A Dual Investigation of Questions in Drug Addiction: Exploring the Role of Neuroinflammation in Opioid Withdrawal and A Novel Measure of Reward-Seeking Behavior in Rodents

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Abstract

Substance abuse disorders are extremely disruptive to the lives and relationships of those who suffer from them and are a burden on society and the healthcare system. Effective treatments are currently lacking and our understanding of the neurobiology driving addictive behaviors remains incomplete. The following animal studies aimed to advance the drug addiction field by 1) investigating neuroimmune interactions as a potential mechanism in opioid withdrawal in mice as well as 2) introducing a novel behavioral test in order to expand the repertoire of tools available to researchers investigating drug or reward-seeking behaviors in rats.

Microglia, the immune cells of the brain have been recently recognized as being important contributors in a wide variety of neuropsychiatric disorders, including drug addiction. Although current views support a microglia role in the physical drug dependence experienced by opioids addicts, it is thought that the emotional effects of withdrawal, such as anxiety and depression, are better predictors of relapse. In the first series of experiment, a genetic mouse model lacking the key microglia immune receptor TLR4 was used to determine the involvement of this protein in the molecular and behavioral responses to opioid withdrawal, in particular those related to negative emotions.

Although technological advances in behavioral testing techniques have improved our ability to model drug addiction in rodents, the low-tech behavioral assays used most frequently have remained largely unchanged over the last several decades. The second set of experiments introduced and validated a novel behavioral task based on the classic measure of reward, conditioned place preference. In the modified test, objects rather than contexts were used as a conditioned cue, which potentially allows for greater flexibility and opens up new ways of analyzing conditioned approach, a commonly used measure for assessing the reward value of a given substance.

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Chapter 1: Introduction to Neuro-immune Interactions in Opioid Addiction and Withdrawal

Prevalence and impact of opioid addiction

Opiate addiction is a major health and societal problem, with a prevalence of 1.4 % for opioid use disorders (Compton et al. 2007) and relapse rates of 90% a year after treatment (Bailey et al. 2013). Currently, there is a lack of proven therapies for treating opioid abuse. However, as our understanding of the neural mechanisms underlying addiction improves, it opens up new opportunities for evidence-based treatments. Over the last few decades, there has been an increasing focus on the potential role of microglia, the innate immune cells of the brain, in modulating the different effects of drugs that contribute to abuse liability. Accumulating evidence indicates that microglia play an important role in drug abuse across a wide range of substances, although this relationship appears to be particularly important for opioids.

Microglia and neuro-immune interactions

Microglia are derived from a population of myeloid cells closely related to macrophages that invade the CNS early in development. These cells comprise around 5-12% of total cells in the mouse brain (Lawson et al. 1990) and roughly 10 - 20% of total glia (Banati 2003). Microglia can adopt a variety of different states depending on the local conditions. Under quiescent conditions, microglia appear in a resting or ramified state characterized by active surveying of the CNS environment (Correa et al. 2013). Microglia can also take on different activation states, most commonly classified as M1 versus M2 phenotypes

(Orihuela et al. 2016; Boche et al. 2013). The M1 phenotype refers to the classical proinflammatory response of microglia and macrophages consisting of phagocytosis and release of cytotoxic cytokines. By contrast the “alternative” M2 phenotype is characterized by anti-inflammatory or repair functions. An immune challenge would likely trigger the M1 phenotype, although even within this category the specific response is highly dependent on the nature of the challenge (Hanisch and Kettenmann 2007). Microglia can be activated by a wide variety of stimuli including pathogens, cytokines, ATP, glutamate, and particles associated with cellular damage or death (Correa et al. 2013).

Sensitivity to such a wide range of stimuli can be attributed in part to the presence of toll-like receptors (TLRs). TLRs are a family of pattern-recognition receptors that recognize and respond to different pathogens as well as other substances, and can trigger a variety of intracellular responses (Takeda and Akira 2004). These include initiation of an innate immune response leading to phagocytosis and/or release of cytokines and chemokines as well as facilitation of the delayed adaptive immune responsive driven by other immune cells (Kawai and Akira 2008). Within the TLR family, one of the best understood receptors is TLR4, which responds to gram-negative bacteria and mediates the microglia response to the classical agonist lipopolysaccharide (LPS). TLR4 signaling can occur via adaptor molecule MyD88- dependent and independent pathways. MyD88- dependent pathways lead to translocation of the transcription factor NF-kB into the nucleus. Many immune factors such as cytokines and chemokines have NF-kB responsive promoter regions and thus are upregulated via a TLR4- MyD88 pathway (Takeda and Akira 2004; Kawai and Akira 2005).

M1 microglia activation occurs in a graded fashion and can result in a variety of different responses that serve to either sensitize the cell to a future insult or actively engage in an inflammatory response. Microglia responses include proliferation, migration, morphological changes, phagocytosis, release of nitrous oxide (NO) or cytokines, and changes in surface receptor expression (Hanisch and Kettenmann 2007; Boche et al. 2013). Microglia activation via a TLR4 pathway typically results in release of pro-inflammatory cytokines such as IL-1β and TNFα These cytokines can bind to receptors on other microglia in order to proliferate a pro-inflammatory response, as well as to receptors on neurons, where they are able to alter excitability among other functions (Beattie et al. 2002).

Addiction and neuro-immune signaling in humans

Although the vast majority of studies supporting a role for microglia in opioid addiction have used animal models, there is some evidence that opioid addiction in humans is also associated with altered immune function. One source of this evidence is gene association studies. Identification of genetic polymorphisms associated with disease states such as drug addiction is becoming an increasingly important tool in identifying potential disease biomarkers or mechanisms. In the case of opioids, a polymorphism of the gene encoding IL-1β that results in increased expression of this pro-inflammatory cytokine is associated with an elevated risk for opioid dependence (Liu et al. 2009). An association between immune factors and addiction was also uncovered when a list of genes implicated in human opioid, nicotine, and ethanol dependence was compiled in a meta-analysis and analyzed for common molecular networks. Genes involved in immune signaling

pathways were found to be associated with addiction across all substances (Reyes-Gibby et al. 2015).

Evidence of altered immune activity can also be found when looking at markers of inflammation in the blood or brain tissue of opioid-addicted individuals. Levels of pro-inflammatory cytokines are elevated in both blood (Chan et al. 2015) and postmortem brain tissue (Dyuizen and Lamash 2009) of heroin addicts. In this last study, postmortem tissue was collected from a patient who died of a heroin overdose and elevated pro-inflammatory cytokines were specifically observed in the locus coeruleus (Dyuizen and Lamash 2009), a brain area important in mediating many of the physical effects of opioids (Maldonado et al. 1992).

Finally, a relationship between drug abuse and immune activity is supported by clinical findings on the efficacy of glial inhibitors as a treatment for addiction. Administration of ibudilast, a glial inhibitor, reduced some subjective symptoms of withdrawal in heroin-dependent subjects (Cooper et al. 2016). This study suggests that not only is there an association between glial activity and drug abuse, but also that glial activity is contributing to the addictive properties of opioids, in this case symptoms of withdrawal. Ibudilast as well as other glial inhibitors are currently being investigated as potential treatments for addiction to opioids as well as other drugs of abuse (https://clinicaltrials.gov).

Other drugs of abuse

There is also a connection between abnormal immune activity and addiction to other drugs of abuse. The same polymorphism of IL-1β described above was also associated

with increased likelihood of ethanol dependence (Liu et al. 2009). Furthermore, levels of pro-inflammatory cytokines in the blood are elevated in alcoholics (González-Quintela et al. 2000) as well as in healthy subjects during an alcohol hangover (Kim et al. 2003). Likewise, pro-inflammatory cytokines were higher in postmortem brains of alcoholics within areas involved in drug reward and withdrawal such as the ventral tegmental area (VTA) and amygdala (He and Crews 2008). Similar findings have been observed for abusers of psychostimulants such as methamphetamine (meth). Abstinent meth addicts had increased binding of a labelled marker for reactive microglia revealed via positron emission tomography (PET) imaging (Sekine et al. 2008). This marker for reactive microglial was higher in the striatum of abstinent meth users versus matched controls and was negatively correlated with the length of abstinence (Sekine et al. 2008). Finally, a clinical study found that the glial inhibitor ibudilast, when given to methamphetamine-dependent subjects, reduced subjective highs following intravenous (i.v.) infusions of meth (Worley et al. 2016). This finding indicates that inhibition of glial activity may have therapeutic potential for multiple drugs of abuse.

Addiction and neuro-immune signaling in animal models

Use of animal models has provided more direct support for microglia activity as an underlying factor in opioid addiction as well as identified TLR4 signaling as a potential mechanism of glial activation. Not only do opioids upregulate microglia expression of TLR4 both *in vivo* (Schwarz and Bilbo 2013; Loram et al. 2012) and *in vitro* (Dutta et al. 2012; Wang et al. 2012), but it has been suggested that opioids directly bind and activate TLR4 receptors (Wang et al. 2012; Hutchinson et al. 2010). Using different genetic and pharmacological techniques, pre-clinical researchers have furthered the field by

demonstrating that microglia activation is 1) increased by opioid exposure via TLR4 signaling, and that this activation contributes to the 2) rewarding effects of opioids, and 3) symptoms of opioid withdrawal.

Microglia activation

Glial markers that increase with activation such as Iba1 (Boche et al. 2013; Ito et al. 1998) allow for quantitative analysis of glial activity in specific brain regions in response to opioid administration. These markers can also be used to identify microglia in combination with another marker of activation such as phosphorylation of the MAPKs or transcription factor NF-kB. Phosphorylation of p38 and ERK MAPKs occur in microglia in response to injury (Kaminska et al. 2009; Ji and Suter 2007; Zhuang et al. 2005) or exposure to pathogens (Kim et al. 2004; Xing et al. 2011), and may serve as a good indicator of TLR4-dependent activation (Okun et al. 2011). In addition to these markers, increased expression (mRNA) or release (ELISA) of soluble cytotoxic factors such as NO, IL-1β, or TNFα in the blood or brain can also be used to infer increased microglia activation.

Morphine exposure both *in vivo* and *in vitro* results in increased microglia activation assessed using a number of different endpoints. Morphine administered to primary or immortalized microglia *in vitro* results in a range of responses that appear to be dose-dependent. While lower morphine doses (<1uM) increase expression of Iba1 (Horvath and DeLeo 2009) and phosphorylation of ERK and ATK (Horvath and DeLeo 2009; Merighi et al. 2013), this dose range is not high enough to increase expression of cytokines (Ghazi et al. 2010; Merighi et al. 2013; Turchan-Cholewo et al. 2009; Wang et

al. 2012). Morphine doses >1uM similarly increase phosphorylation of ERK, but also upregulate expression of IL-1β and increase activation of NF-kB (Wang et al. 2012).

Likewise, the effects of *in vivo* morphine treatment on microglia activation are also treatment- and/or timing- dependent. Many of these studies focus specifically on the nucleus accumbens (NAc), a brain region critical in addictive behavior (see below). Within the NAc, expression of Iba1 was found to be increased by repeated but not acute morphine in mice (Zhang et al. 2012, 2011), whereas a similar study found *decreased* Iba1 density in the rat NAc following repeated morphine treatments (Campbell et al. 2013). Similarly, levels of a marker for reactive microglia have been reported to either increase (Campbell et al. 2013) or decrease during morphine withdrawal (Hutchinson et al. 2009), although this discrepancy may be due to the use of spontaneous versus naloxone precipitated withdrawal, respectively. Findings concerning levels of pro-inflammatory cytokines are somewhat more consistent. Multiple studies reported increased expression of cytokines or chemokines within the NAc following repeated morphine treatment (Zhang et al. 2011; Chen et al. 2011; Schwarz et al. 2013). Although a separate study reported no change in the NAc following acute or chronic morphine exposure, they observed elevated pro-inflammatory cytokines during opioid withdrawal (Campbell et al. 2013), which is consistent with other findings (Hutchinson et al. 2009). Finally, increased phosphorylation of p38 in NAc was observed following repeated but not acute morphine treatment (Zhang et al. 2012).

Reward

The rewarding effect of a drug is thought to be among the strongest factors in determining addiction liability (Wise 1996; Robinson and Berridge 2001). Thus,

measures of drug reward are central to the field of addiction biology. Drug reward or reinforcement can be assessed using multiple approaches, the most common being self-administration and conditioned place preference. Studies exploring whether microglia activation contributes to opioid reward have blocked microglia activity either via administration of glial inhibitors (e.g. minocycline), knockout of TLR4 or downstream accessory protein MyD88, or antagonism of TLR4 via an inactive stereoisomer of opioid antagonists (e.g. +Naloxone).

Conditioned place preference for morphine was dose-dependently reduced by glial inhibitor minocycline delivered prior to each morphine treatment either systemically via gavage (Hutchinson et al. 2008) or infused directly to the NAc (Zhang et al. 2012). However, morphine CPP was not affected by a single dose given immediately before testing (Zhang et al. 2012), indicating that glial activity is contributing the rewarding effects of opioid throughout treatment and conditioning. A role for glial inhibition, and specifically TLR4 signaling, on opioid reward was further supported by findings that CPP for Oxycodone is reduced in TLR4 and MyD88 knockout mice (Hutchinson et al. 2012). Pharmacological antagonism of the TLR4 receptor can be achieved using the + isomer of naloxone, which is inactive at classical opioid receptors but acts as an antagonist at TLR4 receptors (Hutchinson et al. 2010).+Naloxone given with morphine blocked expression of conditioned placed preference for morphine and dose-dependently reduced responses for the opioid remifentanil in a self-administration procedure (Hutchinson et al. 2012).

Other drugs of abuse

Similar to the effects of morphine, *in vitro* exposure to methamphetamine (Tocharus et al. 2010), cocaine (Liao et al. 2016; Northcutt et al. 2015), and ethanol (Fernandez-Lizarbe et al. 2009) all increased microglia activation measured as increased cytokine release. Additional measures of activation include increased MAPK phosphorylation (Fernandez-Lizarbe et al. 2009), or translocation of NFkB to the nucleus (Liao et al. 2016). Repeated administration of methamphetamine (Asanuma et al. 2004; Fantegrossi et al. 2008), cocaine (Liao et al. 2016), and ethanol (Fernandez-Lizarbe et al. 2009) *in vivo* also resulted in elevated glial activation measures, such as increased density of reactive microglia in the striatum (Asanuma et al. 2004; Liao et al. 2016) or cortex (Fernandez-Lizarbe et al. 2009).

Measures of reward for non-opioid drugs of abuse are also affected by inhibition of microglia. Glia inhibition using pretreatment of systemic propentofylline disrupted methamphetamine conditioned place preference (CPP) (Narita et al. 2006). Likewise, concurrent injections of minocycline reduced self-administration of ethanol in a free choice drinking test (Agrawal et al. 2011). Finally, minocycline (gavage) and +naloxone (subcutaneous, s.c.) pre-treatment both blocked cocaine CPP while TLR4 knockout animals exhibited reduced cocaine self-administration (Northcutt et al. 2015).

Neuro-immune activation and withdrawal

Somatic vs. Affective withdrawal

Opioid withdrawal triggers a variety of aversive physical and emotional symptoms that largely oppose the acute effects of the drug. According to the opponent process theory of

addiction, withdrawal symptoms arise due to neuroadaptations over the course of prolonged drug use (Solomon and Corbit 1974). These adaptations are thought counter the acute effects of opioid which eventually leads to tolerance during continued drug use and severe withdrawal effects following abstinence from the drug. The withdrawal syndrome is comprised of both physical as well as emotional or affective symptoms, which are hypothesized to arise from distinct neurobiological processes and may differentially contribute to the likelihood of relapse. While somatic symptoms are thought to derive from neuroadaptations within peripheral tissues or the brain stem, affective symptoms are predicted to originate from adaptions within the reward and motivation circuitry of the brain, in particular the amygdala and NAc (Koob and Le Moal 2008; Aston-Jones and Harris 2004). This classification is supported by evidence from animal studies in which acute microinjections of opioid antagonist methylnaloxonium were used to precipitate morphine withdrawal in distinct brain regions. Naloxone delivered the NAc and amygdala were able to trigger affective symptoms at the lowest dose while infusions into the PAG were most effective in generating somatic symptoms (Maldonado et al. 1992).

The affective symptoms of withdrawal, which include anxiety and depression (anhedonia), are hypothesized to be more important factors driving sustained opioid addiction than physical symptoms for several reasons. First, unlike physical signs, negative affective symptoms are common to all classes of addictive drugs (Koob and Le Moal 2008). Second, while changes in physical withdrawal signs dissipate rapidly, changes in negative affect often persist well beyond cessation of drug intake (Heilig et al. 2010). Former drug users report experiencing these emotional symptoms well into drug

abstinence and alleviation or avoidance of these symptoms can encourage a return to drug-taking behavior. As such, the severity of affective withdrawal symptoms can predict likelihood of relapse (Piasecki et al. 1998).

Behavioral measures of withdrawal

As stated in the previous section, physical symptoms of withdrawal differ across drugs of abuse as well as across species. In rodents there are an array of different motor and autonomic effects that make up the physical symptoms of opioid withdrawal, including weight loss, wet-dog shakes, jumping, diarrhea, rearing, vocalizations, and teeth chattering (Blasig et al. 1973). A potential addition to this list is hyperalgesia, which is an opioid withdrawal symptom mediated by neuroadaptations in the spinal cord which is strongly linked to immune activity (Johnston et al. 2004). Relative to affective symptoms, somatic effects tend to require higher doses of morphine and/or naloxone (Schulteis et al. 1994), and tend to resolve more quickly (Martin and Jasinski 1969).

Affective withdrawal symptoms largely consist of elevated anxiety and/or depressive-like phenotype in rodents, which can be assessed using a variety of behavioral assays. Measures of anxiety in rodents typically attempt to capture heightened neophobia or avoidance behavior in response to fearful stimulus such as light or exposed space. These features are present in behavioral tests such as light/dark box or elevated plus maze. Alternatively, anxiety tests may assess elevated arousal or active avoidance responses to threat, such as acoustic startle or defensive burying, respectively. All the above measures have been previously used to capture the elevated anxiety-like state during acute or precipitated withdrawal from drugs of abuse (Harris and Aston-Jones 1993; Harris and Gewirtz 2003; Hage et al. 2012; Kliethermes et al. 2004).

Anhedonia or depressive-like behavior is often measured as a diminished motivation to work for a reward or avoid a punishment. Anhedonia/dysphoria can be observed as increased threshold for intracranial self-stimulation (ICSS) (Carlezon and Chartoff 2007), reduced sucrose preference or breakpoint for sucrose responding, and increased conditioned avoidance within a conditioned place paradigm (Cryan and Holmes 2005). A depression-like phenotype can also be assessed as conditioned defeat in a forced swim or tail suspension test (Cryan and Holmes 2005). Similar to anxiety, all these measures are sensitive to the anhedonic state observed post-withdrawal (Schaefer and Michael 1986; Orsini et al. 2001; Wei et al. 2011; Stevenson et al. 2008).

