neuroadaptation is thought to underlie addiction, where inadequate dopamine signaling drives drug seeking in an effort to restore dopaminergic activity within the reward circuitry (Wise, 1996). With repeated use, the brain develops a tolerance to the rewarding effects of a drug so that the addict transitions from liking the drug for its reward to compulsively wanting or needing it just to feel normal (Nestler et al., 1997).

The cocaine addict, especially during abstinence, suffers from anhedonia and intense craving due to aberrant NAc activity and signaling (Koob & Mora, 1997). Meanwhile the PFC, although not implicated in cocaine reward, is highly involved in the development of other behaviors associated with addiction that make resisting drug cravings extremely difficult. Regions that are anatomically connected to the PFC limbic system, such as the ventral medial and orbitofrontal cortex, display altered functionality and activity in response to repeated cocaine exposure which drives the development of impulsivity and low self-control which worsen during withdrawal (Winstanley et al., 2009; Goldstein & Volkow, 2005). These behaviors, arising from aberrant PFC activity, increase susceptibility to addiction and relapse.

According to NIDA, there is currently no effective treatment for cocaine addiction. Due to the nature of this affliction, there are numerous sociological and psychological factors involved that make it extremely difficult to treat. Effective psychotherapy involves a community based “resocialization” aspect to support recovery. However, this leaves the patient susceptible to relapsing under the immense pressure from cocaine cravings once they leave the treatment program. Psychotherapy is a promising treatment, yet on its own it is often unable to help addicts resist cravings that can last years. This is why NIDA considers an ideal treatment to be comprised of both psychotherapeutic and pharmacological components (NIDA). In order to combat the drug cravings associated with cocaine addiction, and therefore alleviate the strains the issue places on society and on the individual, a pharmacological intervention must be found.
Background

Historically, drug abuse research has focused on neuronal mechanisms in order to understand the neurobiology of reward and addiction and develop pharmacotherapeutic interventions. Unfortunately, no pharmaceutical intervention has yet proven to be an effective treatment. However, in recent decades, the role of non-neuronal cells in the central nervous system, particularly astrocytes and microglia, have earned attention for their contribution to a wide-range of disorders of the nervous system (Yong, 2010). Interestingly, microglia activation and the subsequent proinflammatory response has been shown to mediate both the initial rewarding effects of morphine as well as the development of a morphine tolerance (Hutchinson et. al., 2007; Watkins et. al., 2007).

Glia cells have long been well known for their traditional, supporting roles in the central nervous system including supplying nutrients and oxygen to neurons and removing dead neurons and related debris. Astrocytes are traditionally understood to provide structural and metabolic support for neurons, help modulate the blood-brain barrier, and react to injury by secreting the intermediate filament protein GFAP to form glial scars, thus preventing aberrant connections from being made (Fawcett et al., 1999). Since 1994, when it was discovered that astrocytes participate in neuron signaling by releasing glutamate as well as by recycling neurotransmitters from the synapse (Parpura et. al., 1994), it has become clear that astrocytes should be considered a part of the brain signaling network. In fact, the term “tripartite synapse” was coined, indicating that astrocytes are an important element of a synapse because of their active role in modulating synaptic excitability (Volterra et. al., 2002). Microglia are an important part of the CNS’s immune system, where they function to detect and destroy pathogens as well as respond to tissue infection and injury. In their quiescent state, microglia constantly survey their environment. When they detect injury, invading pathogens, or other xenobiotics (foreign chemical substance not normally found in the organism), microglia will become activated and localize to the afflicted site to remove the bacteria, dead cells, or other debris. In an activated state, microglia will release a variety of proinflammatory molecules that initiate further glial activation of both microglia and astrocytes, as well as microglial migration in what is termed neuroinflammation (Hanisch & Kettenmann, 2007).

The proinflammatory cytokines released due to glia activation include interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα) (Watkins et. al., 2001). The existence of a glia-to-neuron signaling network is supported by research indicating that each of these proinflammatory cytokines is neuroexcitatory through numerous mechanisms (Stellwagen et. al., 2005; Vezzani & Viviani, 2014; Viviani et. al., 2003). Because drugs of abuse can be considered xenobiotics, investigation into the neuroimmune response to various drugs was initiated. In response to morphine, microglia cells become activated and release proinflammatory cytokines, a subset of which directly interact with receptors expressed on neurons (Schwarz et. al., 2013). An important cytokine involved in the proinflammatory cascade, IL-1β, has receptors
expressed on dopamine neurons that, when activated, can cause hyper-excitation of the cells (Ho & Blum, 1998; Long-Smith et. al., 2010). Furthermore, microglia can respond directly to dopamine release in the NAc (Schwarz et. al., 2013), which could perpetuate the neuroexcitatory effects of morphine-induced neuroinflammation.

These effects of morphine-induced glia activation could explain why microglia modulate the initial rewarding effects of morphine, measured by conditioned–place preference (CPP) and in vivo extracellular NAc dopamine quantification by microdialysis (Hutchinson et. al., 2007 & 2012). Repeated administration of morphine further activates microglia, resulting in proinflammatory progressions that are responsible for developing both an opioid tolerance counteracting opioid analgesia, and an opioid dependence (Watkins et. al., 2007). Increased expression of the anti-inflammatory cytokine IL-10 in the NAc is shown to prevent reinstatement to morphine CPP, highlighting the role of neuroinflammation in opioid reward and reinforcement (Schwarz et. al., 2011). Furthermore, microglia activation inhibition by minocycline suppressed morphine-induced CPP (Hutchinson et. al., 2008), and glia activation inhibition by AV411 attenuated morphine withdrawal symptoms in rats (Bland et. al., 2009).

Investigation into how morphine prompts glia activation revealed interesting results. Although microglia and astrocytes express classical opioid receptors, they can still become activated by morphine in classic opioid receptor knockout mice (Juni et. al., 2007), suggesting that morphine must activate glia by other means. One study revealed that glial activation by lipopolysaccharide (LPS) could suppress opioid analgesia, and that this effect could be blocked by (+)-opiod antagonists that have no binding affinity for the classic opioid receptors (Wu et. al., 2006). The classic receptor for LPS, toll-like receptor-4 (TLR4), is found in the innate immune system and is preferentially expressed on microglia and astrocytes (Dauphinee et. al., 2006). Along with its soluble co-receptor MD-2, this pattern recognition receptor binds LPS (Shimazu et. al., 1999), which initiates a proinflammatory cascade involving activation of the proinflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), leading to the production and release of proinflammatory cytokines such as IL-1β (Lee et. al., 2013). Further investigation into TLR4 mediated signaling demonstrated that morphine activates the TLR4/MD-2 complex, and that the selective TLR4 antagonist (+)-naloxone suppresses behavioral and neurochemical measures associated with morphine reward and reinforcement (Hutchinson et. al., 2010 & 2012). Blocking morphine CPP and morphine-induced extracellular dopamine increases in the NAc by TLR4 antagonism indicates that TLR4 serves as a primary modulator of morphine reward and reinforcement (Hutchinson et. al., 2012).

Because morphine initiates a TLR4 dependent innate immune response that facilitates its rewarding, and possibly reinforcing effects, it was conjectured that other addictive drugs such as cocaine could utilize a similar mechanism to exert their rewarding and reinforcing effects since TLR4 is capable of recognizing a wide range of molecules. In fact, recent work using computer models has revealed that cocaine also interacts with the TLR4/MD-2 complex in silico (Northcutt et. al., 2015), suggesting that some of the effects of cocaine may be TLR4 mediated.
The influence of TLR4 activation on morphine reward prompts further investigation into the contribution of cocaine-induced/TLR4 activation underlying cocaine reward. It was found that both the blockade of TLR4 activation by (+)-naloxone and the blockade of microglia activation by minocycline could inhibit cocaine-induced CPP, a behavioral measure of reward (Northcutt et. al., 2015). This supported the prediction that TLR4 mediated neuroinflammation might serve an important role contributing to cocaine reward. Further investigation into the role of TLR4 in cocaine reward was assessed using microdialysis to measure extracellular dopamine signaling in the NAc. TLR4 blockade with (+)-naloxone and LPS-RS suppressed the cocaine-induced increase in extracellular NAc dopamine, further supporting the claim that TLR4 activation is necessary for cocaine to exert its rewarding effects (Northcutt et. al., 2015). Additionally, collaborative efforts with Bachtell et al., demonstrated the involvement of TLR4 signaling in a paradigm involving chronic exposure to cocaine: administration of the TLR4 antagonist, (+)-naltrexone, dose-dependently suppressed cocaine-primed reinstatement to cocaine seeking in self-administering animals.

This thesis reviews my contribution to much of this recently published work as well as to the following investigations, as represented by the data presented in the following experiments. Seven total aims are included here, intended to further characterize the role of neuroinflammation in both cocaine reward and reinforcement: (1) to determine the effects of cocaine treatment on mRNA markers of proinflammatory activation in rapidly isolated microglia cells; (2) to examine the proinflammatory response to a single cocaine injection within key regions in the mesolimbic dopamine pathway at different timepoints, achieving time and region specificity; (3) to examine the role of the key proinflammatory cytokine IL-1β in modulating dopamine signaling within the NAc, a well-supported neurochemical measure of cocaine reward and possible predictor of reinforcement; (4) to extend findings indicating TLR4 mediation of cocaine-primed reinstatement to cocaine seeking through examination of proinflammatory activation within the mesolimbic dopamine pathway; (5) to investigate the potential of cocaine-induced microglial priming through examination of the proinflammatory response to a cocaine reinstatement challenge following saline or cocaine self-administration and extinction; (6) to establish which cell types express of TLR4/MD2 within key regions of the mesolimbic dopamine pathway and further explore microglia priming and reactivity in naïve, saline self-administering, and cocaine self-administering rats using immunohistochemistry; (7) to expand the investigation of TLR4-mediated neuroinflammatory responses underlying rewarding and reinforcing effects of drugs into meth by examining the proinflammatory response to acute meth administration.
Methods and Materials

Subjects

Pathogen-free, adult male Sprague Dawley rats (Harlan Inc.) weighing approximately 275-350 g were used for all rat studies at the University of Colorado at Boulder. Rats were pair-housed in standard Plexiglas cages and provided with free choice food and water. The environment in the colony room was maintained at 21-23°C with a 12 hour light-dark cycle. Upon arrival, rats were allowed to acclimate for one week. All procedures were approved by the National Institute on Drug Abuse Intramural Research Program Institutional Animal Care and Use Committee. All studies were performed during the light phase.

Drugs

Cocaine HCl was obtained from the National Institute on Drug Abuse (NIDA; Research Triangle Park, NC and Bethesda, MD, USA). Interleukin-1 receptor antagonist was purchased from Sigma (St Louis, MO, USA). Drugs were self-administered or given as ip injections in all studies as described below.