Microglia & TLR4- withdrawal effects in opioids

The first evidence that immune cells contributed to opioid withdrawal came in 1986 from a study by Dafny & Pellis. In this experiment, immune cells were ablated in rats using whole body irradiation either before or after chronic morphine treatment and withdrawal symptoms were observed following naloxone treatment. Irradiation drastically reduced withdrawal signs regardless of when irradiation occurred, indicating that glial activity potentiates opioid withdrawal symptoms (Dafny and Pellis 1986). These findings have since been replicated using systemic administration of glial inhibitor AV411 (Bland et al. 2009; Hutchinson et al. 2009). This effect was subsequently demonstrated to involve TLR4 using concurrent administration of TLR4 antagonist, +naloxone, with morphine (Hutchinson et al. 2010). +Naloxone reduced overall expression of somatic withdrawal symptoms including hyperalgesia (Hutchinson et al. 2010). Furthermore, +naloxone given continuously during withdrawal reduced subsequent incubation of heroin seeking in a self-administration study (Theberge et al. 2013), suggesting that alleviation of

withdrawal symptoms with +naloxone may reduce the likelihood of reinstatement or relapse.

When combined, these studies provide strong evidence that TLR-4 mediated activation of microglia potentiates the severity of opioid withdrawal in rodents. However, all of these studies assessed physical withdrawal symptoms. Thus, it remains unclear whether TLR4 signaling or microglia activation generally alter *affective* withdrawal symptoms. Considering that affective withdrawal symptoms may be better predictors or relapse, this remains an important question when considering the value of TLR4 as a therapeutic target in potential treatments of addiction. However, there are parallels between TLR-mediated "sickness behavior" and the heightened anxiety and anhedonia experienced during opioid withdrawal, suggesting that TLR4 pathways engaged during opioid withdrawal could potential alter these behaviors.

TLR4 and sickness behavior

Sickness behavior is a term that describes the non-specific physiological and behavioral responses to infection, including fever and muscle weakness as well as anxiety and malaise (Kelley et al. 2003). Following infection, peripheral inflammatory factors trigger an immune response in the CNS via afferent connections such as the vagus nerve as well as other pathways (Dantzer 2009). Neuro-immune signaling within the CNS is thought to drive many of the sickness responses, in particular the affective behaviors, which similar to opioid withdrawal syndrome, typically last longer than other symptoms (Frenois et al. 2007). This hypothesis is supported by findings that activation of TLR4 or release of proinflammatory cytokines within the brain is associated with increased anxiety (Engler

et al. 2011) and depressive-like behaviors (Frenois et al. 2007). Furthermore, central administration of LPS is sufficient to elicit depressive-like behavior (Fu et al. 2010) and the behavioral effects of peripheral IL-1β can be blocked via NF-kB antagonist infused into the lateral ventricle (Nadjar et al. 2005). Finally, the increased anxiety observed following peripheral inflammation corresponded with enhanced cellular activity in brain regions implicated in mediating the affective response to opioid withdrawal (see next section) (Engler et al. 2011). Based on these findings, it is possible that TLR4 activation and cytokine release may play an important role in the experience of anxiety and anhedonia during opioid withdrawal.

Mechanisms underlying effects of microglia activation on opioid reward and withdrawal

Although there is substantial evidence indicating that TLR4-mediated microglia activation contributes to the expression of opioid-seeking and withdrawal behaviors, the mechanism underlying this relationship is currently unknown. Thus a second critical question that remains to be addressed is how microglia activity interacts with the mesolimbic circuitry driving opioid reward and withdrawal.

The NAc and TLR4

The NAc is considered to be a central nexus for motivated behavior (Ikemoto and Panksepp 1999; Salamone 1994) and a major component of the neural circuitry driving the expression of opioid-seeking behaviors (Hutcheson et al. 2000) and affective withdrawal (Valverde et al. 1996). Many of the studies cited in previous sections focused exclusively on the role of the NAc in microglia- and TLR4-mediated behaviors. For

example, morphine treatment and withdrawal have been shown to increase the density of reactive microglia and to upregulate pro-inflammatory cytokines specifically in the NAc (Zhang et al. 2011; Campbell et al. 2013; Hutchinson et al. 2009). Furthermore, bilateral infusion of either a glial or p38 inhibitor into the NAc during morphine treatment blocked morphine CPP (Zhang et al. 2012). This study suggests that microglia activity within the NAc is sufficient for changes in opioid-seeking behavior.

Additional evidence supporting the importance of the NAc as a central site of opioid and microglia interaction come from the results of microdialysis studies measuring dopamine (DA) release. Morphine treatment results in an acute elevation in extracellular DA in the NAc shell which is reduced by a glial inhibitor AV411 (Bland et al. 2009) or the TLR4 antagonist +naloxone (Hutchinson et al. 2012). Interestingly, extracellular DA release in response to morphine was correlated with the severity of withdrawal behaviors upon naloxone-precipitated withdrawal (Bland et al. 2009). Thus, microglia activity appears to potentiate the VTA-NAc DA pathway during acute opioid exposure.

Molecular mechanisms of opioid withdrawal

The symptoms of opiate withdrawal, including anxiety and anhedonia, are linked to changes in the neural circuitry of reward and motivated behavior, including the NAc and amygdala. In these regions, acute morphine binds to a G protein-coupled receptor which suppresses adenylyl cyclase (AC) and downstream signaling targets such as cyclic AMP (cAMP) and protein kinase A (PKA). Prolonged suppression of cAMP during chronic morphine treatment can lead to compensatory upregulation of different components in this pathway. Cessation of opioid binding during withdrawal triggers a superactivation of the cAMP signaling pathway which leads to increased phosphorylation of MAPKs and

subsequent activation of downstream transcription factors (Fan et al. 2009). These adaptations in turn can lead to changes in synaptic plasticity and neuronal excitability through altered receptor trafficking and gene expression (Williams et al. 2001).

Although many different molecules are engaged by these pathways (Waltereit and Weller 2003; Hyman et al. 2006), notable targets implicated in affective withdrawal symptoms include MAPKs ERK1/2 and p38 as well as transcription factors cAMP response element binding protein (pCREB) (Guitart et al. 1992; Li et al. 2008) and fos proteins including ΔFosB (Núñez et al. 2010) and c-Fos (Hayward et al. 1990). However, altered expression of some of these factors may be specific to particular brain regions and withdrawal conditions. For example, while withdrawal-induced increases in pCREB (Chartoff 2006; Guitart et al. 1992; Shaw-Lutchman et al. 2002), ΔFosB (McClung 2006; Núñez et al. 2010) and c-Fos (Georges et al. 2000) appear to be common to the NAc, amygdala, and LC, pERK is upregulated in the NAc and amgydala but decreased in the LC following chronic morphine (Eitan et al. 2003). Likewise, pCREB is increased in the NAc following naloxone-precipitated withdrawal (Chartoff 2006) but not during spontaneous withdrawal from heroin (Edwards et al. 2009). Other downstream processes that have been previously associated with affective withdrawal include altered expression of neurotrophic factors such as BDNF (Numan et al. 1998) and increased phosphorylation of the GluA1 AMPA receptor subunit (Edwards et al. 2009).

Although we are far from a complete understanding of the cellular effects of opioid withdrawal, the adaptions listed above have been consistently associated with affective symptoms including anxiety and anhedonia/dysphoria (Pliakas et al. 2001; Pandey 2003; Valverde et al. 2004; Ailing et al. 2008). Thus, these targets would provide a good

starting point for an analysis of how the cellular mechanisms of opioid withdrawal are altered by microglia activity.

Activation of TLR4s by opioid withdrawal

As addressed in a previous section, the interaction between opioids and TLR4-mediated microglia activation is hypothesized to occur via non-stereoselective binding of opioids to TLR4 receptors (Wang et al. 2012; Hutchinson et al. 2010). This hypothesis provides a simple yet well supported mechanism for activation of microglia following opioid exposure. However, several findings from the field are at odds with this proposed mechanism, allowing for the possibility of an alternative means by which opioid withdrawal leads to increased microglia activation.

There is abundant evidence that opioids *can* bind and activate TLR4s, however there is also evidence against direct opioid activation of TLR4 as a *primary mechanism* for the various effects of opioids on immune function. First, there are a large number of *in vitro* studies that support a facilitatory rather than independent role of opioids in neuroinflammtion. In these studies opioids did not elicit strong effects on microglia when administered alone but did greatly potentiate the effects of classical agonists such as LPS (Roy et al. 1998; Merighi et al. 2013; Stevens et al. 2013) or other inflammatory factors such as Tat (Bruce-Keller et al. 2008). It has been hypothesized that this effect is due to the ability of morphine signaling via classical opioid receptors to upregulate TLRs (Dutta et al. 2012; Zhang et al. 2011) or co-activate a common downstream target such as ERK (Merighi et al. 2013). This prediction is further supported by the finding that some of the neuroimmune opioid effects are dependent on mu-opioid receptors (Meng et al. 2013;

Zhang et al. 2011; Franchi et al. 2012). Thus, opioids can also contribute to neuroinflammation by sensitizing microglia to future insults via upregulation of TLR4.

Second, although direct binding of opioids to TLR4 receptors could mediate acute activation of microglia, it is less clear how this would change over repeated injections or withdrawal. Effects of *in vivo* morphine administration on markers of microglia activation were not typically observed following a single dose but rather required multiple days of exposure (Zhang et al. 2012, 2011). This finding raises the question of how repeated administration of opioids affects the TLR4 signaling pathway and whether opioid signaling at TLR4 is also prone to tolerance or sensitization observed in classical MOR signaling pathways. Likewise, microglia activation is observed during both spontaneous and precipitated withdrawal (Campbell et al. 2013; Hutchinson et al. 2009) and withdrawal symptoms are reduced by microglia irradiation immediately prior to testing (Dafny and Pellis 1986). These findings suggest that microglia activation is either initiated or sustained during withdrawal and that this activation contributes to symptoms. However, if direct opioid-TLR4 binding is responsible for activation of microglia, how is this maintained during withdrawal? Opioid binding at the TLR4 site would gradually end as the peptide is metabolized or be competitively replaced by an antagonist, which should inhibit glial responses.

Taken together, these findings suggest that an alternative mechanism of opioid-mediated microglia activation is required. Considering that TLR4 is upregulated by opioids (Dutta et al. 2012) and is known to respond to a variety of pathogen- or damage-associated

molecules (Correa et al. 2013; Beg 2002), opioid exposure may sensitize microglia to a secondary insult. One potential source of this insult could be damage or stress-associated factors released by neurons or astrocytes in response to chronic opioids or withdrawal. Such a mechanism would support the delayed microglia response to repeated morphine injections as well as the sustained activation observed during withdrawal. However, this possibility remains to be tested.

Chapter 2: The Role of TLR4 in the Affective Symptoms of Opioid Withdrawal

In order to address some of the remaining questions posed in the previous chapter, a series of *in vivo* and *in vitro* experiments were conducted to explore the role of TLR4 signaling in opioid withdrawal. First, affective responses during spontaneous opioid withdrawal were evaluated in wildtype (WT) and TLR4 KO mice using the well-established behavioral paradigms conditioned place aversion (CPA), light/dark test, and withdrawal potentiated startle (WPS). To further determine whether behavioral differences observed in the TLR4 KO result correspond to molecular changes in reward/motivation circuitry, we measured levels of phosphorylated CREB and ERK proteins in the nucleus accumbens (NAc) following precipitated withdrawal using

Western Blots. Phospho-ERK levels were additionally investigated using immunohistochemistry to determine whether any changes in pERK were localized to specific subregions of the NAc. Finally, an *in vitro* model of opioid withdrawal was used in order to explore whether soluble factors released from withdrawn cells are able to trigger microglia activation in primary cultures.

Methods/materials:

Animal subjects:

Mice carrying a spontaneous deletion of TLR4 (C57BL/10ScNJ) were purchased from Jackson Laboratories (Bar Harbor, Maine). Knockout (KO) mice were on a C57BL/10J background and were crossed with C57BL/6J mice to generate heterozygous mice on a mixed C57BL strain. Heterozygous male and female mice were then mated to produce TLR4 KO and WT offspring. Mice were genotyped at postnatal day (P) 21 using PCR amplification of the following oligonucelotides; TLR4 KO forward: GCA AGT TTC TAT ATG CAT TCT C, reverse: CCT CCA TTT CCA ATA GGT AG; WT forward: ATA TGC ATG ATC AAC ACC ACA G, WT reverse TTT CCA TTG CTG CCC TAT AG. Mice were housed in mixed groups of 4 per cage on a 12 hour light-dark schedule and food and water were provided *ad libitum*. Behavioral studies were conducted on adult male mice. All procedures performed were in compliance with the National Institutes of Health's animal care standards and were approved by the University of Minnesota's Institutional Animal Care and Use Committee (IACUC).

Drugs:

Morphine sulfate was obtained from Mallinckrodt (St. Louis, Mo) and naloxone from Sigma Pharmaceuticals (Croydon, UK). All drugs were dissolved in 0.9% saline and injected in a 1ml/kg volume subcutaneously (s.c.).

Testing procedures:

Adult WT and TLR4 KO male mice were assigned to either morphine-treated or saline-control groups. Following two days of handling, mice received daily injections of either morphine (20mg/kg) or saline over six consecutive days (Figure 1, Days 1-6). This morphine dose and treatment schedule is similar to other morphine regimens that induce tolerance and dependence in mice (Fukui and Takagi 1972; Abdel-Zaher et al. 2006) and should have been sufficient to increase microglia activity (Zhang et al. 2012). Following this injection regimen, mice were tested on conditioned place aversion (CPA), light/dark, and withdrawal potentiated startle (WPS) procedures. To avoid carry-over effects on behavioral testing and potential health issues resulting from prolonged morphine treatment, mice received a two week drug-free interval between the starting points of each behavioral task. To maintain a state of acute morphine withdrawal during testing, mice received two daily injections of either morphine or saline leading up to light/dark and WPS testing. Separate groups of animals were used for additional behavior testing (CPP, jumping) as well as tissue collection for protein analysis in Western blot and immunohistochemistry.

Conditioned Place Aversion (CPA):

The conditioned place aversion apparatus consists of a rectangular plastic cage (40 cm x 20 cm x 20 cm) divided into two sides by a central partition. Contextual features of each side included a distinct floor texture and wall pattern: metal bars paired with white walls,

and wire mesh paired with black-striped walls. CPA chambers were housed in a rack below an overhead camera in a dimly lit room (Lux = 30-40). The position of mice within the chamber was analyzed in real-time using AnyMaze software (Stoelting, Wood Dale, IL). Prior to testing, animals were placed into an empty test chamber lacking any distinctive contextual cues for 18 minutes in order to allow animals to acclimate to the test chambers. Preliminary testing following this procedure revealed a strong inherent bias for the "bar" side or aversion to the "mesh" side (Figure 2A). To account for this difference in baseline preferences, a biased design was adopted such that morphine withdrawal was always paired with the preferred "bar" side of the CPA chamber.

The CPA procedure was loosely based on a previous study (Contarino and Papaleo 2005) and consisted of pre-test, conditioning, and post-test phases. For the pre-test, mice were placed near the center of the test chamber with the central divider removed to allow free access to both sides for 18 minutes. Conditioning sessions were 30 minutes in length and occurred at 8 hours post-injection. In morphine-treated animals, injections prior to conditioning alternated daily between morphine and saline over 4 days while saline controls received saline prior to all conditioning sessions. Mice were always confined to the "bar" side during morphine days and the "grid" side during saline days. In order to assess morphine withdrawal-induced weight loss, mice were weighed prior to morning injections and immediately following conclusion of conditioning on each day. The post-test was conducted in a similar fashion as the pre-test and occurred 3 days following the final conditioning day in order to avoid acute morphine withdrawal effects on exploratory behavior (Rothwell et al. 2009).

Light/Dark:

Light/dark testing was modified from previous studies (Kliethermes et al. 2004; Crawley and Goodwin 1980) and was performed in a novel chamber containing a large (27 cm x 20 cm x 20 cm) brightly lit (Lux = 760) side and a small (13 cm x 20 cm x 20 cm) covered side (Lux = 20) connected by a small opening (5 x 5 cm). Mice were first given 30 minutes to acclimate to the testing room under dim lighting and were then placed into the dark side of the chamber for a 10 minute test. Overhead cameras recorded movement of the animal, and time spent in each side was determined by raters blind to experimental conditions using computer-assisted software (Button Box, Madison, WI).

Withdrawal-Potentiated Startle (WPS):

Startle measurements were conducted using four ventilated, sound-attenuated acoustic chambers (51 cm × 55 cm × 31 cm; Med Associates, Fairfax, VT), each equipped with two speakers, a Plexiglas startle cage (8.6 cm x 7.6 cm x 5.1 cm) and a load cell transducer system. The transducers were connected via an amplifier to a computer equipped with Advanced Startle software (MED Associates). Startle was computed as peak-to-peak voltage detected by the transducer. Startle responses were measured over three consecutive days, with two days of baseline readings and a final test session 4.5 hours after an s.c. injection of morphine (20 mg/kg). Startle responses on baseline day 2 were used as pretest values to determine potentiation of the startle reflex during morphine withdrawal calculated as percent change (post-pre/pre). All sessions commenced with a 5-minute acclimation period following placement of the mice in the chambers. The startle-eliciting stimulus was a 20-ms white-noise burst (rise time = 3 msec). Baseline and withdrawal startle testing comprised 50 pseudorandomized trials, with 10 each at the following dB levels: 70, 80, 85, 90 and 100 dB (30s inter-trial interval [ITI]).

Conditioned place preference:

Morphine conditioned place preference (CPP) was assessed using a similar procedure as CPA. Prior to testing, mice received 6 daily injections of morphine (20 mg/kg, s.c.) to induce dependence and the pre- and post-tests procedures were identical to those used for CPA (18 minutes of free-access). Conditioning was conducted over 4 days and alternated daily between morphine and saline treatments. Following an injection of either morphine (5 mg/kg, s.c.) or saline mice were immediately placed into the respective conditioning environment for 30 minutes. In order to account for the baseline bias for the “bar” side, morphine was always paired with the non-preferred “grid” environment. Similar to CPA, the post-test was performed three days following the final conditioning day.

Jumping:

Jumping behavior was measured during naloxone-precipitated morphine withdrawal with testing procedures adapted from Kest et al. (2002). Leading up to the day of testing, mice received daily morphine injections over four days escalating in the following sequence 10, 20, 30, 40 mg/kg (s.c.). On the test day, mice were given 40 mg/kg morphine followed three hours later by naloxone (30mg/kg, s.c.). Immediately after the naloxone injection, mice were placed into a cylindrical plastic chamber (13 cm diameter x 26 cm height) and video recorded for 9 minutes. Videos were rated by blind observers for the total number of jumps, defined as all four paws leaving the bottom of the chamber.

Tissue collection:

Western blots: Naïve mice received the same 6 day saline or morphine (20 mg/kg, s.c.) injection regimen described above. On day 7, mice received a final morphine or saline

injection followed by an injection of naloxone (1 mg/kg, s.c.) after a 1 hour delay. An additional control group received two saline injections. One hour after the second injection (2 hrs from the first injection), mice were euthanized and brains were removed and sectioned in a 1mm matrix. Sections containing striatal tissue were further dissected to extract bilateral NAc tissue which was flash frozen using the rapid freezing spray Flash Freeze (Decon Laboratories, King of Prussia, PA).

Immunohistochemistry: A subset of behaviorally-tested WT and TLR4 KO mice were used for immunohistochemistry. Following the completion of WPS, mice were given two week delay followed by three daily injections of morphine (20 mg/kg, s.c.) or saline leading up to the dissection day, identical to the procedures used for repeated behavioral testing described above. Following the third and final morphine injection, mice received an injection of naloxone (1mg/kg, s.c.) after a one hour delay. One hour after the final injection, mice were anesthetized with isoflurane and perfused as described below.

Western blots:

Tissue samples were processed for Western blots as previously described (Been et al. 2016). Briefly, tissues were homogenized in 1% SDS processing buffer and protein was quantified using the Bio-Rad protein DC assay (Bio-Rad Laboratories, Berkeley, CA, USA). Approximately 35 μg of total protein was loaded onto a 12% polyacylamide gradient gel (Mini-PROTEAN TGX Precast Mini Gel, Bio-Rad Laboratories) and run in a 1x SDS running buffer at 100 V for 90 min at RT. Gels were transferred to a nitrocellulose membrane in a 1x SDS running buffer in 20% Methanol at 100V for 50 min at 4C°. Membranes were blocked in 5% BSA in TBST (TBS + 0.1% Tween 20, pH

7.6), and incubated overnight at 4 degrees in primary antibodies against phospho-CREB (1:2,000; Cell Signaling), phospho-ERK (1:1000,Cell Signaling), and GAPDH (1:30,000; Millipore) diluted in 5% BSA in TBS + 0.1% Tween 20 (Sigma-Aldrich). The following day, membranes were washed 5x for 5 min in TBST, and then incubated for 1 hour at RT in the appropriate secondary antibodies (pERK/pCREB, anti-rabbit 680; GAPDH, anti-mouse 800; 1:20,000; Li-Cor Biosciences, Lincoln, NE, USA) and then imaged and band intensities quantified using the Odyssey imaging system (LiCor Biosciences). Bands for each protein were distinguished by color and MW.

Immunohistochemistry (IHC):

Mice were sacrificed by perfusion fixation as previously described (Vulchanova et al. 1997). Briefly, animals were deeply anaesthetized using isoflurane and perfused with calcium-free tyrode solution (in mM:NaCl 116, KCl 5.4, $MgCl_2 \cdot 6H_2O$ 1.6, $MgSO_4 \cdot 7H_2O$ 0.4, NaH_2PO_4 1.4, glucose 5.6, and $NaHCO_3$ 26) followed by fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9). Fixed brains were removed and stored in 10% sucrose solution (PBS with 0.05% sodium azide) at 4ºC. Brains were cut on a freezing microtome into 40 micron coronal sections. Brain sections containing the NAc were transferred to a cyroprotectant (1% PVP, 30% ethylene glycol, 30% sucrose, 25mM PBS) and stored at -20 ºC for later processing. For staining, sections were washed 3x in TBS for 1 hour each then incubated overnight at 4° C in the reagent buffer (1% BSA, 0.2% Triton, 0.2% Tween in TBS). All TBS washes were for 1hour unless otherwise stated. Sections were washed twice in TBS before 48 hour (24 hours at RT, 24 hours at 4° C) incubation in primary antibodies (goat anti-Iba1 1:500, mouse anti-GFAP 1:500, and rabbit anti-pERK 1:500) prepared in the reagent buffer.

Sections were then washed once in TBS for four hours and incubated in secondary antibodies (Cy3 donkey anti- goat 1:150, Cy2 donkey anti-mouse 1:100, and DyLight405 donkey anti-rabbit 1:150) prepared in the reagent buffer for 24 hours at 4° C. Sections were washed with TBS 3x and incubated with NeuroTrace (1:50 in TBS) overnight at 4° C. Sections were washed in 0.1% Triton in TBS for an hour followed by 7x TBS washes. Tissue sections were mounted on gelatin-coated slides and allowed to dry overnight. Slides were quickly rinsed with milliQ H2O before undergoing an alcohol dehydration series (50%, 80%, 100%, and 100%, 15 mins each) and immersed in Limonene for 15 mins. Tissue sections were mounted with DPX and covered with glass coverslips.

Section imaging:

Slides were imaged on a Zeiss Axiovert 200 M microscope at 20x using the open source Micro-Manager-ImageJ software package. One to three sections containing caudal NAc were imaged per animal. Caudal NAc was defined as sections for which the anterior commissure was located 30 – 75 um lateral to a vertical axis originating at the ventral tip of the lateral ventricle. One hemisphere was imaged per brain and exposure settings were consistent across sections from all treatment conditions. Counts of pERK+ and Iba1+ cells were obtained by thresholding images using ImageJ and counting all particles above a pre-set size cutoff. All cells identified by ImageJ were verified by visual inspection. The NAc core was sampled using a 250 x 166 um window (horizontal orientation) located directly above the anterior commissure while the NAc shell was sampled using a 166 x 250 um (vertical orientation) window located medial to the anterior commissure and bordering the diagonal band nucleus (see Fig 6A). The lateral septum was sampled

by counting cells within an area 220 um medial to the lateral ventricle along the length of the ventricle between the corpus callosum and NAc.

Neuron Cultures:

Striatal/cortical co-culture and glia cultures were prepared as previously described (Penrod et al. 2011). Briefly, 12mm coverslips used for both neuronal and microglia cultures were acid washed (1 M HCl) overnight at 55° C then treated in an ethanol series (50-75-90%) for 30 min each before storage at 225ºC. Prior to plating coverslips were coated with poly-D-lysine (PDL, 100ug/ml) and laminin (4ug/ml) overnight at RT then rinsed with sterile water. Timed-pregnant embryonic day 16 (E16) CD1 mice were euthanized and embryos removed. The brains of the embryonic mice were dissected in a sterile hood in Ca2+/Mg2+ free Hank's Balanced Salt Solution (CMF-HBSS) with 10mM HEPES. The cortex and striatum were transferred to separate dishes, minced, and digested with 0.25% trypsin-EDTA and Benzonase at 37ºC for 30 minutes. The tissue was then centrifuged for 5 minutes at 1,000 rpm, washed 3x in CMF-HBSS, and dissociated in plating media. Cells were then plated at a 2:3 (striatal:cortical) ratio in 35 mm dishes each containing 5 coated coverslips with a total density of 175,000 cells per dish. After 1-3 hours, plating media was replaced with neural growth media (Neurobasal containing 1X B27, 0.5mM glutamine, and 1X penicillin/streptomycin) conditioned overnight in mixed glial cultures (glial-conditioned media, GCM). GCM was prepared by providing confluent mixed glia cultures (see below) with neural growth media overnight. Neuronal cultures received fresh, non-glia conditioned neural growth media at 4 days-*in vitro* (DIV) and were maintained with media changes once per week.

Mixed glia and microglia cultures

Newborn mice (P1-3) were decapitated and brains dissected in CMF-HBSS. Cortices were minced, digested in 0.25% trypsin-EDTA and Benzonase at 37ºC for 30 min, and dissociated in glia media (EMEM, 10% FBS, 1X penstrep, 10mM glutamine). Cells were plated in 10 cm dishes for GCM as described in Penrod et al. (2011) or in 75m2 flasks (Corning, Corning, NY) at a cell density equivalent to ~2 brains per flask for isolation of microglia. Glia media was changed 24 hours after plating and once per week thereafter. Approximately 2 weeks after plating, Microglia were isolated from the flasks containing mixed glia via shaking (200 rpm, 1.5 hours, 37°C). Microglia that detached from the astrocyte bed were collected by removing the media. Media containing the cells was centrifuged at 2,000 rpm for 10 minutes and plated in a 24-well plate containing coverslips pretreated with PDL/laminin at a density of 20,000 cells/well. This procedure resulted in high purity of Iba1+ microglia based on almost complete absence of GFAP+ astrocytes (>99% purity). Microglia treatments or neuronal media transfers (see below) were conducted at 24 hr post-plating.

Morphine withdrawal and media transfer:

Neuronal cultures received a media change containing either 0 or 1uM morphine sulfate dissolved in sterile water at 13 DIV, and a second dose given directly into the existing media on 17 DIV. Twenty-four hours following the second dose, the media was removed and cells were washed once with fresh neural growth media to remove any remaining morphine. This media was then removed and replaced with neural growth media conditioned in an age-matched set of untreated neuronal cultures. Cultures were left undisturbed for 24 hours at which point media was collected and supplemented with 10% FBS. Media collected from neuronal cultures treated with water (control) or morphine

(withdrawal-conditioned, WC) was either directly transferred to plated microglia or filtered (0.8 um) and frozen at -20ºC for later use. Following the media collection, neuronal cultures were fixed with 4% paraformaldehyde/PHEM (60 mM PIPES pH 7.0, 25 mM K-HEPES pH 7.0, 10 mM EGTA, 2 mM $MgCl_2$)/0.12M sucrose (PPS) and stored in 3% BSA in TBS. On the day of the media transfer, glia media was removed from the plated microglia and replaced with 0.5 ml/well of one of five treatments: fresh glia media (GM), fresh neural growth media alone (NB), neural growth media containing 100 ng/ml lipopolysaccharide (LPS), or the control (Con) or withdrawal-conditioned (WC) neural growth media collected from neuronal cultures. All neural growth media treatments were supplemented with 10% FBS. Following the media transfer, microglia were returned to the incubator (37° C) for 30 minutes, at which point cells were fixed in PPS and stored in 3% BSA in TBS for immunocytochemistry.

Immunocytochemistry (ICC):

Fixed neurons or microglia were first permeabilized in the reagent buffer (see above) for 20 minutes, washed for 5 min in TBS, and blocked in 3% BSA in TBS for 30 min at RT. Primary antibodies were diluted in 1% BSA in reagent buffer and directly applied to each coverslip for overnight incubation at 4° C. Glia were stained using respective microglia and astrocyte markers goat anti-Iba1 (1:500, Novus, Littleton, CO) and mouse anti-GFAP (1:1000). Microglia activation was assessed using rabbit anti-pERK (1:250, Cell Signaling) and neuron cultures were stained for dopaminergic striatal neurons using mouse anti-DARP32 (1:1000) to verify the presence and health of striatal cells. The following day, coverslips were washed for 5 minutes in TBS prior to application of the appropriate Cy-2, -3, or -5 conjugated secondary antibody (1:150) for 1 hour at RT. Cells

were then washed 2x in TBS for 5 minutes each, incubated for 30 seconds in a 1ug/ml DAPI nuclear stain, and washed for 5 minutes in TBS before mounting. Coverslips were mounted on glass slides with a glycerol-based mounting media (80% glycerol, 2.5% 1,4-diazabicyclo-[2.2.2]cctane, 150 mM Tris pH 8.0) for subsequent fluorescent imaging.

Cell imaging

Coverslips were imaged on a Zeiss Axiovert 200 M microscope with 20x plan-apo objective using the open source Micro-Manager-ImageJ software package. For microglia imaging, six fields were taken per coverslip with exposure times for each channel standardized across each condition within a given experiment. Further processing of images was performed using ImageJ. Outlines of Iba1+ cells obtained via thresholding were overlaid on the pERK channel to determine mean pERK intensity values for each Iba1+ cell. DAPI staining was used to identify artifacts or cell outlines containing multiple nuclei which were excluded from further analysis. Background pERK staining for each field was subtracted from mean pERK intensity to obtain a normalized intensity value for each Iba1+ cell. For neuronal coverslips, six DARPP32+ cells were imaged per coverslip and images were visually inspected for signs of cell damage or death including nuclear blebbing (DAPI) and severe loss or fragmentation of dendrites.

Statistical analysis:

Pretest preferences scores in the CPA test were combined across treatment groups for each genotype in order to reduce variation. CPA and CPP data were assessed using a 3x2 (CPA) or 2x2 (CPP) ANOVA with Test and Genotype as the two variables. Results from the light/dark test, WPS, and jumping were analyzing using 2x2 Genotype x Treatment

ANOVAs as well as planned contrasts between morphine-treated and saline-control animals for each genotype. Weight loss was analyzed using a 2x2x2 Genotype x Treatment x Injection day ANOVA. Unpaired t-tests between saline-treated and morphine-withdrawal treatment groups within each genotype were used to assess pERK Western blot intensity and pERK/Iba1 density data. For cultures studies three replicate experiments were performed using different neuron cultures, glia cultures or both and all replicates contained three triplicate coverslips for the neuron-conditioned media treatment groups. An unpaired t-test was used to compare pERK intensity between Con and WC groups. Where used, posthoc tests were Tukey's HSD.

Results:

Conditioned place aversion (CPA):

WT and TLR4 KO mice exhibited equivalent locomotion in the pretest (distance travelled, $T = 0.12$, $df = 33$, $P = 0.91$), suggesting normal motor function and exploratory behavior in KO mice. As expected, there was a significant baseline bias in WT mice such that mice greatly preferred the "bar" side (**Fig 2A**, versus equal preference, $T = 8.77$, $df = 16$, $P < 0.0001$). Although a "bar" preference was observed in TLR4 KO mice ($T = 2.21$, $df = 16$, $P = 0.04$), this bias was significantly less than that observed in WT animals (WT = 291.4 sec, KO = 74.4; $P < 0.0001$).

There was a significant effect of treatment (**Fig 2B**, $F[2, 71] = 5.01$, $P = 0.009$) on preferences for the drug-paired environment and this effect was dependent on genotype (treatment x genotype interaction, $F[2, 71] = 3.81$, $P = 0.027$). *Post hoc* comparisons

revealed that morphine-treated WT mice exhibit significant aversion to the drug-paired environment (versus pre-test, $P = 0.0037$) while TLR4 KO animals did not exhibit any change in preference ($P = 0.65$). As expected, preferences did not change relative to pre-test values in the saline-only animals from both genotypes (WT: $P = 0.91$, KO: $P = 0.64$).

Light/Dark:

Similar to CPA, there was a significant treatment effect on preference for the dark chamber (**Fig 2C**, $F[1,42] = 11.83$, $P = 0.0014$) such that morphine withdrawal reduced time spent in the dark chamber. This effect appeared to be dependent on genotype, although this interaction failed to reach significance ($F[1,42] = 3.5$, $P = 0.069$). Planned contrasts between dark preferences for morphine-withdrawn and saline-controls for WT and TLR4 KO mice supported a genotype-dependent effect.

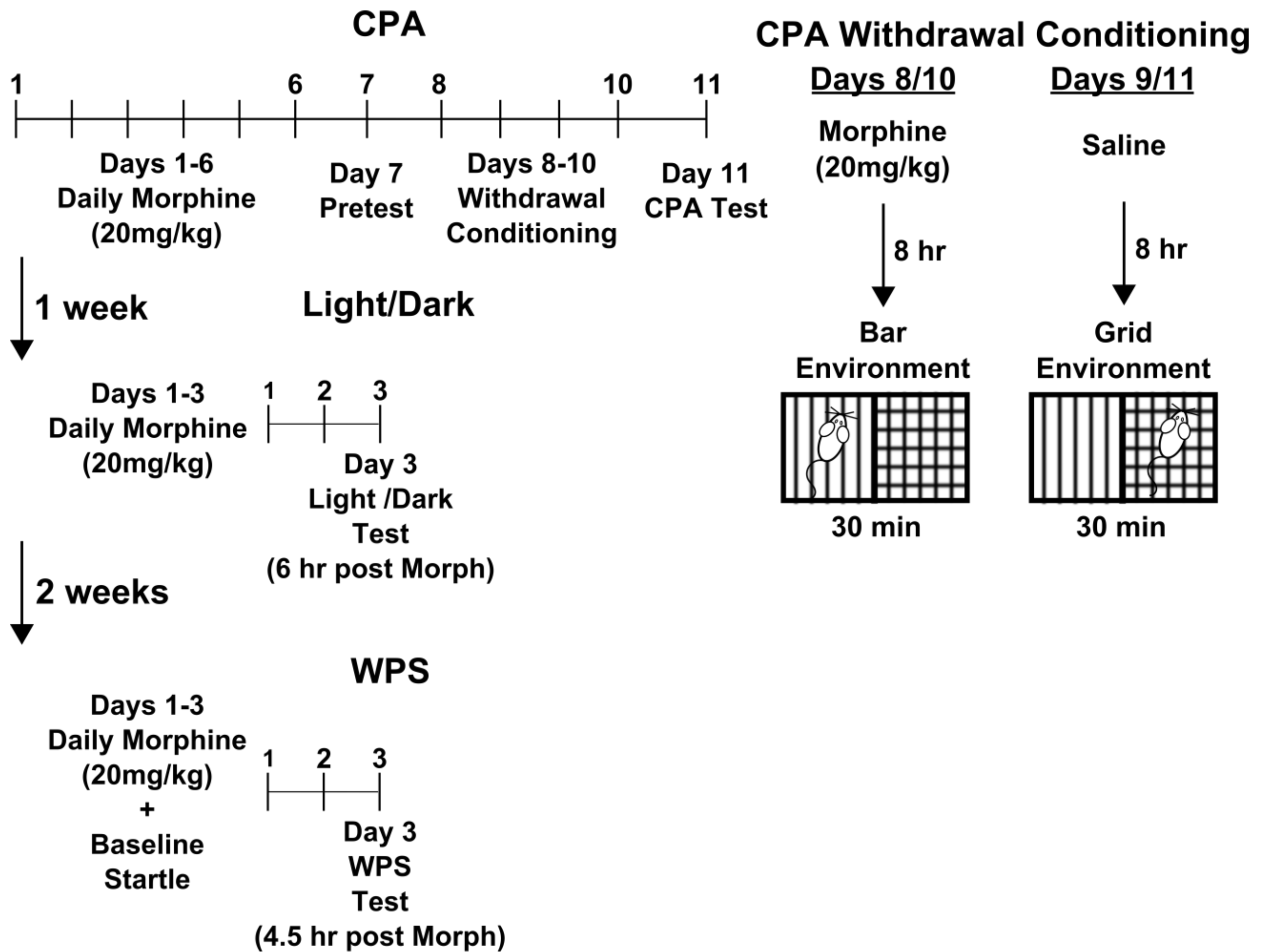

Figure 1. Morphine regimen and testing schedule. Following six daily morphine injections, mice were tested conditioned place aversion (CPA). Subsequent behavioral testing was conducted two weeks following the beginning of the previous test and was preceded by two daily morphine injections. CPA, light/dark test, and withdrawal-potentiated startle (WPS) were performed during spontaneous withdrawal at the indicated time point.

Morphine withdrawn WT mice spent less time spent in the dark chamber ($F[1,42] = 13.69$, $P = 0.0007$), while this effect was not observed for TLR4 KO mice ($F[1,42] = 1.27$, $P = 0.27$). Although WT controls exhibited a higher preference for the dark chamber compared to TLR4 KO controls (WT = 173.5 sec, KO = 94.9 sec), this effect was not significant in an unpaired t-test ($T = 1.41$, $df = 17$, $P = 0.177$).

Withdrawal-Potentiated Startle (WPS)

At baseline testing, startle responses to the 100dB stimulus were not affected by either morphine treatment (data not shown, $F[1,48] = 1.44$, $P = 0.24$) or genotype ($F[1,48] = 0.1.67$, $P = 0.20$).

On the test day, startle responses at 100dB were strongly potentiated by morphine withdrawal (**Fig 2D**, $F[1,48] = 13.69$, $P = 0.0006$) and similar to the results of the light/dark test, there was evidence of an interaction with genotype but this comparison failed to reach significance ($F[1,48] = 3.47$, $P = 0.069$). A genotype-dependent effect was further supported by planned contrasts of startle responses between morphine and saline treatment groups within each genotype. While an increase in startle potentiation was observed in the morphine-withdrawn WT animals ($F[1,48] = 15.67$, $P = 0.0003$), no difference was found in TLR4 KO mice ($F[1,48] = 1.67$, $P = 0.203$).