Rapid Microglia Isolations

Tissue Preparation

Rats were euthanized with a lethal i.p. injection of 65 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA) and trancardially perfused with ice cold 0.9% saline for 3 minutes. The brains were removed and diced with a scalpel on a glass petri dish over ice and placed, without the cerebellum, in a glass homogenizer containing 3 mLs 0.2% glucose in phosphate buffer saline (PBS). The tissue was gently and thoroughly homogenized before being filtered through a 70 μM filter into a 50 mL conical. The filter was then rinsed with 1 mL of PBS into the conical to collect the maximum number of cells possible. The filtrate was then pipetted into a 15 mL conical to be centrifuged at 350 x g for 10 minutes at RT. The supernatant was removed with a glass pipet and discarded.

Density Gradient Microglia Isolation

Stock Percoll was diluted 1:10 in 10x (0.1M) PBS to make 100% isotonic percoll. 1x (0.01M) PBS was used to dilute the 100% percoll to 70% and 40% percoll. The pellet was resuspended and mixed in the room temperature 70% percoll. Next, 4 mLs 40% percoll was carefully layered onto the 70% percoll homogenate layer. Finally, 2 mLs 1x PBS was layered onto the 40% layer. Tubes were centrifuged at 1200 x g for 45 minutes at RT with minimal acceleration and no break. The density gradient centrifugation yielded two organic layers: the thicker, top layer within the 40% percoll and 1x PBS interface contained all CNS components minus microglia; the bottom, faint layer within the 40% and 70% percoll interface contained
isolated microglia cells. The top layer was carefully aspirated with a glass pipet without disturbing the microglia layer and discarded. The microglia cells were carefully aspirated from all conicals with a glass pipet and transferred into a single 50 mL conical where they were washed with 5 mL DPBS per brain. The cells were then centrifuged at 350 x g at RT for 10 minutes with normal break and acceleration. Supernatant was pipetted away from the faint pellet and discarded.

**Microglia Plating and Treatment**

The microglia pellet was immediately suspended in 5 mLs per brain DMEM (100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 0.6%glucose and 2 mM L-glutamine) supplemented with 10% FBS. A hemocytometer was used to count stock cells so they could be diluted in DMEM/10% FBS media so that x cells could be plated in each of the 100 μl 96 V-bottom wells. Cells were incubated for 24 h at 37 °C. Then, cell cultures were treated with various cocaine concentrations and incubated for 4 h at 37°C. After treatment, cells were centrifuged at 100 x g at 4°C for 10 minutes. After the supernatant was removed, the cells were removed with 100 μl Trizol and transferred to 1.5 mL microcentrifuge tubes to be processed for total mRNA extraction, cDNA synthesis, and PCR amplification as described below.

**Real Time RT-PCR**

**Collection of Tissue Micro-punches**

Two hours following acute drug injection (Experiment 2) or reinstatement challenge drug injection (Experiment 4,5), rats were euthanized with i.p. 65 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA) followed by transcardial perfusion with ice cold 0.9% saline. The brains were flash frozen in chilled isopentane, frozen on dry ice and stored at -80°C until the collection of tissue micro-punches. Brains were mounted on a cryostat and sectioned (30μm) at -20°C. The location of each region (VTA, NAc shell, vmPFC, BG) was determined using a brain atlas (Paxinos and Watson). Circular micro-punches of approximately 0.25 cm in length were taken from each region bilaterally using the blunt-end of 18-guage, stainless steel hypodermic tubing. Tissue micro-punches were stored in 1.5 ml microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80°C until the mRNA extraction. Slices were taken near the beginning and end of each micro-punched section, mounted onto gel-treated slides, stained with cresyl violet, cover-slipped, and viewed under a light microscope to verify the accuracy of the micro-punch collection.

**Total RNA Extraction**

Total RNA was isolated from each tissue micro-punch with the standard method of phenol:chloroform extraction (Chomczynski and Sacchi, 1987). Tissue micro-punches were first homogenized in 800 μl Trizol reagent (Invitrogen, Carlsbad, CA) for 30 seconds. For isolated microglia cells suspended in 100 μl Trizol, 700 μl Trizol was added and cells were homogenized.
The samples were incubated for 5 minutes at room temperature before 160 μl of chloroform was added. Tubes were vortexed for 2 minutes followed by centrifugation (11,900 x g) for 15 minutes at 4°C. The clear supernatant was pipetted out of the tubes without disturbing the aqueous, nucleic acid containing phase. 8 μl of Glycogen (Invitrogen) was added to bind the RNA and vortexed, followed by 400 μl of isopropyl alcohol which was added to facilitate nucleic acid precipitation. Samples were then briefly vortexed and incubated for 10 minutes at room temperature before centrifugation (11,900 x g) for 10 minutes at 4°C. The RNA pellet was washed twice in 1 ml 75% ethanol with centrifugation (7,500 x g) for 5 minutes at 4°C for each wash before being inverted and air dried for about 20 minutes. The pellet was finally resuspended in 11 μl nuclease free water and frozen at -80°C until cDNA synthesis.

**cDNA Synthesis**

Extracted total RNA was reverse transcribed into cDNA using the SuperScript II First Strand Synthesis System for RT-PCR (Invitrogen). RNA samples were transferred to 1 ml microcentrifuge tubes and had 1 μl random hexamer primers (5 ng/μl) and 1 μl dNTPs (mM) added making the total reaction volume 13 μl. After a brief vortex and spin, samples were moved to the iCycler and incubated at 65°C for 5 minutes. Next, 4 μl of RT 5X buffer and 2 μl DTT (0.1M) was added to each tube, bringing the reaction volume to 19 μl, and incubated at 25°C for 2 minutes. Finally, 1 μl of SuperScript III RT (200 U/μl) was added and the reaction was carried out at 25°C for 10 minutes then 42°C for 50 minutes. The reaction was inactivated at 70°C for 15 minutes. The cDNA was diluted in 20 μl nuclease free water and stored at -20°C.

**Quantitative real time polymerase chain reaction (qRT-PCR)**

PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). Reactions were run in 96-well plates and the formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA). Each well contained 25 μl aliquots of a master mix containing (x, order?). For experimental cDNA samples, triplicate reactions were run so that 1 μl of each sample was added to three wells. PCR cycling conditions were as follows: 94°C for 15 minutes for Taq DNA polymerase hot-start activation; 40 cycles of denaturation at 95°C for 15s, annealing at 57°C for 30s, and extension at 72°C for 30s, at which temperature SYBR Green I fluorescence was captured. Relative gene expression was determined using the 2^-ΔΔCT method (Livak et al., 2001). Mean CT of triplicate measures was computed for each sample. Sample mean CT of the internal control (GAPDH) was subtracted from the sample mean CT of the respective gene of interest (ΔCT). The sample with the absolute highest mean ΔCT was selected as a calibrator and subtracted from the mean ΔCT of each experimental sample (ΔΔCT). 2^-ΔΔCT yields fold change in gene expression of the gene of interest normalized to the internal control gene expression. A full outline of the total RNA extraction, cDNA synthesis, PCR amplification protocols, and primer sequences, can be found in prior publication (Frank et al., 2006).
Primer Specification

Primer sequences for mRNA of genes of interest expressed in glia and endothelial cells were designed by Dr. Matthew Frank at the University of Colorado at Boulder (Table 1).

Table 1:

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<tr>
<th>Oligo Name</th>
<th>Direction</th>
<th>Oligo Sequence (5’ to 3’)</th>
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<tr>
<td>GAPDH ei</td>
<td>Forward</td>
<td>TCTTCCAGGAGCGAGATCCC</td>
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In-vivo Microdialysis

Guide Cannula Placement Surgical Procedure

Each rat was anesthetized with isoflurane and placed in a stereotaxic apparatus. Sterile 12 gauge CMA microdialysis guide cannula (CMA Microdialysis) were implanted into each rat aimed at the left or right nucleus accumbens shell (relative to bregma: Anterior/Posterior = +1.7 mm; Medial/Lateral= ± 0.8 mm; relative to dura: Dorsal/Ventral= -5.6 mm, bite bar= 0) using a counterbalanced technique (Paxinos and Watson, 1998). For the purpose of intra-VTA injections, an injection guide cannula (Plastics One) was implanted into the VTA (relative to bregma: Anterior/Posterior = -5.0 Medial/Lateral= ± 0.9 mm; relative to dura: Dorsal/Ventral= -7.0 mm, bite bar= 0). Guide cannula and a tether screw (CMA Microdialysis) were attached to the skull using three jeweler’s screws and dental cement. Rats recovered for a minimum of one week before beginning in-vivo microdialysis.
Collection of Dialysate

On the afternoon preceding the microdialysis procedure, rats were transferred to the dialysis room that had the same light/dark cycle as the colony room. Rats were individually placed in Plexiglas bowls with *ad libitum* food and water. Once microdialysis probes (CMA 12, MW cut-off 20,000 Da, 2 mm active membrane) were inserted through each guide cannula, artificial cerebrospinal fluid (aCSF: 145 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl, 1.0 mM KCl) was perfused through the probes at 0.2 μl/min overnight using a CMA infusion pump. In the morning on the day of the experiment the flow rate was turned up to 1.5 μl/min where it remained for the duration of the experiment. After the rats were allowed to acclimate to the higher flow rate for 2 hours, three baseline DA samples were collected. After the last baseline sample was collected, subjects began receiving injections. All samples were manually collected every 20 minutes for 4 hours (12 samples total) and stored at -80°C until HPLC analysis.

Systemic Administration of Drugs

All rats received two subcutaneous (s.c.) injections of either 2.5 mg kg\(^{-1}\) (+)-naloxone, for a total of 5 mg kg\(^{-1}\) (+)-naloxone, or equivolume saline. At 10 min following the first s.c. injection, rats received the second identical s.c. injection along with an intraperitoneal injection of either 10 mg kg\(^{-1}\) cocaine HCL or saline.

VTA Microinjections

For studies requiring an intra-VTA microinjection, rats received 1 μl of drug (10 μg IL-1 receptor antagonist in sterile saline) 10 min before an intraperitoneal administration of 10 mg kg\(^{-1}\) cocaine or equivolume saline.

High Performance Liquid Chromatography

A high performance liquid chromatography (HPLC) system was used to quantify dopamine concentrations within the NAc shell. The system consisted of an ESA 5600A Coularray detector with an ESA 5014B analytical cell and an ESA 5020 guard cell. The reverse phase liquid chromatography column was an ESA MD-150 (C-18, 3um, 150 x 3.2 mm), and the mobile phase was ESA buffer MD-TM (Chelmsford, MA, USA). The analytical cell potentials were kept at -100 mV and +250 mV and the guard cell at +300 mV. A standard curve of increasing dopamine concentrations was measured (0.5μL, 1μL, 2μL, 4μL, 8μL dopamine per 27μL injection). Each dialysate sample was injected into the system, where the concentration of dopamine within each dialysate was determined by comparing the amount of dopamine separated in the column to the standard curve of dopamine concentrations.