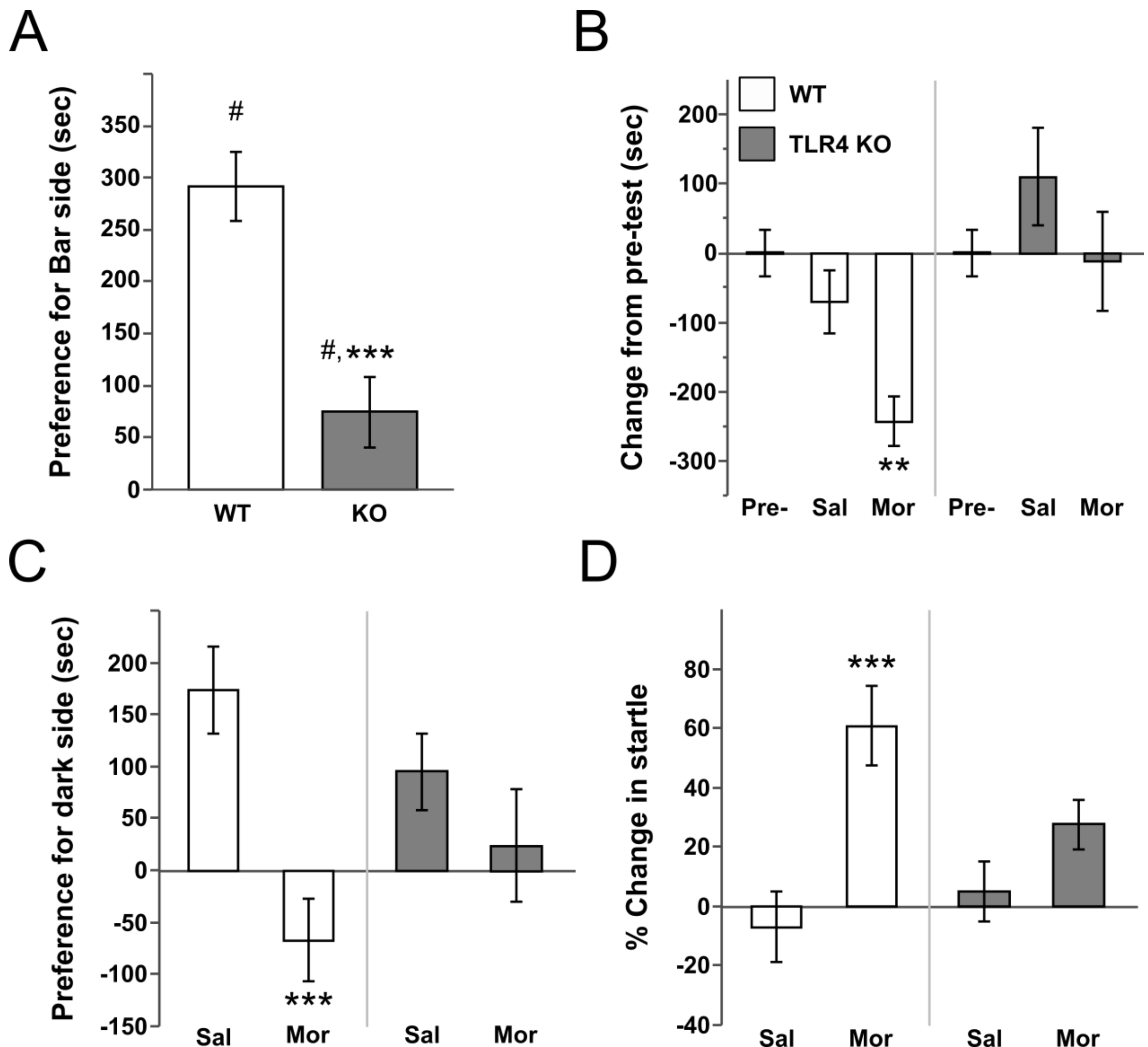

Figure 2: Effects of morphine withdrawal on affective behaviors in WT and TLR4 KO mice. A) WT and KO mice both exhibited an inherent bias toward the "Bar" location during the pre-test. The strength of preference was significantly greater in WT mice. (# $P < 0.05$ versus no preference, *** $P < 0.001$ versus WT). B) Following conditioning, WT but not TLR4 KO mice exhibited a reduction in preference for the morphine withdrawal-paired environment relative to averaged pre-test values. N's: WT Sal = 8, CPA = 10; KO Sal = 9, CPA =10. C) Preference for the dark chamber in the light/dark test was significantly reduced by morphine withdrawal in WT but not TLR4 KO mice. N's: WT Mor = 12, Sal = 9; KO Mor = 12, Sal = 10. D) Morphine withdrawal potentiated the startle response of WT but not TLR4 KO mice relative to saline-treated controls. N's: WT Mor = 16, Sal = 9; KO Mor = 12, Sal = 10. Bars depict mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus pretest preferences (B) or saline-treated controls (C,D).

Conditioned place preference (CPP)

A pre-test bias toward the "bar" side was again observed in WT mice (data not shown; bar pref = 140.3 sec; versus equal preference, t= 4.617, df = 9, P = 0.0013) and although KO mice also preferred the bar side (bar pref = 64.9 sec) it was not significantly different from equal preference (t =1.48, df = 6, P = 0.19). Preference for the morphine-paired "grid" environment was highly dependent on test phase (**Fig 3**, F[1, 35] = 82.08, P < 0.0001), such that all mice exhibited a strong preference for the grid side at the post-test (versus pre-test, P <0.0001). There was no effect of genotype (F[1, 35] = 0.0002, P = 0.99) although genotype x test-day interaction approached significance (F[1,35] = 4.038, P = 0.064). Despite the evidence for an interaction, both WT and TLR4 KO exhibited stronger preferences for the drug-paired side in the post- versus pre-test (WT: P < 0.0001, KO: P = 0.002).

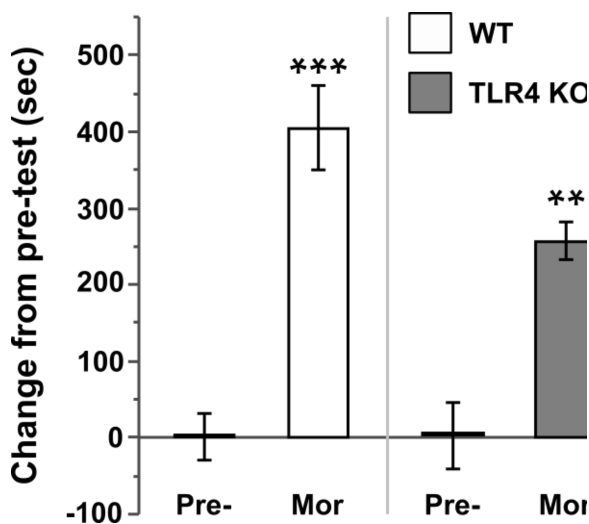

Figure 3: Conditioned place preference for morphine in WT and TLR4 KO mice. Place preference was measured as change in preference for morphine-paired environment normalized to averaged pre-test preference scores. Mice from both genetic backgrounds exhibited a significant increase in preference for the morphine-paired environment following conditioning. Bars depict mean ± SEM. N's: WT = 10, KO = 8. * P < 0.05, ** P < 0.01, *** P < 0.001 versus pre-test preferences.

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Naloxone-precipitated withdrawal jumping:

Naloxone-precipitated jumping was significantly higher in mice receiving morphine prior to naloxone injection versus saline (**Fig 4A**, $F[1,33] = 97.4$, $P < 0.0001$). Both WT and TLR4 KO mice exhibited significantly more jumps following naloxone-precipitated morphine withdrawal versus morphine alone ($P<0.0001$). There was no main effect ($F[1,33] = 1.0$, $P = 0.35$) or interaction (Txt x Genotype, $F[1,33] = 1.13$, $P = 0.29$) for genotype.

Spontaneous withdrawal weight loss:

Animal weights were measured before morning injection and at the end of CPA conditioning to assess the weight loss effects of morphine withdrawal. As expected, weight loss over this period was greater on days when mice received a morphine injection (**Fig 4B-C**, $F[1,101] = 6.75$, $P = 0.0125$) and injection day only affected weight loss in morphine-treated mice (treatment x injection interaction, $F[1, 101] = 12.31$, $P = 0.001$; morph-treated $P < 0.0001$; saline-control $P = 0.939$). Both WT and TL4KO mice lost more weight during morphine days versus saline days (WT: $P = 0.0003$, KO: $P = 0.001$). This interaction was not influenced by genotype ($F[1, 101] = 0.27$, $P = 0.60$) nor was there a main effect of genotype on weight loss ($F[1,101] = 0.0005$, $P = 0.98$). The lack of a genotype-specific effect was further confirmed with planned contrasts between weight losses following morphine vs. saline injections within each genotype. Significantly greater weight loss was observed following morphine in both WT ($F[1, 101] = 15.1$, $P = 0.0003$) and TLR4 KO mice ($F[1,101] = 10.19$, $P = 0.0025$).

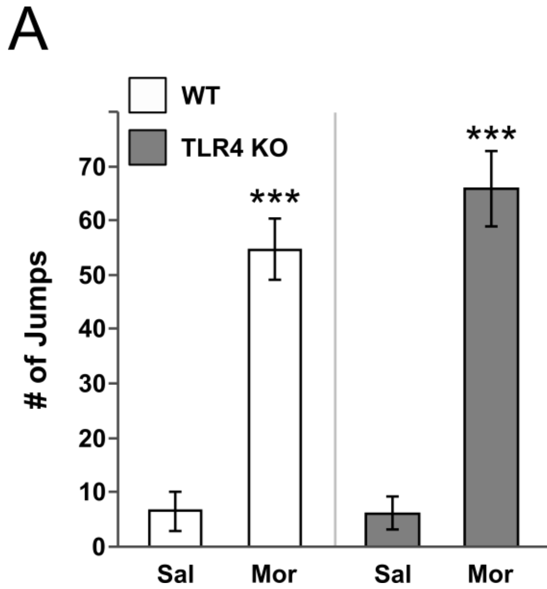
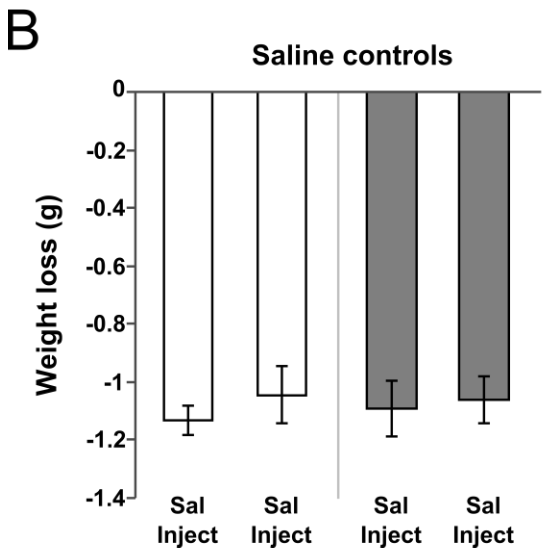
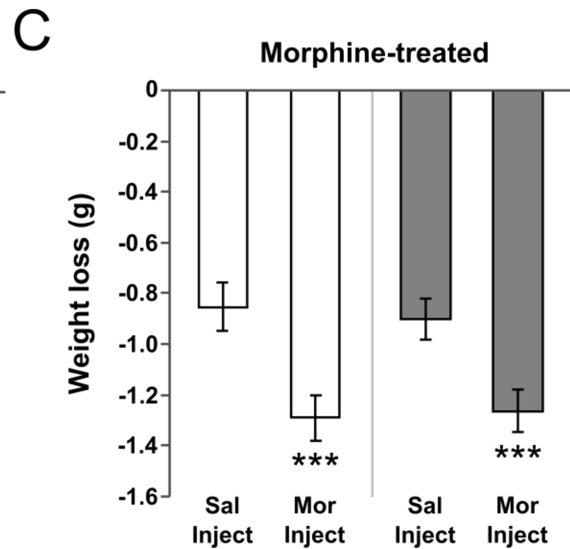


Figure 4: Physical withdrawal signs in WT and TLR4 KO mice. A) WT and TLR4 KO animals display a similar increase in jumping responses following naloxone-precipitated morphine withdrawal relative to saline-treated controls. N's: WT Sal = 8, Mor = 10; KO Sal = 7, Mor = 9. B) – C) Weight loss in grams eight hours post-injection of either saline or morphine. B) No differences in daily weight change were observed between saline-treated WT and TLR4 KO mice. N's: WT = 16, KO = 16. C) Morphine treated WT and TLR4 KO mice exhibited similar withdrawal-induced weight loss. N's: WT = 9, KO = 10. Bars depict mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 versus Saline-treated controls (A) or saline injection days (C)

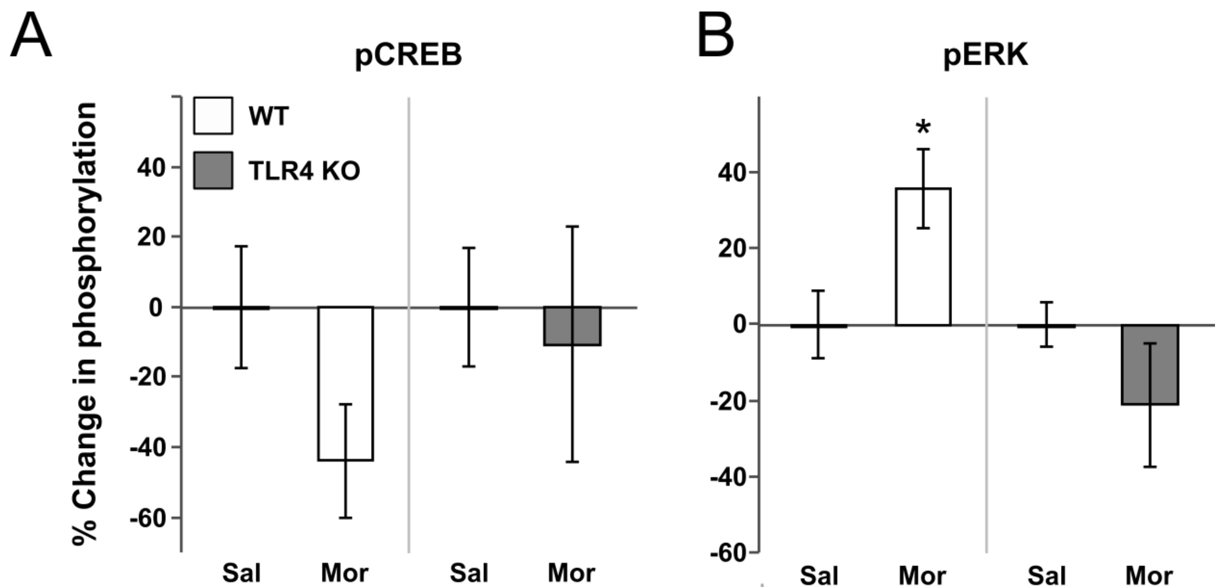




Western blot:

Due to the lack of differences between saline and saline + naloxone groups for both pCREB (KO: t= 0.516, df = 10, P = 0.62; WT: t = 0.97, df = 9, P = 0.359) and pERK (KO: t = 0.856, df = 12, P = 0.411; WT: t = 0.01, df = 8, P = 0.99) expression within NAc

tissue, all saline animals were combined for analysis. Morphine withdrawal resulted in a trend toward reduced pCREB in withdrawn WT mice versus saline controls (**Fig 5A**, t = 1.84, df = 15, P = 0.086) and did not alter pCREB expression in the NAc of KO mice (t = 0.23, df = 17, P = 0.78). Morphine withdrawal resulted in an increase in pERK versus saline control groups in WT NAc tissues (**Fig 5B**, t = 2.62, df = 14, P = 0.022) but not TLR4 KO tissues (t = 1.23, df = 19, P = 0.25).



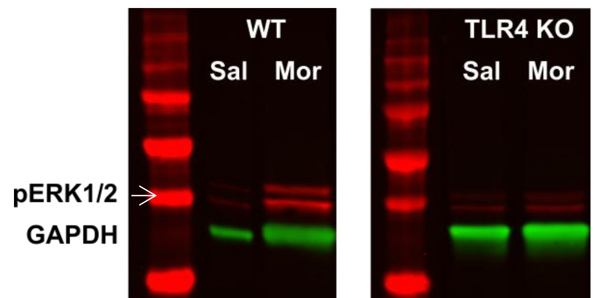


Figure 5: Effects of naloxone-precipitated withdrawal on levels of phosphorylated ERK and CREB in the NAc of WT and TLR4 KO mice. A) Morphine withdrawal did not significantly alter NAc pCREB levels in either WT or TLR4 KO mice. N's: WT Sal = 11, Mor = 6; KO Sal = 12, Mor = 7. B) Naloxone-precipitated morphine withdrawal increased phosphorylation of ERK in the NAc of WT but not TLR4 KO mice relative to saline-treated controls. N's: WT Sal = 10, Mor = 6; KO Sal = 14, Mor = 7. Bars depict mean ± SEM. * P < 0.05 vs. saline-treated controls. Representative blots from each genotype and condition and shown below the graph in B. PERK1/2 bands (MW42/44) are shown in red near the MW 40 marker (arrowhead) while GAPDH (MW 36) is shown in green.

Immunohistochemistry:

Due to the lack of difference in pERK staining in the core versus shell of the accumbens (Regions shown in **Fig 6A**, WT: $P = 0.62$, KO: $P = 0.92$), these areas were combined in the analysis of pERK+ cell count density. Density of pERK+ cells varied by region in WT ($F[1, 23] = 4.87$, $P = 0.044$) but not TLR4 KO ($F[1,23] = 0.03$, $P = 0.86$) mice. The region effect in WT mice was dependent on treatment (Region x treatment interaction, $F[1,23] = 5.95$, $P = 0.29$) such that there was a significantly higher density of pERK+ cells in the lateral septum of morphine withdrawn mice versus saline controls (**Fig 6B**; $P = 0.013$) that was not observed in the accumbens ($P = 0.49$). No effect of treatment ($F[1,23] = 0.57$, $P = 0.47$) or region x treatment interaction ($F[1,23] = 1.15$, $P = 0.3$) was observed in TLR4 KO mice.

In contrast to pERK, Iba1 staining varied significantly between the core and shell regions (WT: $P < 0.0001$, KO: $P < 0.0001$) with higher density of microglia in the core. In order to account for this difference, core and shell regions were considered separately in a 3x2 ANOVA for region and treatment performed for WT and TLR4 KO mice. As expected, there was a significant effect of region for both WT (data not shown, $F[2,20] = 166.75$, $P < 0.0001$) and KO ($F[2, 21] = 134.54$, $P < 0.0001$) mice, in which microglia density was significantly higher in the core versus shell and lateral septum in both genotypes ($P < 0.001$ for all comparisons). Microglia density was greater in NAc shell versus lateral septum in TLR4 KO mice ($P = 0.004$) while these values were equivalent in WT mice ($P = 0.41$). There was no effect of treatment for mice of either genotype (WT: $F[1,23] = 0.032$, $P = 0.86$; KO: $F[1,23] = 0.103$, $P = 0.76$). Given the lack of treatment effects, we further investigated whether microglia density combined across treatments varied by genotype. A 3x2 (region x genotype) ANOVA revealed a significant region effect (

$F[2,43] = 328.18$, $P < 0.0001$) and a genotype x region interaction ($F[2,43] = 12.62$, $P = 0.0001$) revealed that microglia density in WT mice was significantly higher in the NAc core (**Fig 7A-C**, $P < 0.0001$) but lower in the NAc shell versus KO mice, although this comparison failed to reach significance ($P = 0.083$). No differences in Iba1 density were observed in the lateral septum ($P = 0.91$).

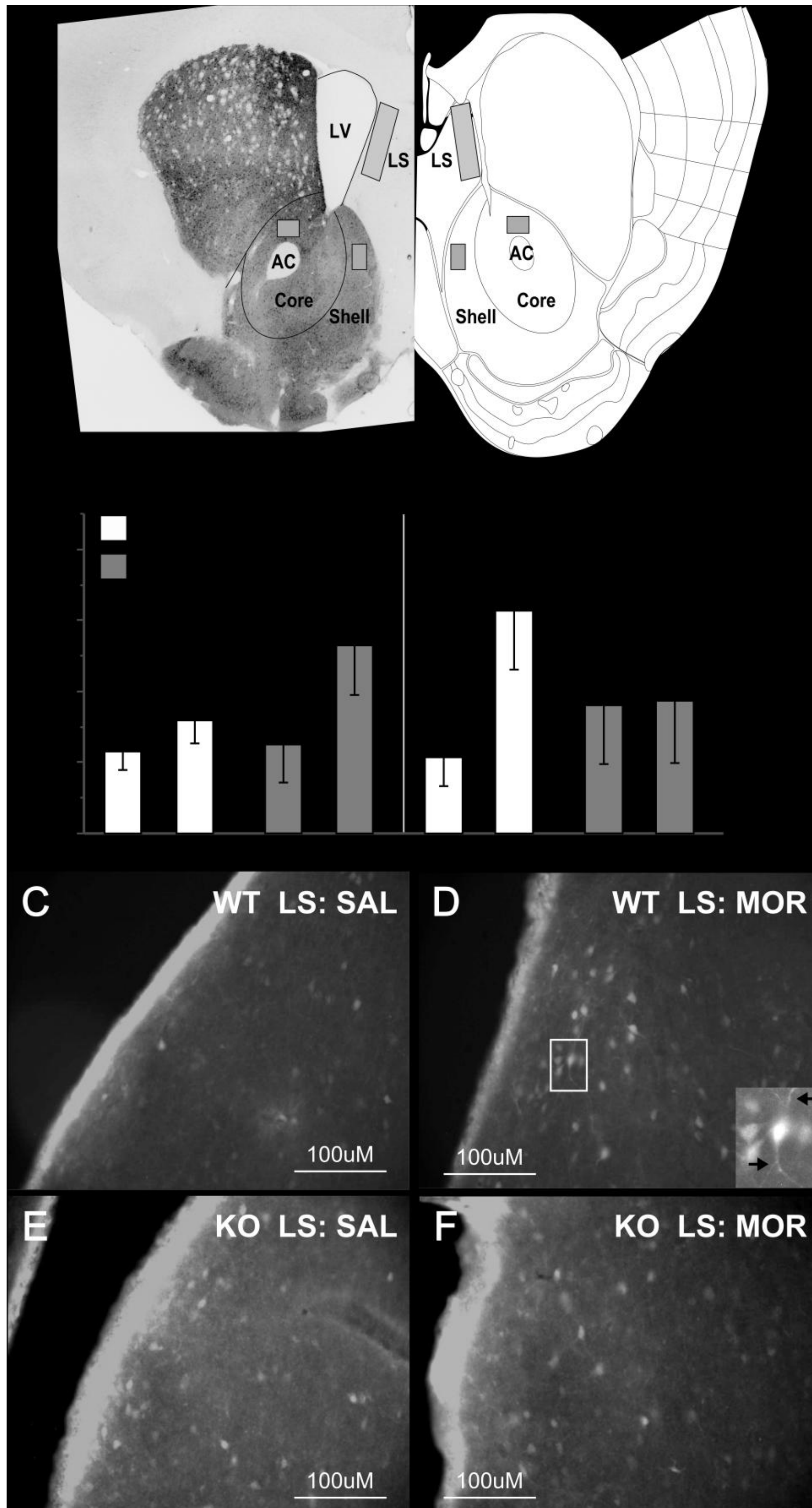


Figure 6: Effects of morphine withdrawal on pERK expression in the NAc and lateral septum (LS) of WT and TLR4 KO mice. A) Location of imaging sites (grey boxes) for the NAc shell/core and LS relative to dopaminergic DARP32+ neurons (left panel) and brain areas defined in an atlas (right panel). B) pERK+ cell densities averaged across the NAc shell and core (left panel) and LS (right panel). Density of pERK+ cells was higher in the LS of morphine-

treated vs. saline-control WT mice. No effect of morphine was found for TLR4 KO mice or for either genotype in the NAc. N's: 4 per each genotype x treatment. Bars depict mean ± SEM. * $P<0.05$. C-F) Representative images of pERK staining in the LS of WT mice (C-D) and TLR4 KO mice (E-F). Inset in D shows magnified image of pERK+ cells in boxed area. Many pERK+ cells appear to have neuron-like projections (arrows, D inset). Abbreviations: AC = anterior commissure, LV = lateral ventricle.

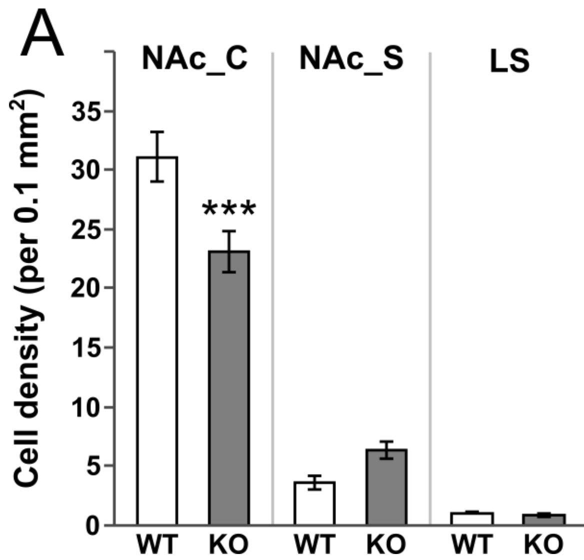
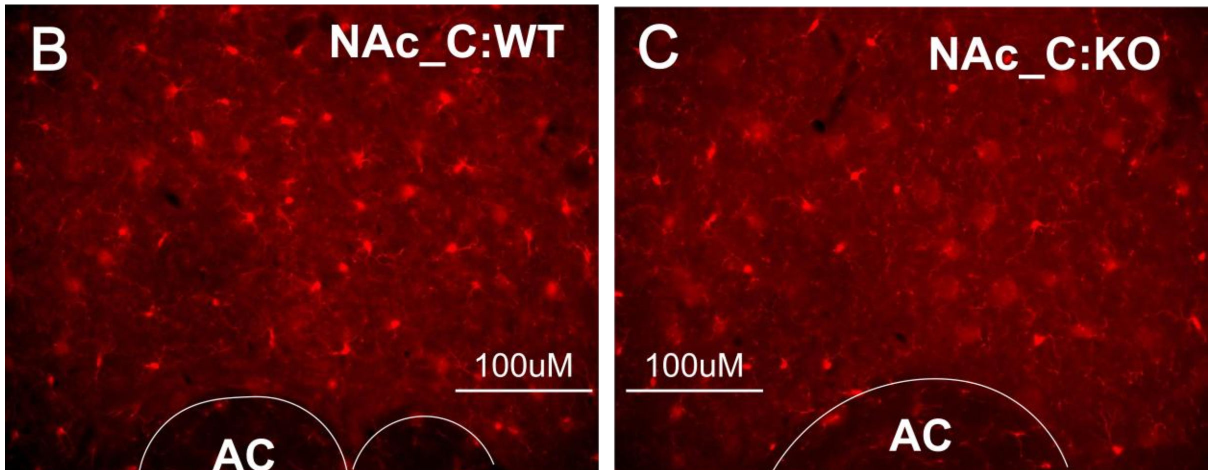


Figure 7: Microglia density in the NAc and LS of WT and TLR4 KO mice. A) Density of Iba1+ microglia within each region. Cell densities were averaged across morphine-treated and saline-controls for each genotype. Significantly fewer microglia were found in the NAc core of TLR4 KO mice relative to WT mice. No differences in microglia density between genotypes were observed for the NAc shell or LS. N's: 4 per each genotype x treatment. Bars depict mean ± SEM. *** $P<0.001$. B-C) Representative images of Iba1 staining in the NAc core of B) WT mice and C) TLR4 KO mice. Abbreviations: AC = anterior commissure, NAc_C = NAc core. NAcS = shell.



Culture:

Phospho-ERK intensity was expressed as percent change from pERK fluorescence of microglia treated with neurobasal media from each respective replicate (**Fig 8A**). Relative to neural growth media (NG), pERK response were lower in microglia given standard supplemented DMEM (GM, -24.6%). As expected, treatment with LPS (100nM) enhanced pERK (146.9%). Neural growth media conditioned with neurons for 24 hours also resulted in an elevated microglial pERK signal (Control = 95.4%, 1uM withdrawal-conditioned = 134.5%). A direct comparison between these groups revealed significantly increased pERK in the microglia exposed to withdrawal-conditioned media (Fig 7B, $t = 2.49$, $df = 14$, $P = 0.03$).

Together these experiments demonstrate that deletion of TLR4 alters the expression of affective behaviors observed under conditions of acute opioid withdrawal. Furthermore, opioid withdrawal was associated with an increased in ERK phosphorylation within the NAc which was not observed in TLR4 KO mice. Additional quantification of pERK-expressing cells within NAc did not support a significant increase in the accumbens core or shell following opioid withdrawal in WT mice, although an increase was observed in the lateral septum. Consistent with the changes in ERK-phosphorylation observed in the Western blots, this increase was only found in WT mice. Finally, media conditioned in striatal-cortical neuron cultures undergoing morphine withdrawal potentiated activation of primary microglia measured as an increased pERK intensity. Overall, these data

indicate that TLR4 contributes to the affective symptoms of withdrawal from morphine and this role may involve downstream effects on ERK phosphorylation with the NAc or neighboring circuitry. Furthermore, these results of the culture experiments indicate that the enhanced glial activity during opioid withdrawal may be in part driven by a soluble pro-inflammatory factor released from neurons.

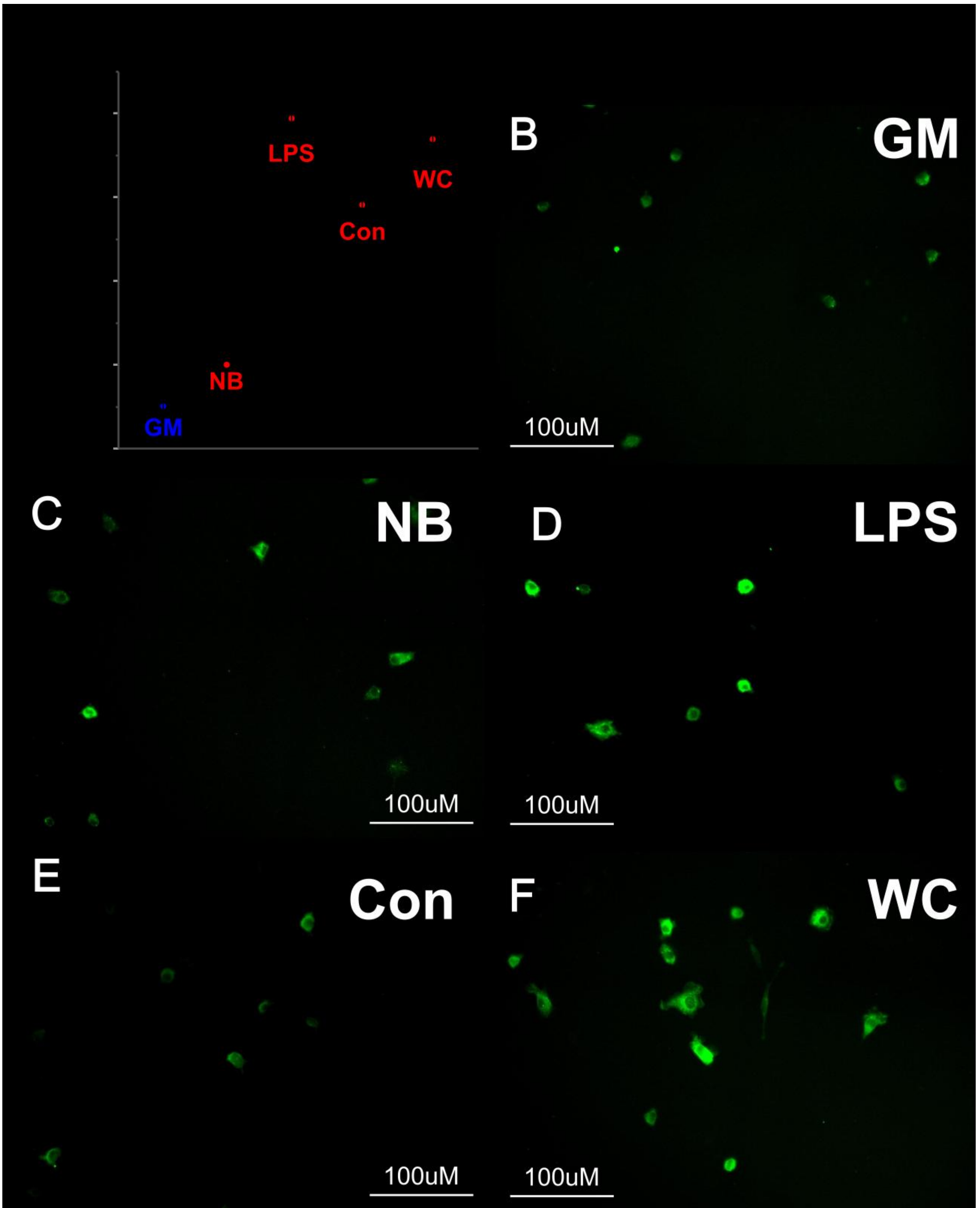

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Figure 8: Effects of withdrawal-conditioned media on microglia ERK phosphorylation. Primary microglia cultures were fixed 30 min after a media transfer and analyzed for ERK phosphorylation A) Percent change in intensity of pERK staining normalized to microglia treated with fresh neural growth media (NG). N's: GM = 4, NG = 5, LPS = 2, Con = 9, WC = 7 coverslips. Bars depict mean ± SEM. * $P<0.05$. Remaining treatment conditions are fresh glia media (GM), neural growth media with lipopolysaccharide (LPS), neural growth media conditioned overnight in control neuron cultures (Con), or withdrawal-conditioned media from neuron cultures withdrawing from 6 days of 1uM morphine treatment (WC). B-F) Representative images of pERK staining in microglia from the various treatment conditions.

Chapter 3: Discussion and Conclusions: TLR4

Affective withdrawal in TLR4 KO mice

Across all three behavioral measures of anxiety and anhedonia/dysphoria there was a significant effect of spontaneous withdrawal in the morphine-treated WT mice relative to saline controls. Furthermore, the results of the CPA and WPS testing were consistent with expected outcome of increased dysphoria and anxiety-like behavior during morphine withdrawal based on previous studies (Contarino and Papaleo 2005; Harris and Gewirtz 2003). By contrast, morphine withdrawal increased time spent in the "light" compartment of the Light/Dark test, indicating an anxiolytic effect or reduction of anxiety in WT mice.

Although these results appear counterintuitive, a similar finding was previously reported in morphine withdrawn mice assessed for anxiety-like behavior in an elevated plus maze. Across two different experiments, morphine-withdrawn mice, but not rats, spent more time in the open arms of an EPM (Hofford et al. 2009; Buckman et al. 2009). Similar to the light compartment of the light/dark box, the open arms are assumed be anxiety-provoking in mice (Belzung and Griebel 2001) such that increased time spent in the open arms would indicate an anxiolytic effect of withdrawal. As suggested by Buckman et al. (2009), the increased time spent in the open arms may actually reflect escape behavior or increased risk-taking rather than reduced anxiety.

Regardless of whether the results of the light/dark test are consistent with increased anxiety in morphine-treated animals, the lack of a withdrawal effect in morphine treated

TLR4 KO mice is still consistent with results of CPA and WPS tasks. Across all three measures, knockout of TLR4 blunted the change in affective behavior that normally accompanies opioid withdrawal. As such, these findings support the prediction that TLR4 is necessary for the expression of affective withdrawal symptoms in mice.

Morphine reward and somatic withdrawal

Despite the blunting of affective responses to opioid withdrawal, TLR4 KO animals exhibited normal preference for the environment paired with morphine in the CPP test. In addition to suggesting that the rewarding effects of morphine are intact in TLR4 KO animals, this finding also rules out the possibility that the lack of CPA in the KO animals is due to impaired Pavlovian learning. Furthermore, TLR4 KO animals displayed normal somatic withdrawal signs including increased weight loss and naloxone-precipitated jumping behavior that were equivalent to WT responses.

The findings of normal opioid CPP contradict the results of a previous study that found reduced preference for an environment paired with oxycondone in TLR4 KO mice (Hutchinson et al. 2012). However, these studies differed not only on the opioid used (oxycondone vs. morphine) but also mouse strain (BALB vs. C57) and morphine treatment prior to conditioning (no pretreatment vs. 6 days of morphine injections). It is worth noting that in the present study morphine preferences appeared to be lower in the KO animals. As such, under different testing circumstances such as those used by Hutchinson et al. (2012) this trend could become a significant genotype effect.

Previous studies have produced mixed results regarding the role of TLR4 in somatic withdrawal. As stated in an earlier chapter, there is abundant evidence that microglia

activity generally is important in somatic opioid withdrawal (Dafny and Pellis 1986; Bland et al. 2009; Hutchinson et al. 2008). Likewise, initial evidence indicated a role of TLR4 based on the ability of theTLR4 antagonist +naloxone to reduced somatic withdrawal signs in rats (Hutchinson et al. 2010). However, consistent with the results of the present study, naloxone-precipitated jumping behavior was reported to be normal in TLR4 KO mice (Liu et al. 2011; Mattioli et al. 2014). Considering the relative importance of affective versus somatic withdrawal symptoms in the maintenance of addiction, the possibility that treatments aimed at disrupting TLR4 signaling may specifically resolve affective symptoms without altering other opioid effects would make this receptor especially attractive as a therapeutic target.

ERK and CREB phosphorylation

Morphine withdrawal is associated with a number of different molecular changes within the NAc including alterations in the phosphorylation states of the transcription factor CREB (Chartoff 2006) and MAPK ERK1/2 (Edwards et al. 2009). Here we report that CREB phosphorylation was unaffected by morphine withdrawal in both WT and KO animals. Although this finding is inconsistent with a previous study in rats which reported increased phospho-CREB during naloxone precipitated withdrawal (Chartoff 2006), other studies have found a lack of effect or decreased pCREB during withdrawal (Edwards et al. 2009; McDaid et al. 2005). Timing appears to be of particular importance, as increased pCREB was observed 30 minutes after naloxone precipitated withdrawal (Chartoff 2006) while pCREB is unchanged at 12 and 24 hour of spontaneous withdrawal (Edwards et al. 2009) and reduced at 3 and 14 days of abstinence (McDaid et al. 2005). Thus, perhaps due to the relatively low morphine dose and longer time post- naloxone (1

hour versus 30 minutes) pCREB had already reached baseline levels at the time of dissection. In such a case, tissue collection at a shorter delay may have captured a pCREB response and allowed for a better evaluation of the effects of TLR4 deletion on CREB activation post-withdrawal.