Microinjection Cannula and Microdialysis Probe Placement Verification
Once the final dialysate sample was collected, rats were euthanized with i.p. 65 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA) to have their brains extracted. Brains were frozen in isopentane and sectioned (30 μm) on a cryostat at -20°C. Brain slices containing the VTA and NAc regions and cannula tracks were mounted onto gel-treated slides, stained with cresyl violet, cover-slipped, and viewed under a light microscope. To be included in the data analysis, 75% of the probe had to be within the NAc shell and microinjections had to be visibly within the VTA region.

**Self-Administration and Reinstatement**

**Apparatus**

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St Albans, VT) equipped with two response levers and an infusion pump system.

**Procedure**

Rats were first trained to press a level to acquire food pellets after 24-48 hours of food deprivation. This served to facilitate their acquisition of cocaine self-administration. Food pellets were received on a fixed ratio 5 (FR5) reinforcement schedule so that every 5 lever presses produced 1 food pellet. Training proceeded until the acquisition criteria of 30 food pellets produced within 3 consecutive sessions was met. Following acquisition training, rats were fed *ad libitum* for at least 1 day before undergoing jugular catheterization surgeries. For the reinstatement paradigm (Experiments 4, 5), rats self-administered saline, or cocaine as described below. They were then given s.c. injections of either saline or cocaine (15 mg/kg) as the reinstatement challenge in their home cages after lever responding was extinguished over 8 daily extinction sessions. 2 hours later, rates were euthanized, saline perfused, and their brain were removed for micro-punching and mRNA processing as described above. For Experiment 6, rats again acquired either saline or cocaine self-administration and underwent extinction training. However, rats received no reinstatement challenge and were sacrificed following the extinction training for immunohistochemistry as described below.

**Jugular Catheterization**

Rats were implanted with intravenous jugular catheters under halothane anesthesia (1-2.5%). The chronic indwelling catheters were implanted in the right jugular vein that exited the animal in the midscapular region. Following surgeries, rats were individually housed in the colony room and allowed to recover for one week.

**Cocaine Self-Administration**

After a 1 week recovery from surgery, cocaine self-administration training was conducted in 15 2-h daily sessions at fixed ratio 1 reinforcement schedule with a cocaine dosage of 0.5mg/kg/infusion. Cocaine injections, delivered over 5 s concurrent with the illumination of a
stimulus light above the active lever, followed by a 15-s timeout period when the house light remained off and responding produced no consequence. Responses on the second lever produced no consequence. The positions of active and inactive levers were counterbalanced. To assess a full range of cocaine doses in a single session, the final phase of training consisted of separating the session into five sequential 20 min components to deliver cocaine injections in an ascending order as follows: no injection (referred to as extinction), 0.03, 0.09, 0.27 and 0.89 mg kg\(^{-1}\) per injection, each preceded by a 2-min timeout period. A sample injection of cocaine at the corresponding dose occurred independently of responding at the end of each timeout. Training continued until: (1) a minimum of 5.0 mg kg\(^{-1}\) cocaine was self-administered within a session with \(<20\%\) variation in the total number of injections compared with the previous session, (2) the dose of cocaine that maintained maximal response rates varied by no more than one-half log unit over two consecutive test sessions and (3) maximal response rates were at least fivefold higher than response rates maintained during extinction.

**Immunohistochemistry**

*Collection of Tissue by Paraformaldehyde Perfusions*

After undergoing saline/cocaine self-administration (Experiment 6), rats were euthanized with i.p. 65 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL, USA). Upon the cessation of motor reflexes, the subjects underwent transcardial perfusions with ice cold 0.9% heparinized saline for 2 minutes, followed by ice cold 4% paraformaldehyde (8% paraformaldehyde cut with 0.2M PB the day of perfusions, pH 7.4) for 3 minutes. The brains were pulled and post-fixed in 4% paraformaldehyde for 4 hours at 4°C before being moved into 22% sucrose at 4°C.

*Collection of Tissue Slices*

Five days later, and once all of the brains have sunk in the sucrose, the brains were ready to be sliced for tissue. Prior to mounting, the brains were placed in -30°C isopentane for approximately 80-90 seconds. In a cryostat at -20°C, 30 μm slices were taken of each brain region (approx. 30 for PFC, 60 for NAc and VTA) and mounted onto SuperFrost gel-subbed slides (Fisher ScientiWc, Pittsburgh, PA). The location of each region (vmPFC, VTA, NAc) was determined using a brain atlas (Paxinos and Watson). Slides were allowed to dry overnight, and were stored at 4°C until staining.

*Antibody Staining*

Tissue sections underwent double immunofluorescence staining for microglial activation marker CD11B (OX42 labeling) and TLR4 binding partner MD-2. Slides were washed in 1x (0.01M) phosphate buffer saline (PBS) and 0.5% Triton X in PBS (PBS-T). The first and third washes were in PBS-T while the second was in PBS, all lasting 5 minutes with slight agitation. Tissue was blocked overnight in 2.5% donkey serum albumin (DSA) in PBS-T at 4°C (Frank et.
al., 2006). On day two the slides were washed as before but for 10 minutes at a time. Tissue was next incubated in 1/100 dilutions of rabbit anti-rat MD-2 and mouse anti-rat OX42 in 2% donkey serum (in PBS-T) for 24 hours at 4°C. The antibody solution was pipetted onto the slides and covered with parafilm in an airtight humidified container. Following incubation the tissue was washed and then incubated in 1/200 dilutions of each secondary (488 donkey anti-rabbit for MD-2; 594 goat anti-mouse for OX42) in 2% normal donkey serum (in PBS-T) for 2 hours at RT. The secondary antibodies were protected from light during incubation. Finally, the tissue was washed 3 times for 5 minutes in PBS. The slides were then briefly dunked in dH2O to remove salts and immediately cover-slipped with hard-set Vectashield with DAPI. Tissue was stored at 4°C in the dark until analysis.

**Statistics**

GraphPad Prism Version 5 (San Diego, CA, USA) was used to calculate statistics and create graphs. The types of statistical analyses done were chosen based on experimental design. Bonferroni post hoc tests were used for one-way analysis of variance (ANOVA), two-way ANOVA, and repeated measures ANOVAs. Specifics regarding the types of analyses used are indicated in the figure keys. Data are presented as mean±s.e.m. Data collection and quantification was performed blinded whenever possible; final analyses were not performed blind to the conditions of the experiments. However, when possible, behavioral analyses and experiments were performed blind to the experimenter.
Results

Experiment 1: Cocaine upregulates Interleukin-1β (IL-1β) mRNA in rapidly isolated microglia

Our laboratory has demonstrated that cocaine activates TLR4 and that blockade of cocaine-induced TLR4 signaling suppresses measures of cocaine reward (Northcutt et. al., 2015). TLR4 is known to be preferentially expressed on microglia cells (Dauphinee et al., 2006). In order to explore whether cocaine-induced TLR4 activation is microglial-dependent, the effects of cocaine on rapidly isolated microglial cells were assessed. As shown in Figure 1, 24 hour incubation in varying concentrations of cocaine produced upregulation of IL-1β mRNA. LPS is an outer-membrane component of gram-negative bacteria that is well characterized as a TLR4 agonist (Watkins et. al., 2001); therefore LPS treatment in a subset of the rapidly isolated microglia served as the positive control. As expected, both the 10 ng/mL and 1 ng/mL concentrations of LPS caused significant IL-1β mRNA upregulation (***p<0.001).

![Rapid Microglia Isolation 24h LPS, Cocaine Dose Response](image)

**Figure 1:** TLR4 activation by lipopolysaccharide (LPS) and cocaine induce upregulation of IL-1β mRNA in rapidly isolated microglia cells treated in culture for 24 hours. mRNA expression is relative to that of GAPDH; shown as % of media control. One way analysis of variance (ANOVA) revealed a significant effect of treatment (***p<0.001). Bonferroni multiple comparisons indicated significant IL-1β mRNA upregulation at various concentrations of cocaine.
upregulation from 10 ng/ml LPS, 1 ng/ml LPS, 100 μM cocaine (**p<0.001), and 10 μM cocaine (**p<0.01). Data are mean±s.e.m. n=3-5/group.

There was significant cocaine-induced IL-1β mRNA upregulation at 100 μM (**p<0.001) and 10 μM (**p<0.01) but not at 1-0.001 μM, indicating a dose-dependent effect. Previous work with cultured neonatal microglia showed IL-1β mRNA was significantly upregulated in response to 1 hour cocaine incubations at 0.1 and 1 μM (Bonferroni post hoc, **p<0.01) and the expected dose-dependent effects of LPS on IL-1β mRNA upregulated expression. Furthermore, this action was shown to be TLR4 dependent as microglia treated in culture for 1 hour with 1 or 0.1 μM cocaine along with the TLR4 antagonist (+)-naloxone at 1 or 10 μM completely suppressed microglial upregulation of IL-1β mRNA (Northcutt et. al., 2015; Sup Figure 1). Together, this data indicate that cocaine causes TLR4 mediated microglia activation leading to the expression of IL-1β.

**Experiment 2: Acute cocaine-induced IL-1β mRNA upregulation is significant within the VTA 2 hours following systemic injection**

After confirming microglia cell reactivity in relation to cocaine-induced upregulation of IL-1β mRNA, this inflammatory response was assessed in regions relevant to cocaine reward in the mesolimbic dopamine pathway. Rats were given cocaine injections (10 mg/kg, i.p.), euthanized, saline perfused, and had their brains extracted 30 minutes or 2 hours following the acute drug administration. mRNA extracted from micro-punches from the vmPFC, NAc, and VTA was converted to cDNA and amplified via RT-PCR. This served to obtain both regional and temporal specificity in the assessment of the cocaine-induced proinflammatory response. It was found that IL-1β mRNA is upregulated at 2 hours, but not 30 minutes, in the VTA (**p<0.01), but not in the NAc or vmPFC after acute cocaine administration (Figure 2). None of the other mRNAs tested (GFAP, TNFα, CD11B, and TLR4) showed significant upregulation. A follow-up study was conducted to assess whether cocaine-induced mRNA upregulation is TLR4-dependent. In a two-by-two design, rats were treated with either vehicle or the selective TLR4 antagonist (+)-naloxone (2.5 mg/kg total in two s.c. injections, spaced 10 minutes apart, with the second injection paired with either cocaine (10mg/kg i.p.) or vehicle), and given either cocaine (10 mg/kg i.p.) or equivolume saline injections. mRNA expression was assessed at 30 minutes and 2 hours following injections. Once again, cocaine induced upregulation of IL-1β mRNA expression and treatment with (+)-naloxone blocked this effect at 30 minutes (*p<0.05) and 2 hours (**p<0.01) (Northcutt et. al., 2015; Sup Figure 2).
Figure 2: Bonferroni multiple comparisons revealed that cocaine (10 mg/kg, intraperitoneal (i.p.)) induced upregulation of interleukin-1β (IL-1β) mRNA in the ventral tegmental area (VTA) (**p<0.01) but not in the nucleus accumbens (NAc) shell or ventral medial prefrontal cortex (vmPFC). Two-way analysis analysis of variance (ANOVA) revealed an effect of region (p=0.034) and time following cocaine injection (p=0.017); data are mean±s.e.m., n=5-6/group. (Northcutt et. al., 2015).

Experiment 3: Intra-VTA Interleukin-1 receptor antagonism (IL-1ra) prevents cocaine-induced dopamine increase within the nucleus accumbens shell

Systemic blockade of TLR4 suppresses cocaine-induced increases of intra-NAc dopamine (Northcutt et al., 2015). Due to the finding that IL-1β mRNA is selectively upregulated in the VTA after an acute cocaine administration, a microdialysis experiment was performed to assess the effects of cocaine-induced IL-1β signaling on the mesolimbic dopamine pathway. A microdialysis probe was implanted into the NAc shell and a microinjection guide cannula was implanted in the VTA. The microdialysis probe has a semi-permeable membrane that allows extracellular dopamine within the NAc shell to diffuse through the probe membrane along its concentration gradient allowing live DA samples to be collected in the aCSF fluid. Dopamine was quantified using high-performance liquid chromatography and analyzed by repeated measures two-way analysis of variance (ANOVA) with Bonferroni *post hocs.*
Following the collection of three baseline samples, rats received intra-VTA injections of 10 μg in 1 μl IL-1 receptor antagonist (IL-1ra) or saline 10 minutes prior to cocaine injections (10 mg/kg i.p.) at time zero. Cocaine paired with intra-VTA saline resulted in significant increases in extracellular NAc shell dopamine at 40, 60, and 100 minutes (Bonferroni, **p<0.01 and ****p<0.0001). Pretreatment with IL-1ra completely suppressed the cocaine-induced dopamine increase in the NAc shell (Figure 3) while IL-1ra treatment alone had no effect on NAc dopamine levels.

![Figure 3](image_url)

**Figure 3:** Cocaine-induced increases in extracellular nucleus accumbens (NAc) shell dopamine are dependent on interleukin-1β (IL-1β) signaling within the ventral tegmental area (VTA). Cocaine (10 mg/kg, i.p.) produces elevated extracellular dopamine in the NAc that are significant at 40, 60, and 100 min (Bonferroni, **p<0.01, ****p<0.0001) following drug administration. Intra-VTA interleukin-1 receptor antagonist (IL-1ra) suppressed this effect. There were no differences between other treatment groups. Data are mean±s.e.m., n=3-4/group. (Northcutt et. al., 2015).

These findings suggest that cocaine-induced increases of dopamine within the NAc shell are IL-1β dependent, implicating a critical role for IL-1β signaling underlying the rewarding effects of cocaine. When taken with the mRNA data indicating that cocaine-induced IL-1β mRNA upregulation is TLR4 dependent, evidence suggests that TLR4 activation is an important component underlying cocaine-reward, providing a possible pharmacological target in further studies of cocaine reward. In fact, in vivo TLR4 blockade by either subcutaneous (+)-naloxone (2.5 mg/kg in two injections spaced 10 min) or LPS-RS (5 μg, intra-VTA) also blocked cocaine-
induced NAc shell extracellular dopamine increases (Northcutt et. al., 2015: Sup Figure 3). It has been shown that IL-1ra, (+)-naloxone, and LPS-RS microinjected into the VTA did not interfere with non-TLR4 activation of dopaminergic signaling (Northcutt et. al., 2015), suggesting that TLR4/IL-1β signaling blockade do not interfere with dopaminergic neuron functioning.

Up to this point, the microglial proinflammatory response to an acute cocaine administration has been fairly well characterized in the context of cocaine reward. The next steps of this investigation were aimed at characterizing cocaine-induced neuroinflammation in a more clinically relevant, chronic model of cocaine administration by utilizing self-administration and reinstatement paradigms.

**Experiment 4: Cocaine-primed reinstatement to cocaine seeking in self-administering rats induces IL-1β mRNA upregulation in the VTA 2 hours following drug challenge.**

Two important findings were described above: first, acute cocaine administration causes a significant, TLR4 dependent upregulation of IL-1β mRNA within the VTA 2 hours following injection; second, IL-1β signaling is crucial in generating the cocaine-induced spike in NAc shell dopamine signaling. This highlights the role of inflammation in mitigating cocaine reward, however, how these inflammatory processes might contribute to the reinforcing effects of chronic cocaine exposure is unknown. Interestingly, a single systemic administration of (+)-naloxone just prior to a cocaine-primed reinstatement trial dose-dependently suppresses cocaine seeking in self-administering rats (Bachtell et. al., unpublished; Sup. Figure 4). Further investigation has revealed that intra-VTA microinjection of IL-1ra attenuates cocaine primed reinstatement (Bachtell et. al., unpublished; Sup. Figure 5). It is important to note that while intra-VTA IL-1ra significantly attenuated reinstatement to cocaine seeking (**p<0.001), lever responding was still approximately 45% that of responding for the untreated cocaine reinstatement group, whereas systemic TLR4 blockade suppressed lever pressing such that it was not significantly different from extinction trails, suggesting that some TLR4 mediated signaling other than IL-1β may also be involved in circumstances of chronic or repeated cocaine exposure.

A follow-up study was conducted in order to examine the neuroinflammatory response within the mesolimbic dopamine pathway in cocaine self-administration and reinstatement model. Rats were subjected to cocaine in a self-administration/reinstatement paradigm in which cocaine was self-administered (0.5mg/kg/infusion) on a fixed ratio schedule 1 of reinforcement in 15 daily sessions. Lever responding was extinguished in 8 daily extinction sessions. Following successful extinction, rats were given a cocaine challenge (15mg/kg, ip.), that served as a reinstatement prime in the previous study. However, instead of undergoing a reinstatement lever-press session, rats were euthanized and underwent saline transcardial perfusions 30 minutes and 2 hours after challenge injections. mRNA was extracted from micro-punches of the vmPFC, NAc, and VTA, and was then used to make cDNA for qRT-PCR as described previously. The control group consisted of animals that self-administered saline and had a saline challenge, while a naïve group was included for comparison.
Figure 4: Interleukin-1β (IL-1β) mRNA is upregulated within the ventral tegmental area (VTA) in response to a cocaine reinstatement challenge. (a-d) A cocaine challenge (15 mg/kg, i.p.) was given to rats in home cages following cocaine self-administration (0.5mg/kg/infusion) on a fixed ratio schedule 1 (FR1) of reinforcement over 15 daily sessions and 8 daily extinction sessions. Control animals self-administered saline and received a saline challenge, and are represented by the grey saline bars. One-way analysis of variance (ANOVA) assessed effect of treatment, bonferroni post hoc multiple comparisons assessed differences across data sets; data are mean±s.e.m., n=3-6/group.
Figure 4 depicts cocaine-induced IL-1β and CD11B mRNA expression at 30 minutes and 2 hours within the VTA and NAc shell in the self-administration and reinstatement model. One way ANOVA analysis revealed an effect of treatment type (including cocaine 2h vs 30 min) for IL-1β expression in the VTA (***p<0.001) while bonferroni post hoc multiple comparisons indicated a significant increase in ventral tegmental IL-1β mRNA expression at 2 hours (**p<0.01), but not 30 minutes. For CD11B or TNFα (data not shown) mRNA expression, there were neither significant upregulations nor significant differences between the two time points within the VTA (p>0.05). The same was true for IL-1β and CD11B in the NAc (p<0.05).

Repeated cocaine exposure resulted in a 251% increase in IL-1β mRNA expression at 2 hours when compared to IL-1β mRNA expression of saline self-administering animals, and a 416% increase at 2 hours when compared to naïve animals (data not shown). This is in contrast to the 150% increase in IL-1β mRNA expression at 2 hours (when compared to naïve animals) found in response to a single cocaine injection in Experiment 2. This suggests that repeated glia activation with cocaine may sensitize them have a more robust proinflammatory response to subsequent cocaine exposure.

**Experiment 5: Chronic cocaine self-administration primes glial proinflammatory response to a cocaine reinstatement challenge.**

The data from experiment 4 suggested that two hours following the cocaine challenge would be the ideal timepoint to further assess site-specific markers of neuroinflammation in the cocaine reinstatement paradigm. Further, the increase in intra-VTA IL-1β mRNA following an acute cocaine administration was exaggerated in self-administering rats when compared to naïve rats receiving a single cocaine injection. This suggests the possibility of priming: a phenomenon observed in glial cells where repeated activation eventually leads the microglia to assume a state where they are not fully quiescent and will display an over-exaggerated proinflammatory response to subsequent activating stimuli (Perry, Cunningham & Holmes, 2007). In order to further explore the possibility of repeated exposure to cocaine leading to a primed inflammatory response, a two-by-two reinstatement follow-up study was conducted. Animals either self-administered cocaine or saline and, following extinction training, received either a saline or cocaine reinstatement challenge. Brains were removed, VTA, NAc, and vmPFC micro-punches were collected and processed for RT-PCR analysis as described in experiment 4. The mRNA expression of a variety of genes was assessed within the mesolimbic dopamine pathway following extinction and a reinstatement challenge in order to paint a more complete picture of the CNS’s inflammatory response to repeated cocaine exposure and reinstatement.