Increased activity of the MEK-ERK pathway following naloxone-precipitated withdrawal have been previously observed within the NAc of mice (Li et al. 2010) and rats (Edwards et al. 2009; Asensio et al. 2006). Furthermore, inhibitors of MEK-ERK pathway reduced affective withdrawal signs when infused into the amygdala (Hofford et al. 2009) and physical withdrawal signs when injected into the spinal cord (Cao et al. 2005, 2006). Thus, the increase in NAc ERK phosphorylation observed in the present study may contribute to affective symptoms of morphine withdrawal. In this case, the lack of increase in NAc pERK in TLR4 KO mice may account for blunted affective withdrawal symptoms.

Although an increase in phospho-ERK was detected in NAc tissue following morphine withdrawal, there was not a corresponding change in the number of cells immunopositive for pERK in either the NAc shell or core of withdrawn WT mice. However, pERK+ cells were elevated medial to the NAc within the lateral septum (LS). This discrepancy may be due to the different behavioral experience and prior morphine exposure of the mice used for each experiment. Tissue used for pERK Western blots was taken from naïve mice given the same morphine regimen used to induce dependence for behavioral testing (6 days of daily 20mg/kg morphine). By contrast, tissue used for pERK immunohistochemistry was collected from mice that had been previously tested for behavior. Thus, in addition to behavioral experience, these animals received additional

morphine injections on the days leading up to testing. Alternatively, the tissue punches used for Westerns may have included portions of the lateral septum. In this case, the increase in pERK following morphine withdrawal may be driven by changes in the LS rather than NAc.

Despite these possibilities, the validity of these results is supported by similar findings reported in a previous study. The expansion of our regions of interest to include the LS was performed based on previous work by Ciccarilli et al. (2013) which reported increased pERK+ cell density in the LS but not NAc in morphine withdrawn rats. In this study, morphine withdrawal also increased levels of cfos in both the NAc and LS which were reduced by systemic administration of an ERK inhibitor (Ciccarelli et al. 2013). This finding suggests that ERK activity is still contributing to cellular responses to opioid withdrawal within the NAc, despite the lack of change in pERK+ cells within this region. In addition, naloxone administered to the lateral septum of morphine-treated animals elicits withdrawal symptoms, indicating that this region is also involved in the circuitry underlying the behavioral effects of opioid withdrawal (Tremblay and Charton 1981).

Glial activation in TLR4 KO mice

Media conditioned for 24 hours in a cortical-striatal co-cultures following morphine withdrawal increased activating of microglia as determined by an elevation in pERK intensity. To our knowledge, this is the first evidence that morphine withdrawal may trigger the release of soluble proinflammatory molecule(s) within an *in vitro* environment. Although the cortical-striatal co-culture is predominantly made up of neurons, the cultures also contain astrocytes (Penrod et al. 2011). In addition, these neuronal cultures consisted of striatal MSNs as well as cortical neurons which are

required in order for the MSNs to develop normal striatal neuron morphology (Penrod et al. 2011).Thus, additional exploration using this model would be required to determine the exact cellular origin of the molecule(s) responsible for the observed increased activation of microglia.

Likewise, there are many soluble proteins released from neurons that can affect glial activity. As previously stated, TLRs fall into the family of pattern recognition receptors (PRRs) that respond to non-self molecular patterns including pathogen associated molecular patterns (PAMPs) or apoptotic cell/damage associated molecular pattern (AC/DAMPs) (Beg 2002; Correa et al. 2013). Indeed, TLR4 is sensitive to factors released by stressed or dying cells (O'Neill 2008). Repeated morphine exposure can lead to apoptosis or excessive excitation of neurons in the cortex and hippocampus (Emeterio et al. 2006) which could in turn stimulate microglia activity through cellular debris and other soluble factors released during damage. One such factor is fractalkine, a cell-surface neuronal chemokine with a soluble form that is released upon strong excitation (Jones et al. 2010). Fractalkine binds to the CX3CR1 receptor predominantly expressed by microglia and promotes the expression of proinflammatory cytokines through p38 MAPK and NF-kB signaling pathway (Zhuang et al. 2007; Zujovic et al. 2000). Intrathecal administration of a fractalkine neutralizing antibody in the spinal cord of rats treated chronically with morphine can reverse the induction of analgesic tolerance, suggesting that prolonged morphine exposure increases soluble fractalkine *in vivo* and fractalkine signaling in microglia contributes to analgesic tolerance (Johnston et al. 2004).

Another factor released by neurons that may be capable of eliciting an immune response

is a-synuclein. This protein has been previously implicated as a proinflammatory immune factor contributing to Parkinson's. Furthermore, a-synuclein protein is upregulated following spontaneous withdrawal from morphine (Ziolkowska et al. 2005) and activates microglia in a TLR4 specific manner, as measured by phagocytic activity, translocation of NFkB, and increased release of pro-inflammatory cytokines including TNFα (Fellner et al. 2013). Considering that some cytokines such as TNFα can induce cell death in neurons (McGuire et al. 2001), increased cytokine expression in response to microglia activation could lead to a positive feedback loop in which neuronal stress leads to activation of microglia which further exacerbates neuronal damage or stress.

Although morphine withdrawal *in vitro* was associated with a pro-inflammatory response by microglia, naloxone-precipitated withdrawal *in vivo* did not alter the density of microglia in the NAc or LS. Previous studies have shown that chronic morphine (Zhang et al. 2012) and withdrawal (Campbell et al. 2013) can increase the density of Iba1+ microglia within the NAc. However, other studies have reported a lack of change or decreased in Iba1+ density during opioid administration or withdrawal (Hutchinson et al. 2009; Campbell et al. 2013). Relative to Iba1+ density, alternative measures of glia activity including expression of pro-inflammatory cytokines are more consistently elevated in response to opioid treatment or withdrawal (Zhang et al. 2011; Chen et al. 2011; Schwarz et al. 2013; Campbell et al. 2013; Hutchinson et al. 2009). This discrepancy may be due to wide variety of microglia responses to immune challenge beyond increased cell density driven by proliferation or aggregation. Rather, microglia activation may take the form of more subtle changes in cell-surface protein expression or

morphology (Soltys et al. 2005; Bianco et al. 2005; Hanisch and Kettenmann 2007) which were not measured in this study.

Although morphine withdrawal did not alter microglia density in the present experiments, microglia density was greater in saline-treated WT versus TLR4 KO animals within the NAc core. A previous study has reported altered Iba1 intensity in the same TLR4 KO line, however this group found reduced Iba1 intensity in the spinal cords of KO animals (Mattioli et al. 2014). Given this finding as well as the fact that there was no change in Iba1+ cell density within NAc Shell or LS of KO mice, it appears that the effects of TLR4 deletion on microglia are likely region-specific. Although the difference in density with the NAc core is relatively small, even a small number of microglia can have a strong influence over the local environment (Saura 2007). However, additional experiments will be required to determine if the reduced microglia density within the NAc core of KO mice may contribute to the differential behavioral and pERK effects of morphine withdrawal in these animals.

Conclusions

Finally, although TLR4 is thought to be mostly limited to glia (Lehnardt et al. 2003; Hutchinson et al. 2012; Olson and Miller 2004), it can be found in subpopulations of neurons, particularly in the cortex (Tang et al. 2007). Thus, it is possible that effects of the TLR4 knockout are partially driven by non-glia responses. Likewise, TLR4 is found in peripheral immune cells and thus a systemic TLR4 knockout would likely affect peripheral immune activity, although TLR4 KO mice did not exhibit any overt signs of sickness or infection. Also, given the large number of TLRs expressed by microglia and the possibility of common downstream signaling pathways, it is possible that one or more

of the remaining TLRs may perform some compensatory functions and mitigate the overall impact of TLR4 deletion. In order to further explore these questions, it will be necessary to implement a conditional knockout to better control the timing and location of TLR4 deletion.

Overall, these experiments offer promising evidence of a role for TLR4-microglia signaling in the affective symptoms of opioid withdrawal. Furthermore, the altered pERK response to morphine withdrawal in the NAc of TLR4 KO animals may hint at a molecular mechanism underlying the blunted behavioral responses. Finally, *in vitro* evidence of a withdrawal-induced release of a pro-inflammatory factor may indicate an alternative pathway leading to microglia activation during withdrawal.

Chapter 4: Introduction to Conditioned Approach and Animal Models of Reward-Seeking Behavior

Conditioned approach

Conditioned approach toward cues previously paired with reward (Brown and Jenkins 1968; Miller 1961; Pavlov 1932) is an evolutionarily conserved behavior observed in many species including Drosophila, zebrafish, mice, rats, and primates (Cunningham and Zerizef 2014; D'amato and Buckiewicz 1980; Kaun et al. 2011; Ninkovic and Bally-Cuif 2006; Spyraki et al. 1982). This behavioral tendency forms the basis of the conditioned place preference (CPP) task (Beach 1957), the most commonly used measure of positive reinforcement in rodents. In this paradigm, a rewarding stimulus such as a drug, food, or

a conspecific, is paired with a distinct environment or context. The efficacy of the reinforcer can be quantified by measuring the animal's preference for the context paired with the reinforcer compared to an alternative (control) context in a choice test. Over the past few decades, the use of the CPP paradigm has increased steadily, with over a thousand cited uses of the task between 1998 and 2007 (Tzschentke 2007).

CPP- Relevance to human addiction

The popularity of the CPP task – especially in the study of drug addiction – may be attributed to the many advantages of this approach (see Bardo and Bevins 2000; Schechter and Calcagnetti 1993). These include control over the drug dose and timing of delivery, low cost of materials, and the capacity for high throughput. Additionally, CPP is appealing for its ethological relevance because environmental cues play an instrumental part in compulsive human behaviors including habitual drug use. For example, smokers experienced cigarette cravings when exposed to laboratory replicates of environments commonly associated with smoking such as bars or coffee shops, even in the absence of more explicit smoking cues like ashtrays or cigarettes (Conklin 2006). Similarly, alcoholics shown images depicting social or physical environments associated with alcohol consumption exhibited elevated arousal and reduced startle responses, even when images lacked explicit alcohol cues (Nees et al. 2012).

Conditioned approach using discrete Cues

Nevertheless, conditioned behavior may also be controlled by more discrete cues. In the case of drug addiction these include the presence of fellow drug users and access to specific objects including drug paraphernalia. For this reason, objects commonly serve as

drug-related stimuli in human and non-human primate imaging studies. Images or replicates of the drug (e.g., cigarettes, cocaine powder) or drug-related paraphernalia, presented alone or as part of a stimulus complex, reliably increase emotional and physiological responses in drug users and induce craving for a variety of drugs of abuse including nicotine (Franklin et al. 2009; Wilson et al. 2012; Engelmann et al. 2011), marijuana (Filbey et al. 2009), cocaine (Grant et al. 1996), alcohol (Myrick et al. 2008), and opioids (Powell et al. 1993).

The few studies that have utilized object cues within a Pavlovian conditioned preference procedure have revealed that animals approach objects previously paired with rewards. Bassareo and Di Chiara (1997) found that rats oriented towards and made contact with an empty box that predicted access to highly palatable food. After the box was repeatedly presented in the absence of food, rats ceased to approach the box, suggesting the approach behavior could be extinguished. A more recent study found that female rats would preferentially explore chambers containing scented objects paired with either amphetamine administration or opportunities for paced mating versus a chamber containing a neutral control object (Guterl et al. 2015). Thus, similarly to environmental cues, objects are both integral to drug use in human addicts and elicit conditioned approach behavior in rodent models of drug-seeking behavior.

Advantages of objects as conditioned cues

The adoption of object cues in studies of positive reinforcement may confer certain methodological advantages. First, rodents experience and discriminate objects using active exploratory behaviors including whisking and sniffing (Diamond et al. 2008; Sullivan et al. 2012) and find interaction with objects rewarding (Peartree et al. 2012).

Vigorous exploration of objects is commonly observed in the novel object recognition (NOR) test, where performance is measured by the duration of investigation toward the more salient, novel object (Antunes and Biala 2012). Guterl et al. measured conditioned object preferences based on time spent in the compartment containing the respective objects (2015), a similar measure as that used for CPP. As such, it remains unclear whether an animal's interaction with drug-paired objects is amenable to measurement during each phase of stimulus exposure (i.e., during conditioning as well as testing), allowing for a detailed analysis of acquisition and expression of conditioned approach. Second, behavioral tests wherein rodents are given simultaneous access to three or more objects are relatively common (Frick and Gresack 2003; Oliveira et al. 2010; Palchykova et al. 2006). Thus object cues may allow for assessment of conditioned responses to three or more stimuli, for example to generate a dose-effect curve, which can be time consuming when using CPP (Bardo and Bevins 2000). Finally, use of objects as conditioned cues allows for the analysis of an additional, and critical, dimension of conditioned reinforcement learning; namely, the context in which conditioning occurs. As well as directly eliciting conditioned responses, contextual cues can act as "occasion-setters" for responses to explicit cues (Bouton and Swartzentruber 1986). For example, a switch in context may produce "renewal" of conditioned responding following extinction (Thewissen et al. 2006). Hence, the presence of objects as conditioned stimuli may allow for further investigation of modulatory influences of contexts on conditioned behavior.

Chapter 5: Conditioned Object Preference: An Alternative Measure of Reward Learning

In a series of experiments (Figure 9) we sought to explore the utility of objects as drug-conditioned stimuli in rats by pairing different objects with cocaine. We first determined

whether rats would exhibit an enduring preference for an object previously experienced following cocaine injection. Next we examined extinction and drug-primed reinstatement of a preference for a cocaine-paired object. This experiment also enabled us to assess whether the strength of a conditioned object preference (COP) was predicted by the animal's engagement with the drug-paired object during conditioning. A third experiment tested the use of three objects simultaneously to assess preference for stimuli paired with different cocaine doses. Finally, we assessed the Pavlovian phenomenon of renewal by extinguishing the rats' preference for the cocaine-paired object in a second environment and then re-testing for COP in the original test chamber. Taken as a whole, these experiments provide strong support for the future incorporation of object cues in tests of appetitive Pavlovian conditioning in rats.

Materials and Methods:

Subjects

Charles River-derived Sprague-Dawley rats (Charles River, Wilmington, VA) were housed in a vivarium on a 12-hr light-dark cycle where they were provided with *ad libitum* food and water. All behavioral tests were conducted during the light phase. Behavioral testing was conducted in adult male rats at P65 or older. All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Conditioned object preference

The conditioned object preference procedure was modified from a novel object recognition (NOR) task used previously (Kennedy et al. 2014). Prior to the start of testing, rats were handled for two days followed by two days of habituation to the test chamber for 10 minutes per day (black ABS plastic, 50 cm x 50 cm x 50 cm). The basic test procedure used for all experiments consisted of a pre-test, conditioning, and a post-test (Figure 9) although test durations and length of conditioning varied by experiment (see experiment-specific sections below). During the pre-test, rats were placed in the test chamber containing either two or three different test objects secured to the floor using fasteners. Following the pre-test, conditioning was conducted by injecting animals with either saline (0.9%) vehicle or cocaine HCl (5 or 20 mg/kg, Research Triangle Institute, Research Triangle Park, NC) and placing them into the test chamber for 25 minutes with two (exp. 1,2,4) or three copies (exp. 3) of the same object. Treatment alternated daily between saline and cocaine and a different object was paired with each treatment. Injections were always delivered intraperitoneally (i.p.) in a 1ml/kg volume. Post-tests were identical to the pre-test and were conducted in drug-free animals 24 hours following the final conditioning day. Both pre- and post-tests, as well as some conditioning sessions (exp. 2B), were video-recorded by a camera positioned directly above the test chamber. All videos were scored for the duration of object investigation (nose < 2 cm from object) by a trained rater blind to group assignment using computer-assisted software (Button box; Behavioral Research Solutions, Madison, WI). Conditioned object preference was analyzed using either the percentage of total investigation directed toward the cocaine-paired object or a comparison of raw investigation duration directed toward the cocaine vs. saline objects. A subset of videos was scored by a second rater to validate object

preference scores. Correlations between independent scoring of object preferences were strong (*Rs* = 0.871 – 0.955).

Chambers were wiped down with 70% ethanol and objects were cleaned with bleach water between testing. Multiple object pairs were used for each experiment but test object pair, as well as conditioning order and drug-paired object location during testing were all counterbalanced across animals. Objects were of similar size but varied in color, material, texture, or shape and included small plastic or rubber dog toys as well as plastic cups and bowls. Across all experiments, each object within a test pair was investigated equally during the pre-test, indicating that no objects were inherently preferred or avoided by the rats (*Ps* > 0.05, data not shown).

Experiment 1:

Rats were conditioned for two days and received one saline and one cocaine (20 mg/kg) conditioning session prior to the post-test 1. Following post-test 1, rats received two additional days of conditioning with one saline and one cocaine session. A second post-test (post-test 2) was conducted 24 hours after the final conditioning day. Pre- and post-tests were 5 minutes in length.

Experiment 2A:

Cocaine-treated animals received six days of conditioning alternating daily between saline and cocaine (20 mg/kg). Conditioning was identical in saline-only groups but animals received daily saline injections (i.p.). After conditioning, animals received a single post-test followed by extinction and cocaine-primed reinstatement. Extinction was

performed with daily, 10 minute tests over six days in which both objects were presented without additional cocaine or saline administration. Cocaine-primed reinstatement was assessed 24 hours after the final extinction day in 10-minute object preference tests conducted over two days. On the first day animals were re-tested for object preference following a saline injection (i.p.). On the second day, all animals, including saline-only groups, received a cocaine prime (10mg/kg, i.p.) immediately prior to testing. All tests including pre- and post-tests were 10 minutes in length.

Experiment 2B:

Saline and cocaine conditioning sessions for the animals used in Experiment 2A were video-recorded and rated for object investigation. Object investigation during saline or cocaine conditioning for each animal was compared to subsequent preference for the cocaine-paired object in the post-test using a linear regression.

Experiment 3:

Three different objects were used for testing and conditioning. Test objects were placed in three of the four corners of the test chamber with consistent object locations across pre- and post-tests. Animals received nine days of conditioning which alternated daily between saline, 5mg/kg, and 20 mg/kg cocaine, with a different object paired with each treatment. Treatments were always given in this order but the treatment received on the first conditioning day was counterbalanced across animals. During conditioning, three identical copies of the designated object were placed in the test chamber. Pre- and post-tests were 10 minutes in length.

Experiment 4:

Rats received a pre-test, conditioning, and post-test, identical to those described in experiment 2. These tests were conducted in the same chamber used in experiments 1-3 (environment A). Animals were then given a second COP post-test in a new chamber (environment B). Environment B was a chamber the same size as the home cage with opaque plastic walls. Rats were tested in these chambers without bedding and objects were secured on the cage floor at opposite sides of the chamber. Environments A and B were thoroughly cleaned with 70% ethanol between tests. After the post-test in environment B, rats received additional tests without cocaine or saline administration to facilitate extinction similar to the extinction performed in experiment 2A. After preference for the cocaine-paired object had returned to baseline values following extinction, rats were given a final post-test in the original environment A. All tests in both environments were 5 minutes in length.