The data summarized in Figures 5-7 depicts the mRNA expression of certain markers of neuroinflammation across different brain regions. The first two columns represent mRNA expression in rats that underwent saline self-administration (striped bars) and were given either a saline challenge (first column) or a cocaine challenge (second column). The next two columns depict (solid bars) mRNA expression for cocaine self-administrating rats presented with a saline (third column) or cocaine challenge (fourth column). An unweighted means analysis two-way
ANOVA revealed the effects of the self-administration drug and reinstatement challenge drug on the results. Bonferroni *post hoc* multiple comparisons assessed the effects of the reinstatement challenge drug on results within self-administration groups. Results are compared to the % mRNA expression of the saline S-A/saline challenge groups.
A. IL-1β

B. IL-6

C. CD11B

D. NFKBIA

E. GFAP

F. TNF α

Saline SA & Saline (i.p.)
Cocaine SA & Saline (i.p.)
Saline SA & Cocaine (15 mg/kg i.p.)
Cocaine SA & Cocaine (15 mg/kg i.p.)
Figure 5 VTA: Chronic cocaine self-administration sensitizes microglia inflammatory response in the ventral tegmental area (VTA) to subsequent cocaine administration after extinction to drug seeking. (a-f) Two by two study consisted of a group of saline self-administering (S-A) rats and cocaine S-A rats (15 2hr daily sessions, 15 mg/kg/infusion) that received a saline or cocaine reinstatement challenge (15 mg/kg i.p.) following extinction training (8 2hr daily sessions). mRNA expression % of saline S-A/saline (saline self-admin. with saline challenge) relative to % of GAPDH expression. (a) IL-1β mRNA expression: two-way analysis of variance (ANOVA) revealed a S-A group difference (**p=0.0013). (b) IL-6 mRNA expression: two-way ANOVA revealed a S-A group difference (*p=0.023) and a reinstatement challenge difference (*p=0.034); bonferroni multiple comparisons revealed significance of cocaine S-A/cocaine (*p<0.05). (c) CD11B mRNA expression: two-way ANOVA revealed a significant interaction (*p=0.012) and a reinstatement challenge difference (**p=0.001); bonferroni multiple comparisons revealed significance of cocaine S-A/cocaine (**p<0.01). (d) NFκBIA mRNA expression: two-way ANOVA revealed a S-A group difference (*p=0.011). (e) GFAP mRNA expression: two-way ANOVA revealed a S-A group difference (*p=0.031). (f) TNFα mRNA expression: no significance to report. Data are mean±s.e.m., n=3-6/group.
A. IL-1β

B. IL-6

C. CD11B

D. NFKBIA

E. GFAP

F. TNFα

- Saline SA & Saline (i.p.)
- Cocaine SA & Saline (i.p.)
- Saline SA & Cocaine (15 mg/kg i.p.)
- Cocaine SA & Cocaine (15 mg/kg i.p.)
**Figure 6** NAc: Acute and reinstated cocaine upregulates nucleus accumbens (NAc) IL-1β mRNA. (a-f) Two by two study consisted of a group of saline self-administering (S-A) rats and cocaine S-A rats (15 2hr daily sessions, 15 mg/kg/infusion) that received a saline or cocaine reinstatement challenge (15 mg/kg i.p.) following extinction training (8 2hr daily sessions). mRNA expression % of saline S-A/saline (saline self-admin. with saline challenge) relative to % of GAPDH expression. (a) IL-1β mRNA expression: two-way analysis of variance (ANOVA) revealed a S-A group difference (**p=0.0087) and a reinstatement challenge difference (**p=0.003); bonferroni multiple comparisons revealed significance of the cocaine challenge in both groups (**p<0.01). (b-d) No significance to report. (e) GFAP mRNA expression: two-way ANOVA revealed a significant interaction (**p=0.0077). (f) TNFα mRNA expression: no significance to report. Data are mean±s.e.m., n=3-6/group.
A. IL-1β

B. IL-6

C. CD11B

D. NFKBIA

E. GFAP

F. TNFα

Saline SA & Saline (i.p.)

Cocaine SA & Saline (i.p.)

Saline SA & Cocaine (15 mg/kg i.p.)

Cocaine SA & Cocaine (15 mg/kg i.p.)
Figure 7 PFC: Cocaine self-administration (S-A) induces IL-1β mRNA upregulation; cocaine reinstatement induces NFκBIA mRNA upregulation in the ventral medial prefrontal cortex (vmPFC). **(a-f)** Two by two study consisted of a group of saline self-administering (S-A) rats and cocaine S-A rats (15 2hr daily sessions, 15 mg/kg infusion) that received a saline or cocaine reinstatement challenge (15 mg/kg i.p.) following extinction training (8 2hr daily sessions). mRNA expression % of saline S-A/saline (saline self-admin. with saline challenge) relative to % of GAPDH expression. **(a)** IL-1β mRNA expression: two-way analysis of variance (ANOVA) revealed a S-A group difference (**p<0.0001). **(b-c)** No significance to report. **(d)** NFκBIA mRNA expression: two-way ANOVA revealed a reinstatement challenge difference (**p=0.014) and a significant interaction (**p=0.019); bonferroni multiple comparisons revealed significance of cocaine S-A/cocaine (**p<0.01). **(e-f)** No significance to report. Data are mean±s.e.m., n=3-6/group.

In the VTA, analyses revealed significant differences in mRNA expression between the self-administration (S-A) groups for IL-1β (**p<0.01), IL-6, NFKBIA, and GFAP (**p<0.05). Within the cocaine S-A groups, the cocaine challenge caused significant mRNA upregulation of IL-6 (**p<0.05) and CD11B (**p<0.01), with a trend toward significance for both cocaine challenge groups for IL-1β. The IL-1β mRNA expression (254.7% of % saline) in this region replicated that of IL-1β in the timecourse experiment (251.5% of % saline; Experiment 4). In the NAc shell, IL-1β mRNA was expressed in the cocaine S-A group significantly more than it was in the saline S-A group (**p<0.01), while the cocaine challenge within groups also caused significant IL-1β mRNA upregulation (**p<0.01) when compared to animals that received a saline challenge. Finally, in the vmPFC, the S-A group difference was extremely significant for IL-1β mRNA expression (**p<0.0001). Within group analysis also showed that the cocaine challenge in cocaine S-A rats caused an upregulation of NFκBIA mRNA (**p<0.01). From this study it can be concluded that chronic cocaine exposure leads to vastly more neuroinflammation than acute cocaine exposure within the mesolimbic dopamine pathway. Interestingly, mRNA expression of some neuroinflammatory markers appear to remain elevated in cocaine S-A animals that only received saline following the 8 day extinction period. The proinflammatory mRNA response in cocaine S-A animals was further exaggerated when they received a cocaine injection following extinction, which also yielded the expression of more neuroinflammatory markers than did a single administration of cocaine following saline S-A. These findings would indicate that repeated cocaine exposure does indeed prime glia, leading to prolonged inflammation and a more robust response to further activation.

Experiment 6: IHC staining of CD11B and MD-2 within the PFC, NAc, and VTA

Experiment 5 revealed that the inflammatory response to chronic cocaine self-administration was substantial yet varied greatly between the different brain regions examined. Additionally, it would appear that chronically administering cocaine sensitizes, or primes, microglia cells to have a more robust response to subsequent cocaine administration. An in-progress immunohistochemistry fluorescence staining experiment aims to determine if this immune response variability and sensitization is due to differential activation of microglia cells and/or differences in expressed concentrations of TLR4-MD-2 complexes across the brain regions of interest. Brains from naïve, saline S-A, and cocaine S-A rats have been collected to
determine how chronic cocaine exposure may alter microglial activation states and localization. This will also reveal if MD-2 localizes primarily to microglia cells and how much of it is present within key mesolimbic dopamine regions. Slices of the PFC, NAc, and VTA were dual stained for the microglial activation marker, CD11B, and for MD-2 protein. CD11B serves to show both the presence and state of microglia cells. The MD-2 antibody was chosen due to the lack availability of an antibody that is selective for TLR4, paired with evidence that the presence of the MD-2 protein is necessary for cocaine to bind to TLR4 (Northcutt et al., 2015). Figure 8 depicts CD11B staining within the NAc from a trail staining run on tissue from one naïve and one cocaine S-A rat.
Figure 8: Repeated cocaine exposure alters microglial morphology. Pictures are taken of the NAc shell under 40X magnification using green-tritsy channel for OX-42 594 to observe CD11B in the region. (a) CD11B staining in a naïve rat. Two examples of quiescent microglia are shown in A.1 and A.2. (b) CD11B staining of a cocaine S-A rat. At least two examples of either activated or primed microglia are shown in both B.1 and B.2. S-A brains were pulled 24 hours following the last S-A session. This preliminary work is assessed only qualitatively in this thesis.

Experiment 7: CD11B, IL-6, TNFα, and GFAP mRNA is upregulated in the VTA in response to an acute meth injection

Previous work has revealed that other drugs of abuse such as morphine (Hutchinson et. al., 2010 & 2012) and meth (Northcutt et. al., in preparation) also interact with the TLR4/MD-2 complex. Given that TLR4 signaling is necessary for both cocaine reward and reinforcement, there was substantial interest in exploring whether or not meth interacts with TLR4 to cause neuroinflammation. Meth, like cocaine, is a psychostimulant that acts on DATs to increase extracellular dopamine, the difference being that meth actually reverses the direction of dopamine through these transporters, rather than antagonizing them. Recently, our laboratory demonstrated that meth does interact with MD-2. Further, we found that systemic TLR4 antagonism with (+)-naloxone suppresses meth CPP (Sup. Figure. 6) and attenuates meth-
induced extracellular NAc dopamine elevations (Northcutt et al., *in preparation*; Sup. Figure 7). These findings suggest that meth may also interact with TLR4 to initiate neuroinflammatory signaling within the mesolimbic dopamine pathway.

Similar to the methods described in experiment 2, the mRNA expression of various neuroinflammatory markers was assessed in the dopamine pathway in response to acute meth administration at 30 minutes and 2 hours (Figure 9). Intriguingly, IL-1β mRNA was not upregulated in any region at any timepoint. In the VTA, CD11B mRNA expression was upregulated at both 30 minutes and 2 hours (*p<0.05) and TNFα mRNA expression was significant at 30 minutes (**p<0.01). Two effects not found in the acute cocaine studies presented here upregulation of IL-6 (*p<0.05 at 2h) and GFAP (**p<0.01 at 30 min; *p<0.05 at 2h) mRNA. These findings, given that TLR4 antagonism attenuates both meth induced NAc dopamine increases and CPP, imply that some TLR-4 mediated signaling other than that of IL-1β may be responsible for this increase and, possibly, the rewarding affect of meth.

![Figure 9](image_url)

**Figure 9:** Acute morphine administration (dose, i.p.) induces inflammatory mRNA upregulation in the ventral tegmental area (VTA). (a) CD11B: Two-way analysis of variance (ANOVA) revealed a
significant effect of brain region (*p=0.016) while bonferroni multiple comparisons revealed significant upregulation in the VTA at 30 min and 2 hr (*p<0.05). (b) TNFα: Two-way ANOVA revealed a significant effect of brain region (*p=0.036) and time (**p=-.006) and an interaction (*p=0.032) while bonferroni multiple comparisons revealed significant upregulation in the VTA at 30 min (**p<0.01). (c) IL-6: Two-way ANOVA revealed a significant effect of brain region (*p=0.0173) while bonferroni multiple comparisons revealed significant upregulation in the VTA at 2 hr (**p<0.05). (d) GFAP: Two-way ANOVA revealed a significant effect of brain region (**p=0.0001) and time (**p=-.0016) and an interaction (*p=0.014) while bonferroni multiple comparisons revealed significant upregulation in the VTA at 30 min (**p<0.01) and 2 hr (*p<0.05). Data are mean±s.e.m., n=3-5/group. (Northcutt et. al., in preparation).

In a two-by-two follow-up study, it was explored if the meth-induced mRNA upregulation of proinflammatory markers found in Experiment 7 is indeed TLR4 mediated. For the data summarized in Figure 10, rats were given either a (+)-naloxone (15 mg/kg, i.p.) or saline pretreatment followed by either a meth (1 mg/kg, i.p.) or saline injection. Brains were removed 2 hours following injections, and VTA micropunches were collected. It was again found that at 2 hours, IL-6 (**p<0.01) and CD11B (*p<0.05) mRNA was significantly upregulated in response to meth with vehicle treatments. However, when there was a (+)-naloxone pretreatment, there were no differences in mRNA expression between rats receiving a meth or saline injection (p>0.05). This experiment demonstrates TLR4 mediation of meth-induced neuroinflammation, which provides insight into the mechanisms underlying the suppression of meth reward with (+)-naloxone found in previous work (Sup Figures 6,7).
Figure 10: Meth-induced upregulation of CD11B and interleukin-6 (IL-6) mRNA in the ventral tegmental area (VTA) is TLR4 dependent. (a) CD11B: Two-way analysis of variance (ANOVA) revealed a significant effect of the meth treatment (*p=0.0336) while meth (1 mg/kg, i.p.) injected with the vehicle caused a significant upregulation of CD11B mRNA (*p<0.05) (Bonferroni post hocs). (b) IL-1β: Meth did not cause significant mRNA upregulation (p>0.05). (c) TNFα: Meth did not cause significant mRNA upregulation (p>0.05). (d) IL-6: Two-way ANOVA revealed a significant interaction (**p=0.0017), and a significant effect of meth (**p=0.0049) and (+)-naloxone (**p=0.015). Bonferroni post hocs revealed that (+)-naloxone (15 mg/kg, i.p.) injected with meth (1 mg/kg, i.p.) caused mRNA expression that was significantly less that mRNA expression resulting from meth with the vehicle (**p<0.01). Data are mean±s.e.m., n=3-5/group. (Northcutt et. al., in preparation).
Discussion

The present studies further support the role of TLR4 mediated proinflammatory signaling in cocaine reward, as well as highlight how it may contribute to reinforcement. Cocaine stimulates microglia cells through a TLR4 dependent mechanism, a consequence of which is the upregulation of IL-1β mRNA that is highly specific to the VTA. Cocaine-induced TLR4 activation is crucial for establishing cocaine CPP, while intra-VTA TLR4 activation and IL-1β signaling are necessary for the cocaine-induced NAc dopamine increase. Furthermore, repeated cocaine exposure exhibits a TLR4 mediated glial priming effect, which is necessary for reinstatement to cocaine seeking. These findings are consistent with the recently proposed xenobiotic hypothesis of drug abuse: TLR4, within the innate immune system of the CNS, identifies addictive drugs such as cocaine as foreign invaders, much like it does for pathogens, and initiates proinflammatory signaling as a response that has downstream effects on the mesolimbic dopamine pathway (Hutchinson & Watkins, 2014). This model already has a great deal of support in regard to morphine: morphine-induced TLR4 mediated signaling results in the expression of neuronal inflammatory markers, counteracting its analgesic effects while resulting in drug reward (Watkins et. al., 2007; Hutchinson et al., 2012)

The xenobiotic hypothesis is not only supported by the findings presented here and in our recent publications (Hutchinson et al., 2012; Hutchinson & Watkins, 2014; Northcutt et al., 2015) but is also supported by existing literature. For example, one study compared the response to reinstatement of cocaine to other DAT antagonists in a self-administration (S-A) and reinstatement paradigm in order to measure the reinforcing effects of the drugs. It was found that other DAT antagonists, such as benztropine analogs, that cause NAc dopamine increases do not instigate significant responding to reinstatement, signifying low abuse liability (Hiranita et. al., 2009). The authors later examined both slow and fast acting benztropine analogs and found that neither caused significant conditioned-place preference, a well-supported behavioral measure of drug reward (Li et. al., 2011). It may be that the DAT antagonists did not share the second mechanism of activating TLR4 and thus did not elicit the same level of reinstatement to cocaine seeking or rewarding effects in CPP paradigms.

The proinflammatory response to cocaine appears to be an important component underlying disruptions to the mesolimbic pathway that contribute to drug reward and reinforcement. Although it is currently unknown exactly how proinflammatory signaling influences the mesolimbic pathway, it has been shown that proinflammatory cytokines are neuroexcitatory. IL-1β, IL-6, and TNFα are now known to be powerful modulators of neuronal excitability (Vezzani & Viviani, 2014). These cytokines can act directly on neurons via specialized receptors to control excitability (Schwarz et. al., 2013). For example, TNFα modulates receptor trafficking, increasing surface AMPA receptor density while decreasing surface GABA receptor density, thus making synapses more excitatory (Stellwagen et. al., 2005). IL-1β can bind IL-1 receptors expressed on dopaminergic neurons causing enhanced
conductivity of NMDA receptors, highlighting its importance in the mesolimbic dopamine circuit (Ho & Blum, 1998; Viviani et al., 2003).

Repeated use of meth, and to a lesser extent, cocaine, is associated with neurotoxicity wherein, through fairly uncharacterized mechanisms, there is damage to the blood-brain barrier and neurons (Sharma et al., 2014). LPS induced IL-1β signaling can contribute to dopaminergic neuronal cell death, indicating that the cytokine may play a role in excitotoxicity (excessive neuron stimulation leading to cell damage or death) (Godoy et al., 2008; Long-Smith et al., 2010). Two to three hours preceding excitotoxic cell death, the chemokine fractalkine is rapidly cleaved from neuron cell membranes so the now soluble chemokine can promote chemotaxis (or migration in response to chemical stimulus) of microglia (Chapman et al., 2000), where it is now free to bind its receptor CX3CR1. IL-6 induces expression of this fractalkine receptor on microglia (Lee et al., 2010) and when bound by fractalkine, CX3R1 activation causes the release of IL-1β (Clark et al., 2015). Evidentially, proinflammatory signals may self-amplify, resulting in greater effects on the mesolimbic dopamine pathway. Interestingly, it was found here that only with repeated administration of cocaine were IL6 mRNA levels upregulated while a single injection of meth was sufficient to upregulate IL6 mRNA. Much remains to be discovered about the complicated cytokine IL-6; however this finding may be relevant to understanding the mechanisms underlying the neurotoxic consequences of psychostimulant use.

In this investigation, it was demonstrated that microglia display a proinflammatory immune response to TLR4 activation by cocaine. This was assessed by isolating whole-brain microglia, treating them in culture with the drug of interest for 24 hours, and quantifying mRNA levels using qRT-PCR. When cultured with varying concentrations of cocaine, microglia concentration-dependently upregulate IL-1β mRNA. When cultured with 1 and 10 ng/mL doses of LPS serving as the positive control, IL-1β mRNA expression was significantly upregulated indicating that the cultured microglia were in good health and fully functional. In cultured neonatal microglia, it was demonstrated that cocaine’s effect on microglial IL-1β mRNA expression is TLR4 mediated because cocaine-induced mRNA upregulation could be suppressed with (+)-naloxone (Northcutt et al., 2015; Sup. Figure 1). These data validated the role of microglia in expressing IL-1β in response to cocaine, yet the role of astrocytes should not be overlooked in future studies as they display proinflammatory and neuroexcitatory activity in response to microglial activation (Pascual et al., 2012).

A single systemic injection of cocaine induced expression of IL-1β mRNA in the mesolimbic dopamine pathway, specifically in the VTA. Real time qRT-PCR analysis of tissue samples collected from the PFC, NAc, and VTA following acute cocaine administration enabled for regional specificity when examining the cocaine-induced proinflammatory response. The VTA sends dopaminergic projections to the NAc, so the finding that cocaine-induced IL-1β mRNA upregulation in the VTA likely has substantial impact on the mesolimbic dopamine pathway. As noted previously, IL-1β is able to bind IL-1 receptors on dopaminergic neurons and make them more prone to excitation (Ho & Blum, 1998; Viviani et al., 2003). Therefore, this
may explain how the IL-1 receptor antagonist (IL-1ra) injected directly into the VTA could inhibit extracellular dopamine increases in the NAc following systemic administration of cocaine. This finding, along with other findings represented in supplementary figure 3, indicates that TLR4 signaling in the VTA is necessary for cocaine to produced elevations of dopamine in the NAc. However, direct activation of TLR4 signaling in the VTA with a microinjection of LPS alone does not increase dopamine signaling to the same extent that cocaine does (200% increase by LPS versus 300% increase by cocaine). This implies that both DAT antagonism and TLR4 agonism by cocaine might be necessary to cause such a large dopamine increase in the NAc.

Behavioral data from cocaine CPP has shown that cocaine-induced TLR4 signaling is necessary for reward (Northcutt et. al., 2015), but how it may be involved in addiction-like behaviors was previously unknown. Recent findings indicate that TLR4 activation is necessary and sufficient for reinstatement to cocaine seeking (Sup. Figure 4). Drug reinforcement was measured using a self-administration and reinstatement paradigm which revealed that reinstatement to cocaine seeking is attenuated by intra-VTA IL-1ra pretreatment to the cocaine challenge (Sup. Figure 5). However, intra-VTA blockade of IL-1β signaling did not fully suppress reinstatement to cocaine seeking as it did with systemic TLR4 antagonism using (+)-naltrexone (Bachtell et. al., unpublished). This implies that while cocaine reward may initially be mediated predominately by IL-1β, cocaine reinstatement may be mediated by other TLR4 dependent signaling molecules.

To explore this possibility, the mRNA expression of a several proinflammatory markers in response to a cocaine challenge following cocaine self-administration (S-A) and extinction was measured in the mesolimbic dopamine pathway. First, a timecourse study was conducted comparing mRNA expression at 30 minutes and 2 hours following the cocaine reinstatement challenge. IL-1β mRNA was significantly upregulated in the VTA at 2 hours following the cocaine prime in cocaine S-A animals, displaying a more exaggerated upregulation than what was observed in response to an acute cocaine injection in naïve animals. This 2 hour timepoint was utilized in the following study designed to explore this enhanced mRNA response observed in cocaine S-A animals. The follow-up two-by-two study compared the effects of saline self-administration versus cocaine self-administration as well as the effects of a saline versus cocaine reinstatement challenge on expression of neuroinflammatory markers within the mesolimbic dopamine pathway. The first comparison could show how chronic cocaine self-administration may alter the expression of neuroinflammatory markers in contrast to absent and acute cocaine administration. Adding groups that received a cocaine challenge allowed further observation of the proinflammatory response to an acute cocaine administration (in the saline self-administering rats) as well as the investigation into a potential microglia priming effect (in cocaine self-administering rats).