Statistical analysis:

Analysis of object preferences, such as in the NOR task, can be performed using multiple different approaches (Antunes and Biala 2012). In order to assess conditioned object preference, the present study adapted two commonly used measures, percent of total investigation time spent with the cocaine-paired object and raw investigation time directed toward the cocaine- and saline-paired objects. Percent preference for the cocaine-paired object and duration of investigation toward each object were compared between groups and across test phases using mixed-effects analysis of variance (ANOVA) to assess within- and between-subjects effects. Significant effects were further explored using Tukey's HSD to determine significant comparisons. Due to the large number of comparisons possible with the addition of a saline-only control group in

experiment 2A, Bonferroni-corrected post hoc tests were used instead to explore the change in object preferences across testing within each treatment group (Bird 1975). Planned contrasts were used to compare investigation times between cocaine- and saline-paired objects at each test phase in experiments 2 and 4. Linear regressions were assessed using Pearson's correlation coefficient. Uncorrected significance levels (α) were set at $P < 0.05$.

Results

Experiment 1

Experiment 1 (**Figure 9**) was an initial test to determine whether rats would preferentially investigate an object paired with cocaine and whether the strength of this preference would vary as a function of the degree of training. To address this question, animals were conditioned for two days and received one saline and one cocaine (20 mg/kg) conditioning session prior to post-test 1. Following post-test 1, rats received two additional days of conditioning with one saline and one cocaine session. A second post-test (post-test 2) was conducted 24 hours after the final conditioning day.

Object preference differed based on the amount of conditioning ($F_{(2,39)} = 5.74$, $P = 0.009$; $\alpha = 0.05$) with increased preference for the cocaine-paired object in the post-tests compared to the pre-test (**Figure 10A**). Tukey's HSD post hoc tests revealed that this effect was not significant for post-test 1 ($P = 0.062$) but a significant increase in preference was found for post-test 2 ($P = 0.008$). Although preference scores at post-test 1 did not differ from the pre-test, they also did not differ from post-test 2 ($P = 0.624$) indicating intermediate preference scores at post-test 1 and thereby supporting the

prediction of a stronger preference for the cocaine-paired object following an additional round of conditioning.

Rats explored the cocaine-paired objects for longer than saline-paired objects (**Figure 10B**, effect of object-pairing, $F_{(1,82)} = 11.368$, $P = 0.005$) and this effect varied according to the amount of conditioning (object-pairing x conditioning interaction, $F_{(2,81)} = 5.835$, $P = 0.0075$). Tukey's HSD posthoc tests showed that investigation of the saline-paired object was consistent across testing (vs. pre-test: post-test 1, $P = 0.39$; post-test 2, $P = 0.60$) whereas investigation of the cocaine-paired object was unchanged at post-test 1 ($P = 0.34$) but increased significantly by post-test 2 ($P = 0.002$).

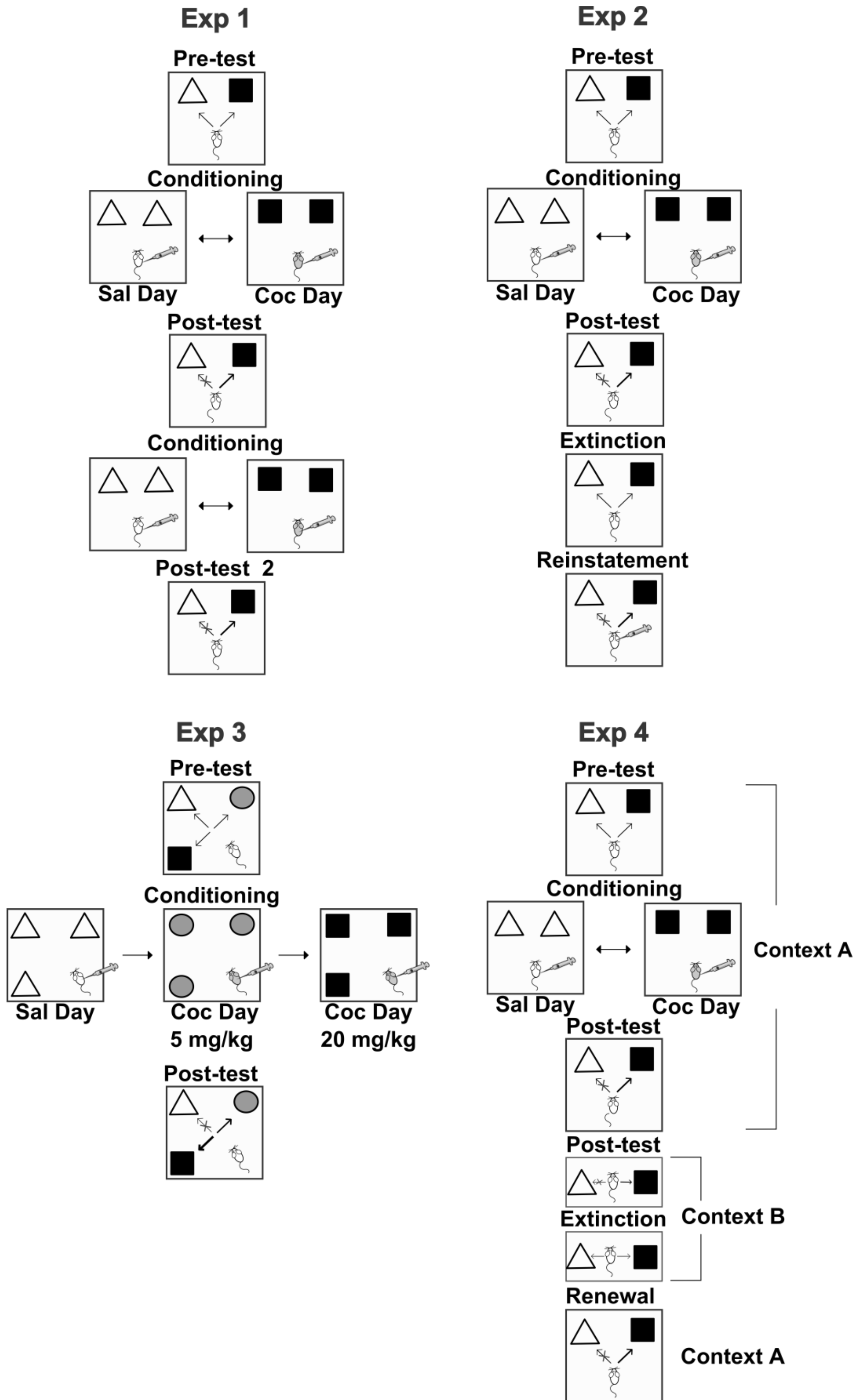


Figure 9: Testing schematic for experiments 1-4. Distinct test objects are displayed using a unique color and shape. Days in which animals receive injections are indicated by a syringe.

Consistent with prefere

nce scores, no difference was found between investigation of the cocaine-paired object between post-test 1 and 2 ($P = 0.28$). Additional analysis using planned contrasts between time spent exploring the saline versus cocaine-paired object revealed that rats spent approximately equal time exploring the cocaine- and saline-paired objects during the pre-test ($F[1,82] = 0.04$, $P = 0.83$) but investigated the cocaine-paired object significantly more during post-test 1 ($F[1,82] = 7.86$, $P = 0.008$) and post-test 2 ($F[1,82] = 17.43$, $P < 0.0002$). Finally, investigation of the cocaine-paired object, but not the saline-paired object, exhibited a linear relationship with the amount of conditioning (Cocaine: rsq = 0.15, $P = 0.01$; Saline: rsq = 0.06, $P = 0.098$), indicating increased investigation of the cocaine-paired objects with additional conditioning.

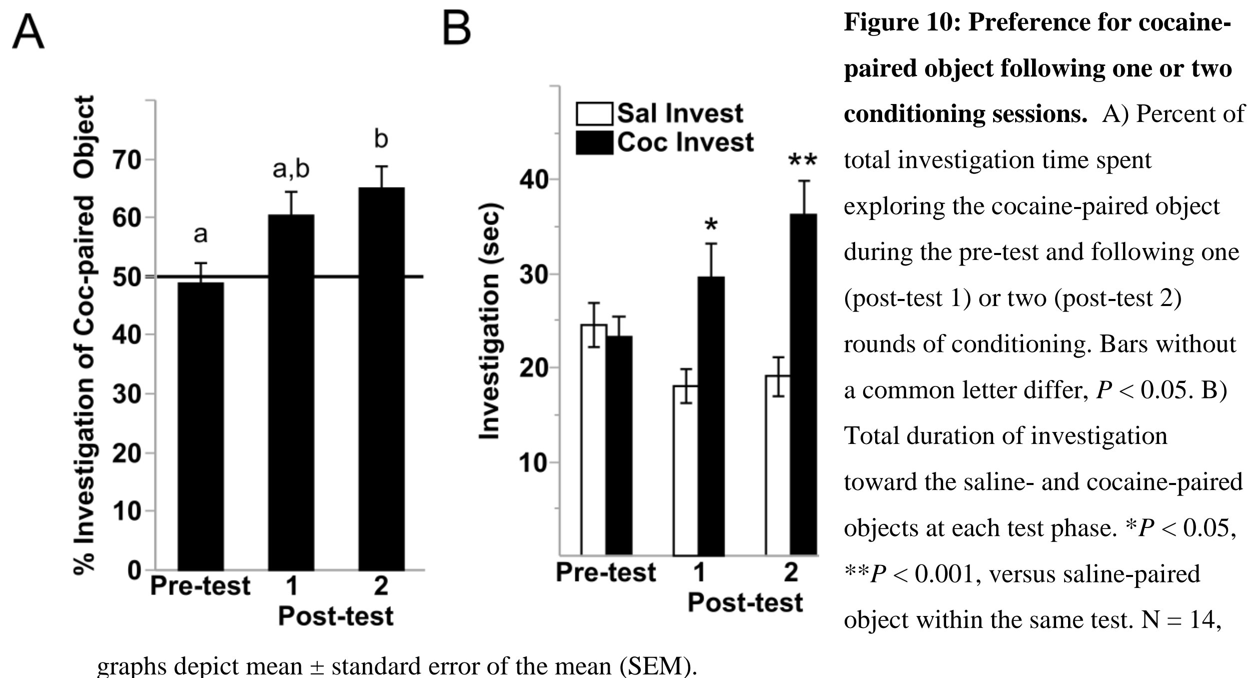


Figure 10: Preference for cocaine-paired object following one or two conditioning sessions. A) Percent of total investigation time spent exploring the cocaine-paired object during the pre-test and following one (post-test 1) or two (post-test 2) rounds of conditioning. Bars without a common letter differ, $P < 0.05$. B) Total duration of investigation toward the saline- and cocaine-paired objects at each test phase. *$P < 0.05$, **$P < 0.001$, versus saline-paired object within the same test. N = 14, graphs depict mean ± standard error of the mean (SEM).

Experiment 2

Experiment 2 (**Figure 9**) was conducted to assess whether conditioned object preferences show extinction and reinstatement and to further validate the results of experiment 1 using a saline-only control group (experiment 2A). Cocaine-treated animals received six days of conditioning alternating daily between saline and cocaine (20 mg/kg). Conditioning was identical in saline-only groups but animals received daily saline injections (i.p.) of equivalent volume. After conditioning, animals received a single post-test followed by extinction and cocaine-primed reinstatement (10mg/kg, i.p). A second aim of experiment 2 was to evaluate the relationship between object investigation during conditioning and preference for the cocaine-paired object in the post-test (experiment 2b).

Experiment 2A

Consistent with the results of experiment 1, object preference in experiment 2A varied across testing (**Figure 11A,** $F_{(4,87)} = 2.58$, $P = 0.046$). Preference scores also differed between cocaine-treated and saline-only animals (**Figure 11B**, effect of treatment, $F_{(1,90)} = 5.18$, $P = 0.037$) with higher overall preference for the cocaine-paired object in cocaine-treated animals. This effect appeared to be dependent on testing phase although the interaction failed to reach significance (test x treatment interaction, $F_{(4,87)} = 2.25$, $P = 0.073$). Post-hoc comparisons (Bonferroni corrected significance set at $P < 0.0125$) between post-tests and pre-test preference scores within each treatment group revealed different patterns of object preference scores across testing. Cocaine-treated ($P = 0.008$) but not saline-only ($P = 0.82$) animals exhibited significantly higher preferences for the cocaine-paired object at post-test 1 versus pre-test values. Following extinction, preference scores of both groups returned to pre-test values (Cocaine-treated, $P = 0.09$; Saline-only, ($P = 0.46$). During reinstatement testing, saline injections alone failed to

alter object preferences in either treatment group (versus pre-test: cocaine-treated, (P = 0.35); saline-only (P = 0.14). However, the cocaine prime (10mg/kg) delivered before testing increased preference for the cocaine-paired object in cocaine-treated rats (versus pre-test: P = 0.0034) but not saline-only rats (P = 0.68).

Cocaine-treated animals exhibited a greater investigation bias toward the cocaine-paired object relative to saline-only animals (**Figure 11D**, object-pairing x treatment interaction, $F_{(4,177)} = 2.561$, $P = 0.046$). This effect was also confirmed by planned contrasts between saline- and cocaine-paired object investigation times for both treatment groups at each test phase. Investigation of the cocaine- versus saline-paired objects was similar during the pre-test in both treatment groups (**Figure 11C**, Cocaine-treated, F[1,180] = 0.92, P =0.34; saline-only (F[1,180] = 0.0006, P = 0.98). Following conditioning, cocaine-treated (F[1,180] = 15.35, P = 0.0002) but not saline-only rats (F[1,180] = 0.35, P = 0.55) investigated the cocaine-paired object significantly more than the control object. All animals investigated both objects equally following extinction (Cocaine-treated, F[1, 180] = 0.89, P = 0.34; Saline-only, (F[1,180] = 0.06, P = 0.79) and following a saline prime during reinstatement testing (Cocaine-treated, F[1,180] = 0.34, P = 0.56; Saline-only (F[1,180] = 2.42, P = 0.12). Finally, no difference in investigation was found between the cocaine- and saline-paired objects following a cocaine prime in either group (Cocaine-treated, F[1,180] = 1.27, P = 0.26; Saline-only , F[1,180] = 0.104, P = 0.75), despite the reinstatement of preference for the cocaine-paired object observed in cocaine-treated animals (**Figure 11A**).

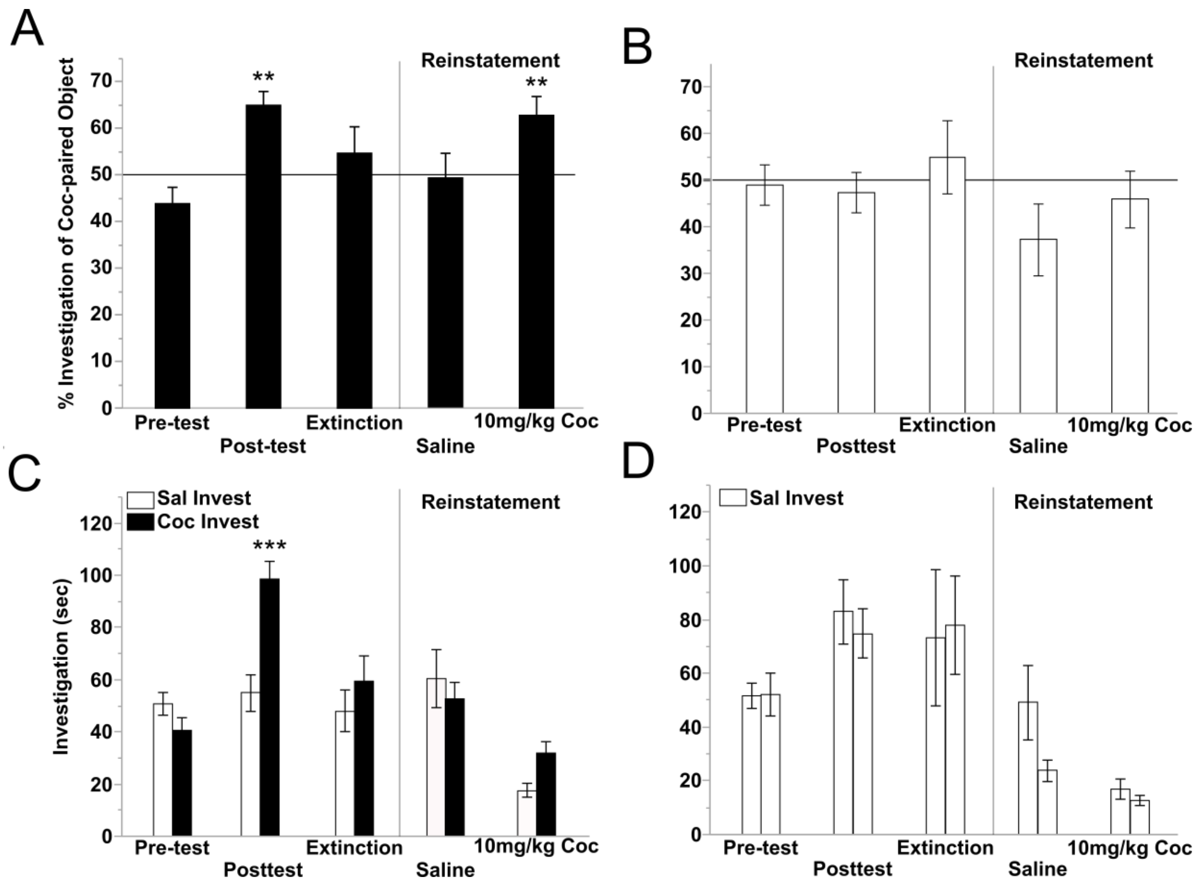


Figure 11: Extinction and cocaine-primed reinstatement of cocaine-paired object preference. Percent of total investigation time spent investigating the A) cocaine-paired object in cocaine-treated animals or B) test object in saline controls. Percentage scores shown at the pre-test, following conditioning (posttest) and extinction, and for saline- or cocaine-primed reinstatement. **$P < 0.01$ versus pre-test percentage. Duration of investigation toward the C) saline/cocaine paired objects for cocaine-treated rats or D) saline/saline paired objects for saline controls across each test phase. *** $P < 0.001$ versus saline-paired object within the same test. N = 12, graphs depict mean ± SEM.