Microglial priming is a well-supported yet poorly understood phenomenon that was first characterized in peripheral macrophages in what was termed the Schwartzman reaction.
(Schwartzman, 1928); wherein acute immune system activation leads to immune system sensitization resulting in an exaggerated response to subsequent activation. The phenomenon was first observed on microglia in the CNS in the aged or diseased brain (Perry, Cunningham & Holmes, 2007; Dilger & Johnson, 2008; Streit & Xue, 2009) and was termed microglia “priming” by Perry, Cunningham & Holmes. It was found that microglia, in the aged or diseased brain, enter a primed state where they are sensitized to subsequent activation resulting in a more robust proinflammatory response and further microglial activation (Perry, Cunningham & Holmes, 2007; Roca et al., 2011; Boche, Perry, & Nicoll, 2013). In a primed state, microglia upregulate IL-1β mRNA expression and then secrete large amounts of the cytokine when activated (Perry, Cunningham & Holmes, 2007; Roca et al., 2011). Whether or not microglia will become primed by a stimulus depends on what the stimulus is. Different doses of LPS, varying degrees of tissue injury, different proinflammatory cytokines, excitotoxicity, and various diseases can all activate microglia cells in different ways, resulting in varied responses that may have beneficial or harmful, neurotoxic effects. It has been shown that primed microglia in a Parkinson’s disease model of neurodegeneration are responsible for upregulated IL-1β mRNA, and a more robust IL-1β release following an LPS challenge, thus facilitating further neurodegeneration (Godoy et al., 2008; Roca et al., 2011). A high dose of LPS will actually result in a suppressed proinflammatory response from microglia cells by increasing RelB (proinflammatory transcription repressor) binding to NFKB, (proinflammatory transcription factor) (Schaafsma et al., 2015). However, an extremely low dose of LPS results in the removal of RelB via interleukin receptor-associated kinase 1 and Toll-interacting protein-dependent mechanisms; a genetic alteration that results in a more proinflammatory microglia phenotype, e. i., primed microglia (Deng et al., 2013; Morris et al., 2014). It is evident that differential activation of microglia, especially through TLR4, can result in a variety of responses, including priming. Therefore, it is possible that cocaine interacts with the TLR4/MD-2 complex in such a way that leads to a priming signaling cascade. If so, this phenomenon could account for chronic cocaine-induced neurotoxicity and the robust proinflammatory response described above.

Evidence for glial priming may be evident in findings presented in Experiment 5; chronic cocaine self-administration caused an extremely significant upregulation of IL-1β mRNA within the vmPFC that, when compared to the saline S-A group, was not observed in response to acute cocaine administration. NFKB mRNA was also upregulated after chronic cocaine S-A and the cocaine challenge, perhaps indicating that repeated cocaine administration primed microglia, causing sustained changes in gene expression leading to IL-1β mRNA upregulation in this region. Perhaps this robust proinflammatory response plays an indirect role in facilitating changes in behaviors like impulsivity and low self-control associated with altered prefrontal cortex activity in the cocaine addict that are thought to contribute to relapse and/or continued cocaine use (Goldstein & Volkow, 2005; Winstanley et al., 2009). Whether IL-1β signaling in the vmPFC serves to drive these behavioral changes remains to be explored and appears to be a worthy target for further research.
Both the cocaine challenge and cocaine self-administration in general prompted a significant IL-1β mRNA upregulation in the NAc. One could hypothesize that this is due, in part, to the cocaine-induced dopamine increase in the NAc because microglia have been shown to directly respond to the release of dopamine in this region (Schwarz et. al., 2013). This suggests that some of the proinflammatory response to cocaine in the mesolimbic dopamine pathway may be due to cocaine’s neuronal actions indirectly, in addition to its direct action on TLR4. There may also be evidence of microglial priming as there is a robust, 450% upregulation of IL-1β mRNA in cocaine self-administering rats following subsequent cocaine administration (reinstatement challenge). One limitation of examining IL-1β in the NAc, however, involves the highly variable IL-1β mRNA response to cocaine administration. Previous work from this lab has shown mRNA expression of this cytokine to be somewhat inconsistent in response to acute cocaine within the NAc (Northcutt et al., 2015). One possible explanation is that the time during which the IL-1β gene is being transcribed varies slightly, and the 2 hour timepoint used captures the mRNA expression at the beginning, middle, or end of a wave. Further studies could incorporate additional timepoints to investigate the cause of this phenomenon.

The VTA remained the site of the most extensive proinflammatory response. Here, chronic cocaine self-administration upregulated mRNA expression of IL-1β, IL-6, NFκB, and GFAP when compared to the saline S-A group. These data demonstrate that repeated cocaine exposure activate glia to drive additional proinflammatory processes not observed with a single dose of cocaine. GFAP mRNA upregulation would indicate that astrocytes are now activated, responding to inflammation and possibly cell damage. As discussed previously, IL6 mRNA is only upregulated in this chronic cocaine exposure paradigm. The upregulation of these mRNAs in addition to IL-1β may reflect the neurotoxic effects of chronic cocaine exposure (Sharma et. al., 2014). Upregulation of NFκB expression could indicate that cells are attempting to further strengthen the immune response to “fight off” the chronic foreign invader, in this case, cocaine. Furthermore, chronic cocaine administration appears to sensitize glia such that subsequent cocaine administration significantly upregulates mRNA expression of IL-6, CD11B (the microglia activation marker), and possibly IL-1β (nearly significant) when compared to cocaine self-administering animals that did not receive a cocaine reinstatement challenge. Together, this data provides evidence as to how cocaine may prime glia to employ a prolonged and exaggerated neuroinflammatory response, which may underlie processes contributing to cocaine reinforcement and relapse.

Current work aims to further explore microglial priming by utilizing IHC staining for CD11B and MD-2 in the mesolimbic dopamine pathway in the brains of cocaine self-administering rats. Comparing the densities of CD11B and MD-2 between regions and between cocaine versus saline self-administering rats will provide insight into why the VTA displays a robust immune response to acute and especially chronic cocaine administration. It will also reveal if chronic cocaine administration causes enhanced chemotaxis and activation of microglia, effectively priming them to be more responsive to cocaine within the mesolimbic dopamine pathway.
pathway. Additionally, this experiment will help identify what cell types within the mesolimbic dopamine pathway express MD2. Although the TLR4-MD2 complex is predominately located on microglial cells throughout the brain, there are specific instances where TLR4 has been described on sensory neurons (Diogenes et al., 2011). Although it is expected that TLR4-MD complexes will also be expressed on microglia, the results of this study and related follow-up studies should be informative. Figure 8 depicts preliminary CD11B staining in the NAc of one naïve (Figure 8a) and one cocaine S-A (Figure 8b) rat. The microglia in these pictures can be qualitatively evaluated using the microglial activation classification of Kreutzberg (1996).

Microglia from naïve rats appear to be in Stage 1 (resting, or quiescent, microglia having a small cell soma and highly ramified processes). Activated microglia retract their processes and have a larger cell soma and thicker processes (Kettenmann et al., 2011). Cocaine S-A microglia appear to be mostly in Stage 3 (amoeboid microglia: round shaped body with short, thick and stout processes) with some in Stage 2 (activated ramified microglia: elongated shaped cell body with long and thicker processes). It cannot be concluded from morphology alone that microglia in Stage 3 are primed and not just activated (Perry, Cunningham & Holmes, 2007); however, primed microglia upregulate IL-1β mRNA expression (as observed in Experiment 5) and do so primarily in Stage 3 (Perry, Cunningham & Holmes, 2007; Boche, Perry, & Nicoll, 2013), which was observed in the cocaine S-A rat NAc. Upon subsequent activation, primed microglia enter Stage 4 (phagocytic cells: round shaped cells with vacuolated cytoplasm, no processes can be observed at light microscopy level) and secrete larger amounts of IL-1β than their unprimed counterparts (Boche, Perry, & Nicoll, 2013). This experiment will evidently unveil clues of cocaine-induced microglial priming as well as cocaine reactivity, while a future IHC experiment could include a cocaine S-A group that received a cocaine reinstatement challenge to see if there are any Stage 4 microglia present.

Another approach will soon be taken to further investigate microglia priming in response to chronic cocaine exposure. Rapidly isolating microglia from a cocaine S-A rat at the end of S-A and after extinction would allow microglia cells specifically to be tested for exaggerated, or primed, inflammatory responses to various treatments. Along with appropriate positive and negative controls, these cells will be treated in culture with cocaine/saline in media or with cocaine/(+)
 naloxone in media. This would reveal if microglia themselves display primed activity following repeated cocaine S-A, and if they remain primed through extinction where intense cravings begin to emerge. If they do indeed display an enhanced proinflammatory response to cocaine treatment, (+)-naloxone in conjunction with cocaine treatment will confirm if the effect is TLR4 dependent and can be suppressed in vitro.

A limitation of the qRT-PCR study on the two-by-two saline and cocaine self-administration study (Experiment 5) was that rats were given their reinstatement challenge in their home cages; the significance of which being that they were not exposed to the cocaine associated cues (placement in self-administration apparatus box or the available lever to press). The presentation of a drug cue is a crucial part of why both rats and humans relapse into cocaine.
use (Lu et al., 2004). Adding a cocaine self-administering group that is exposed to cocaine-paired cues prior to receiving the reinstatement challenge would provide insight into how drug-paired cues may influence neuroinflammation within the mesolimbic dopamine pathway. The proinflammatory effects of cocaine reinstatement paired with the drug cue and without it may prove to be additive within the NAc (Fuchs et al., 2004). In examining this possibility, it would be important to examine the proinflammatory response within the NAc core, as there is evidence that cue-induced cocaine reinstatement is driven by the NAc core rather than the shell (Fuchs et al., 2004; Welberg, 2013).

The xenobiotic hypothesis likely also applies to meth, as it is shown to interact with the TLR4/MD-2 complex (Northcutt et al., in preparation). It has been demonstrated that TLR4 blockade by (+)-naloxone inhibits meth-induced CPP (Sup. Figure 6). Additionally, meth-induced dopamine increases in the NAc can be attenuated by (+)-naloxone (15 mg/kg, i.p.) (Northcutt et al., in preparation; Sup. Figure 7). In contrast to findings where (+)-naloxone completely suppressed cocaine-induced NAc dopamine elevations, (+)-naloxone demonstrates a partial attenuation. This is likely due to the fact that much of this dopamine increase could be attributed to meth’s reversal of the dopamine transporter (DAT) and interference with the vesicular monoamine transporter-2 (VMAT-2), resulting in more free dopamine in the axon terminal that may now flow out of DATs. However, TLR4 signaling still appears to result in approximately half of the meth-induced NAc dopamine increase. Other differences should be noted between cocaine and meth findings discussed here; notably, IL-1β mRNA expression is not upregulated in response to an acute meth administration in the PFC, NAc, nor, and most importantly, in the VTA. However, upregulation of CD11b, GFAP, IL6, and TNFα was suppressed with systemic (+)-naloxone, indicating that meth-induced neuroinflammation is TLR4-dependent. This implies that other TLR4 mediated signaling may be responsible for contributing to the rewarding effects of meth. In fact, mRNA for IL-6, along with GFAP, is expressed in response to acute meth. Note that IL-6 and GFAP mRNA were only upregulated in the VTA in response to cocaine in a chronic, self-administration model (Figure 5). Because meth is highly neurotoxic, while cocaine neurotoxicity is only evident with more chronic exposure (Sharma et al., 2014), IL-6 and GFAP may be causal to, or indicative of, this neurotoxicity. Meanwhile, TLR4 blockade could completely suppress cocaine-induced reinstatement; while intra-VTA IL-1ra could only attenuate it (Sup. Figures 4,5). If IL-1β signaling alone cannot take full credit for reinforcement of cocaine, and seemingly is not involved in TLR4 mediated meth reward, then some other TLR4 dependent mechanisms must be involved. These mechanisms may relate to processes underlying neurotoxicity, as chronic cocaine exposure and acute meth exposure both display neurotoxicity as well as share IL-6 and GFAP mRNA upregulation. Additionally, acute meth exposure leads to the upregulation of TNFα mRNA. As these proteins respond to neurotoxic events, possibly leading to additional microglial activation (Vilhardt, 2005), they may play a large role in NAc dopamine signaling. For instance, IL-6 has been shown to lead to IL-1β release from microglia through an excitotoxicity-sensitive mechanism requiring the initiation of CX3C1 receptor expression; a process that could contribute to robust and
prolonged meth-induced NAc dopamine increases. It is possible that IL-6 has a delayed response in mediating dopamine release that is highly reactive to acute meth administration, but only reactive to chronic cocaine administration. This is suggestive of a highly involved role of IL-6 within the mesolimbic dopamine pathway. Future experiments will explore the effects of intra-VTA IL-6 antibodies on meth-induced NAc dopamine increase. If the treatment proves successful in inhibiting this NAc dopamine increase, it should then be tested in a cocaine self-administration and reinstatement experiment.

Together, the data presented in this thesis further the understanding that drugs of abuse, especially the psychostimulants cocaine and meth, must be examined in regards to both their neuronal and neuroimmune actions within the brain. Cocaine reward and reinforcement may require both the antagonism of dopamine transporters on neurons and the agonism of TLR4. This research explored how neuroinflammation contributes to cocaine reward, reinforcement and relapse. The collaboration that allowed for study of cocaine-primed reinstatement allowed for cocaine-induced neuroinflammation to be studied in a clinically relevant model of relapse to cocaine use. This provided insight into how the brain’s immune system might respond to repeated cocaine use in an addict. Current evidence suggests that Perry, Cunningham, & Holmes’ (2007) glial priming phenomenon could be combined with Hutchinson & Watkin’s (2014) xenobiotic hypothesis of TLR4 mediated drug reward to include reinforcement as follows: (1) TLR4 within the innate immune system recognizes addictive drugs, such as cocaine, as foreign invaders and initiates glial proinflammatory cascades; (2) drug-induced proinflammatory signaling can drive acute drug reward; (3) proinflammatory signaling can be amplified by repeated drug exposure that primes glial activation resulting in an over-exaggerated inflammatory response; (4) this effect may prolong and enhance chronic drug-induced neuroinflammation that may drive drug reinforcement; (5) these rewarding and reinforcing effects are the result of downstream proinflammatory signaling on the mesolimbic dopamine pathway. Further investigation, in a self-administration and reinstatement model, into cocaine-induced glial priming, meth-induced neuroinflammation, psychostimulant neurotoxicity, and psychostimulant-responsive cytokines such as IL-1β and IL-6, may lead to the characterization of an effective pharmacological intervention (such as (+)-naloxone) for psychostimulant addiction that could regulate proinflammatory cascades to possibly halt the reinforcing actions of these drugs or reduce drug craving during recovery.
References


Puhl, M. D., Blum, J. S., Acosta-Torres, S., & Grigson, P. S. (2012). Environmental enrichment protects against the acquisition of cocaine self-administration in adult male rats, but does


Supplemental Figures

**Sup. Figure 1**: Cocaine-induced signaling in isolated neonatal microglial cells is Toll-like receptor 4 (TLR4) dependent. (a) Lipopolysaccharide (LPS) dose-dependently upregulated mRNA expression of interleukin-1beta (IL-1β; ***p<0.01 and ****p<0.001, Bonferroni post hoc) in neonatal microglia following 1h incubation (one-way analysis of variance (ANOVA), F(5, 23)=62.29, p<0.003). (b) Cocaine (0.1 and 1 μM) upregulates mRNA expression of IL-1β (**p<0.01, Bonferroni post hoc) in neonatal microglia following 1h incubation (one-way ANOVA, F(4, 19)=11.56, p=0.0002). (c) (+)-naloxone (1, 10, and 100 μM) suppresses cocaine-induced upregulation of IL-1β mRNA (p<0.0001; two-way ANOVA, interaction F(6, 33)=19.68, p<0.0001, Bonferroni post hocs). Incubation with (+)-naloxone alone had no effect on IL-1β mRNA expression (p>0.05). (Northcutt el. al., 2015; reprinted with permission).
Sup. Figure 2: (+)-Naloxone suppresses cocaine-induced upregulation of interleukin-1beta (IL-1β) mRNA in the ventral tegmental area (VTA) (b) Cocaine-induced (10 mg/kg, i.p.) upregulation compared with saline (p=0.01) of IL-1β mRNA within the VTA 30 minutes following injection is attenuated by (+)-naloxone (2.5 mg/kg given in two subcutaneous (s.c.) injections, the first given 10 minutes before the second, which is paired with cocaine) administration (*p<0.05). Bonferroni post hocs were preceded by two-way ANOVA, indicating the main effects of cocaine ($F_{(1, 17)}=4.61$, p=0.0001) and (+)-naloxone ($F_{(1, 17)}=4.61$, p=0.0447). Data are mean±s.e.m., n=5-6/group. (Northcutt et. al., 2015; reprinted with permission).
Sup. Figure 3: Cocaine-induced increases of nucleus accumbens (NAc) dopamine are dependent on Toll-like receptor 4 (TLR4) signaling within the ventral tegmental area (VTA). (a) Cocaine (10 mg/kg, subcutaneous (s.c.)) produces elevated extracellular dopamine in the NAc 40 min (****p<0.0001) and 60 min (*p<0.01) following drug administration (repeated measures two-way analysis of variance (ANOVA) with Bonferroni post hocs, time and treatment interaction p<0.001). Administration of (+)-naloxone (2.5 mg/kg in two s.c. injections spaced 10 min apart) blocked this effect; there were no differences between this group and the saline or (+)-naloxone-treated rats. Before drug treatment, there were no differences in extracellular dopamine concentrations in the NAc shell across all groups. Data are mean±s.e.m., n=4/group. (b) Cocaine (10 mg/kg, intraperitoneal (i.p.)) produces elevated extracellular dopamine in the NAc that sustains for 40-100 min (Bonferroni, *p<0.05, **p<0.01 and ****p<0.0001) following drug administration. Intra-VTA LPS-RS (lipopolysaccharide, a TLR4 antagonist naturally produced by Rhodobacter sphaeroides) blocked this effect. There were no differences between other treatment groups. Data are mean±s.e.m., n=4/group. (c) LPS (10 ng in 1 μL) microinjected into the VTA produces increased extracellular dopamine within the NAc compared with vehicle microinjection controls (two-way ANOVA, effect of treatment F(1, 144)=35.83, p<0.0001 and time F(11, 144)=2.89, p=0.0018) 40 and 100 min after microinjection (*p<0.05, Bonferroni post hoc). Data are mean±s.e.m., n=5-7/group. (Northcutt et. al., 2015; reprinted with permission).
Sup. Figure 4: Toll-like receptor 4 (TLR4) blockade dose-dependently suppresses reinstatement to cocaine seeking. Rats self-administered saline or cocaine (0.5 mg/kg/infusion) on a fixed ratio 1 schedule of reinforcement in 15 daily sessions. Lever responding was extinguished in 8 daily extinction sessions. Reinstatement testing consisted of two phases. The first was a 2 hr extinction phase immediately followed by a 2 hr reinstatement phase. (+)-Naltrexone (3.75, 7.5, 15 mg/kg) was administered as a pretreatment to a cocaine prime (15 mg/kg, ip). (* p < 0.05). (Bachtell et. al., unpublished).
Sup. Figure 5: Interleukin-1 receptor antagonist (IL-1ra) injected into the ventral tegmental area (VTA) attenuates reinstatement to cocaine seeking. Rats, with surgically implanted bilateral injection guide cannula aimed at the VTA, self-administered saline or cocaine (0.5 mg/kg/infusion) on a fixed ratio 1 schedule of reinforcement in 15 daily sessions. Lever responding was extinguished in 8 daily extinction sessions. Reinstatement testing consisted of two phases. The first was a 2 hr extinction phase immediately followed by a 2 hr reinstatement phase. IL1ra was microinjected into the VTA (10 µg/side) as a pretreatment to a cocaine prime (15 mg/kg, ip). (** p < 0.001). (Bachtell et. al., unpublished).
Sup. Figure 6: TLR4 signaling is necessary for meth-induced conditioned-place preference (CPP). Two-way analysis of variance (ANOVA) revealed a significant effect of meth (****p<0.0001) and (+)-naloxone (*p<0.05) while bonferroni post hocs revealed a significant effect of (+)-naloxone suppressing preference within the meth group (**p<0.01). Data are mean±s.e.m., n=5-6/group. (Northcutt et. al., in preparation).
Sup. Figure 7: Large meth-induced increases in nucleus accumbens (NAc) dopamine rely on TLR4 signaling. There is a significant effect of time and treatment (****p<0.0001) as well as a significant interaction (****p<0.0001) (two-way analysis of variance (ANOVA)). Meth (1mg/kg, i.p.) with vehicle causes significant dopamine increases at 40, 60, 80 min (****p<0.0001), at 100 min (***p<0.001), and 120 min (*p<0.05) (Bonferroni post hocs). (+)-naloxone (15 mg/kg, i.p.) with meth (1 mg/kg, i.p.) suppressed the meth-induced dopamine increase at 40 min (**p<0.01). The (+)-naloxone (15 mg/kg, i.p.) with meth (1 mg/kg, i.p.) group displayed significantly more dopamine than the saline/saline control at 100 min (**p<0.01) and 120 min (*p<0.05) (Bonferroni post hocs). Data are mean±s.e.m., n=3-5/group. (Northcutt et. al., in preparation).