Experiment 2B

Investigation of objects by cocaine-treated animals during conditioning was significantly lower for cocaine versus saline sessions (**Figure 12A**, $F_{(1,70)} = 8.11$, P = 0.016). Object investigation trended toward a decrease across conditioning days ($F_{(2,69)} = 2.94$, $P =$ 0.073) but only for cocaine conditioning sessions (conditioning day x drug interaction,

$F_{(2,69)} = 5.05$, $P = 0.016$). While the duration of object investigation during cocaine conditioning was similar to saline sessions on day 1 (Tukey's HSD, $P = 0.99$), object investigation on cocaine days 2 and 3 was significantly lower than both cocaine day 1 (versus day 2, $P = 0.041$; versus day 3, $P = 0.01$) and the respective saline day on day 3 ($P = 0.02$) with this comparison approaching significance on day 2 ($P = 0.079$) . Comparisons of object investigation during cocaine conditioning sessions with preference for the cocaine-paired object in post-test 1 revealed a strong linear relationship that was dependent on the conditioning day. Whereas object investigation during cocaine conditioning days 2 (**Figure 12B**, $R = 0.728$, $P = 0.011$) and 3 ($R = 0.746$, $P < 0.008$) was positively correlated with preference for the cocaine-paired object at post-test 1, this was not true on cocaine day 1 ($R = 0.49$, $P = 0.12$). Likewise, object investigation during saline conditioning was not correlated with preference for the cocaine-paired object on conditioning days 1-2 (Saline day 1, $R = 0.083$, $P = 0.79$; day 2, $R = 0.158$, $P = 0.64$) and exhibited a trend toward a negative relationship on day 3 ($R = -0.597$, $P = 0.052$).

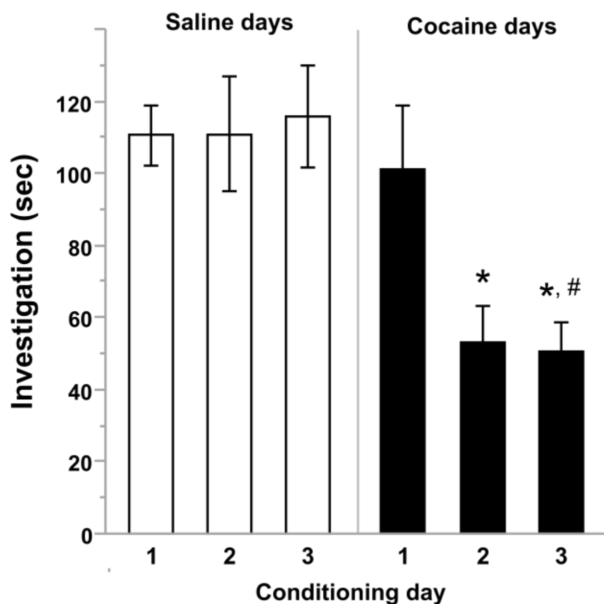
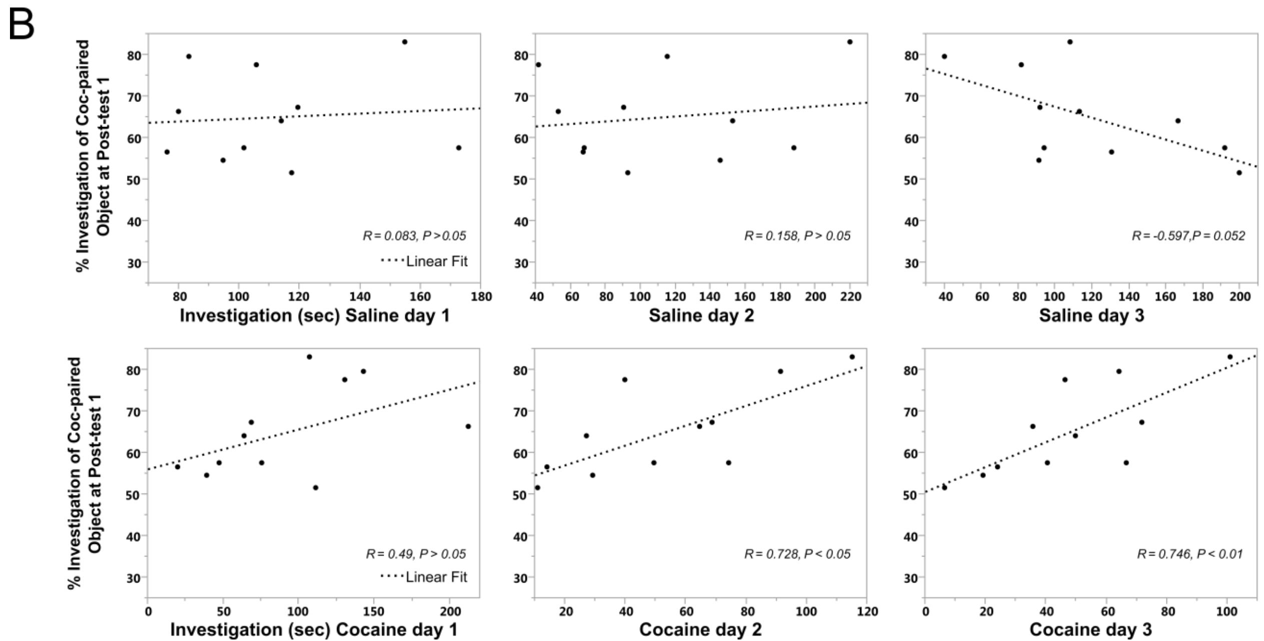


Figure 12: Object exploration during saline and cocaine conditioning in cocaine-treated animals. A) Total duration time spent investigating objects during each of the three saline and cocaine conditioning days. *$P < 0.05$ versus cocaine conditioning day 1, #$P < 0.01$ versus saline conditioning day 3. B) Linear regressions between total duration of object investigation during each saline (top) and cocaine (bottom) conditioning day and cocaine-paired object preference during the subsequent

posttest. Dotted lines indicate best linear fit. N = 12, graphs depict mean ± SEM.



Experiment 3

Experiment 3 (**Figure 9**) was designed to test whether three objects could be used simultaneously within the COP test. This experiment compared preferences for three different objects, two paired with different doses of cocaine (5mg/kg and 20 mg/kg) and one paired with saline. Animals were tested using three different test objects placed in three of the four corners of the test chamber with consistent object locations across pre- and post-tests.

Generally, preference for cocaine-paired objects was higher in the post- versus pre-test (combined 5 and 20mg/kg, $F_{(1,32)}$ =10.6, P = 0.0047). As expected, this effect was

dependent on cocaine dose. Whereas preference for the 20 mg/kg cocaine-paired object increased during the post-test (**Figure 13A**, versus pre-test, $F_{(1,32)} = 5.36$, $P = 0.034$), preference for the 5mg/kg dose remained unchanged across testing [$F_{(1,32)} = 0.122$, $P = 0.73$). Thus, while preference for each object was equivalent in the pre-test (20mg/kg: 32.75%, 5mg/kg: 34.79%, saline: 32.45%), object preference in the post-test was highest for the 20mg/kg cocaine dose (44.13%), followed by the 5mg/kg dose (33.43%), and lowest for the saline-paired object (22.34%).

Object investigations times were dependent on drug-dose [$F_{(2,97)} = 4.77$, $P = 0.015$] but this effect was only observed in the post-test (dose x test interaction, $F_{(2,97)} = 5.28$, $P = 0.01$). In the post-test, object investigation followed a similar pattern as object preference with highest-to-lowest investigation durations: 20mg/kg (70.78 sec), 5mg/kg (51.31 sec), and saline (36.75 sec). Post-test investigation of the 20 mg/kg cocaine-paired object was significantly higher relative to both investigation of the same object during the pre-test (**Figure 13B**, Tukey's HSD, $P < 0.0001$) as well as the saline-paired object at the post-test ($P = 0.001$). The 5mg/kg cocaine-paired object was explored at an intermediate level that was not different from exploration levels of either the saline ($P = 0.48$) or 20mg/kg cocaine-paired objects ($P = 0.157$). Regression analysis of object investigation across increasing cocaine doses revealed a significant linear relationship during the post-test (rsq = 0.16, $P = 0.006$) but not the pre-test (rsq = 0.0003, $P = 0.90$). No differences in object investigation were observed in the pre-test ($Ps > 0.99$).

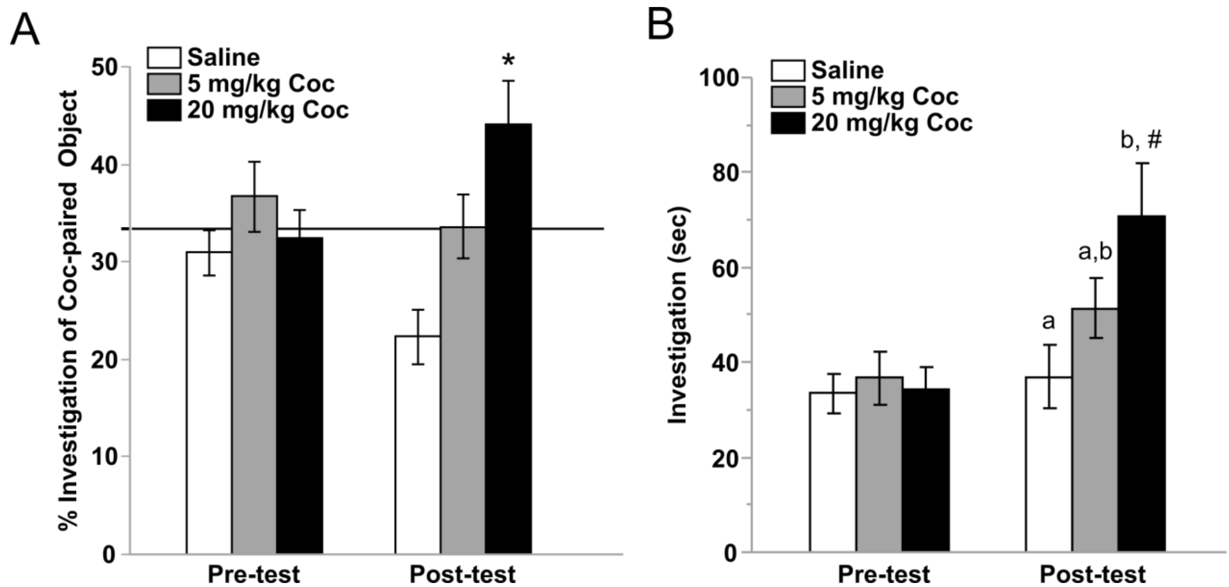


Figure 13: Preference for cocaine-paired objects in three-object preference test. A) Percent of total investigation spent exploring the objects paired with 5mg/kg and 20mg/kg cocaine. *$P < 0.05$ versus pre-test percentage for that dose. B) Duration of investigation toward saline, 5mg/kg, and 20 mg/kg cocaine-paired objects. Posttest durations without a common letter differ. $P < 0.05$, #$P < 0.001$ versus pre-test duration for that object. N = 18, graphs depict mean ± SEM.

Experiment 4

A final experiment (**Figure 9**) was conducted to examine whether context can alter preference for a cocaine-paired object. Rats were tested for cocaine COP in the classic A-B-A design used to assess renewal of drug-seeking behavior (Bouton, 1983; Thewiseen, 2006). Animals were first given a pre-test, conditioning, and post-test, identical to those described in experiment 2. These tests were conducted in the same chamber used in experiments 1-3 (environment A). Animals were then extinguished within a new chamber (environment B), prior to a final post-test in environment A.

Preference for the cocaine-paired object did not differ across test phases (**Figure 14A**, $F_{(4,56)} = 1.52$, $P = 0.21$]. However, object investigation times were affected by object pairing [$F_{(1,119)} = 10.20$, $P = 0.008$] and this effect varied by test phase (**Figure 14B**, object-pairing x test phase interaction, $F_{(4,116)} = 2.92$, $P = 0.032$). Planned contrasts between saline- and cocaine-paired object investigation times at each test revealed equivalent levels of exploration in the pre-test (F[1,119] = 0.009, $P = 0.92$) but increased investigation for the cocaine-paired object in the post-test in environment A (F[1,119] = 13.36, $P < 0.0006$). Significantly higher exploration of the cocaine-paired object was also observed in environment B (F[1, 119] = 7.19, $P = 0.009$) but this effect was lost following extinction (F[1,119] = 0.018, $P = 0.89$). Finally, investigation for the cocaine-paired object was again higher when animals were re-tested in environment A (F[1,119] = 5.19, $P = 0.027$).

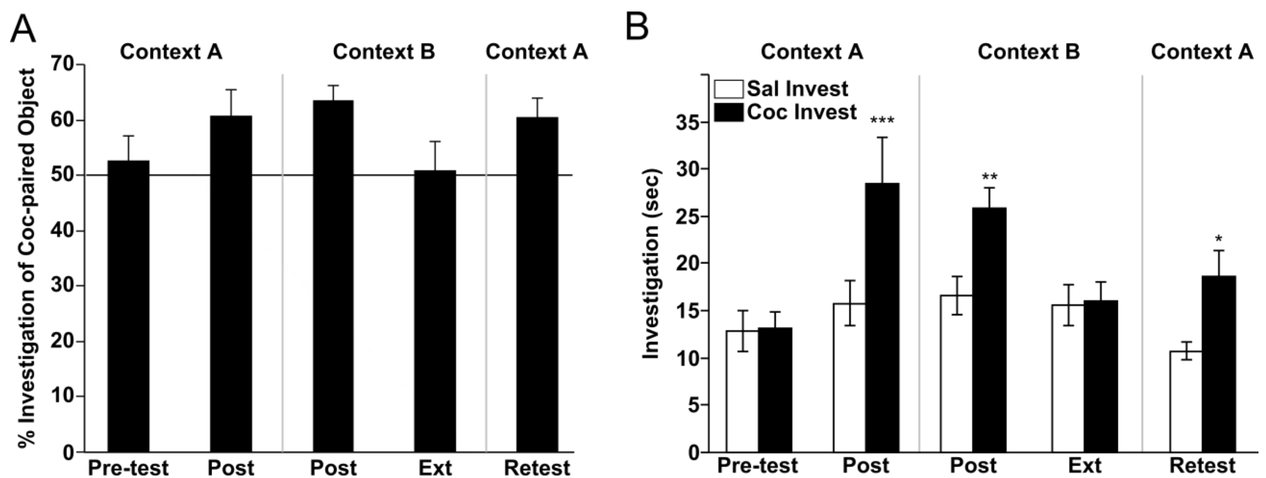


Figure 14: Renewal of cocaine conditioned object preference. Rats received pre- and posttests in one environment (A), and a further posttest in a second, novel environment (B). Following extinction within environment B, animals were returned to environment A for a final posttest (retest). Cocaine-paired object preferences were assessed using A) percentage of total investigation spent investigating the cocaine-paired

object at each test phase and B) duration of investigation toward saline- and cocaine-paired objects. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus saline-paired object within the same test. N = 12, graphs depict mean ± SEM.

Chapter 6: Discussion and Conclusions: Conditioned Object Preference

These experiments demonstrated that rats exhibit a preference for an object or objects that have been paired with cocaine exposure. The animals' preferences were similar in magnitude to those commonly reported toward an environment paired with the same dose of cocaine (Adams et al. 2001; Botreau et al. 2006; Brenhouse and Andersen 2008). Not only were object preferences robust, but they could be acquired to a variety of different objects. This behavioral measure was also sensitive to the dose of cocaine, with preferences for three different objects varying as a function of the cocaine dose associated with each. Finally, the use of objects as conditioned stimuli proved amenable to the demonstration of the Pavlovian phenomena of extinction, reinstatement, and renewal. Taken together, these findings point to the potential utility of COP as an alternative paradigm for assessing reward-related learning and behavior.

Pavlovian learning

The strength of the association between cues after Pavlovian learning is largely determined by the degree of training (i.e. number of stimulus pairings) and the magnitude of reward (Rescorla and Wagner 1972). In the current experiments, we found that the strength of the cocaine-paired object preference varied predictably as a function of both these parameters. Rats exhibited a stronger cocaine preference following two conditioning periods versus one and their preferences were more pronounced when

higher (20mg/kg) versus lower (5mg/kg) doses of cocaine were given. Hence, within the parameters of the current study, COP faithfully reflected the strength of the underlying Pavlovian association between the conditioned object and the signal for reward.

COP as a direct measure of conditioned approach

It is important to note that despite evidence of clear preferences for one object over another, the amount of time allocated to engagement with the objects as a whole still made up a relatively small proportion of the total time spent in the test environment. This feature of COP has at least three implications for the application of this paradigm in future experimental studies. First, it rules out the possibility that preference for a cocaine-paired object simply reflects an aversion to the alternate choice (i.e, the saline-paired object). Animals are free to avoid both objects and thus preference behavior does not reflect a forced choice between the two object cues. This alternative explanation cannot be discounted as easily when it comes to place preference, except in designs that include a third (middle) compartment. The short duration of object exploration relative to the totality of behaviors exhibited also means that the rat's interaction with the objects during the training sessions themselves becomes a potentially valuable source of data. An indication that this measure is meaningful is the fact that the animals' engagement with the cocaine-paired objects over the course of training correlated significantly with the degree of object preference exhibited in the post-test. Again, such an online readout of motivated behavior during training is not available to the experimenter conducting a CPP experiment, in which an animal is necessarily confined to the to-be-paired environment for the entirety of each training session.

A third implication of the small proportion of time rats spend in proximity to the object cues is that object-specific approach behavior can be disambiguated from nonspecific effects, such as reduced or increased locomotor behavior (that would result from sedation or psychomotor sensitization respectively), or increases in competing behaviors (e.g., exploration, rearing, grooming). Nonspecific behavioral effects may reduce overall object exploration but not the relative preference for one object over another. For example, although a 10mg/kg priming injection of cocaine drastically reduced overall levels of object investigation in the test for reinstatement, we were still able to detect a discernable reinstatement of the rats' preference for the cocaine-paired object. These results highlight a potential limitation of the COP test. Treatments or conditions that interfere with object investigation may make it difficult to assess accurate object preferences due to a floor effect. Extremely low investigation values may exaggerate both high and low preferences, leading to greater variability. However, it is precisely the ability of the COP measure to distinguish raw investigation from relative preference that allows for detection of such issues. Overall, therefore, object-directed conditioned approach behavior offers a striking degree of behavioral specificity.

Additional Advantages of COP

A further valuable feature of an object preference task is that learning and retrieval occur within a context that can be systematically manipulated. Expression of object preferences should therefore be subject to "occasion-setting" by contextual cues, as has been demonstrated in many other Pavlovian paradigms (Bouton and Swartzentruber 1986; Bouton 1988). In the present study we determined whether animals exhibited "renewal" of a preference for a cocaine-paired object. Renewal was assessed by returning

animals to the environment in which cocaine was first experienced following extinction within an alternative environment. A preference for the cocaine-paired object, which diminished to pre-test values over the course of extinction in a new context, re-emerged when the rats were placed back in the conditioning context. This suggests that context can serve as an occasion-setter for the expression of object preferences, making COP a useful paradigm for studying the modulatory role of context in functional and dysfunctional reward-related learning and behavior.

Motivation behind COP

Although rats exhibited clear preferences for cocaine-paired objects, the motivation driving this behavior is less clear. It is well established that animals reliably exhibit consummatory-like behaviors, referred to as sign-tracking or autoshaping, toward cues paired with rewards (Brown and Jenkins 1968), even if this behavior results in withholding of the reward (Williams and Williams 1969). This non-teleological form of behavior contrasts with goal-tracking, or behavior directed toward the source of the reward, such as a food hopper (Farwell and Ayres 1979). A body of recent work suggests that rats can be categorized according to whether they predominantly acquire sign- or goal-tracking behavior. "Sign-trackers" are more likely to acquire addiction-like behavior (Flagel et al. 2008), and cues to which sign-tracking behavior is directed (e.g., the response lever in an operant chamber) are more likely to acquire incentive salience, i.e., the ability to serve as reinforcers themselves (for review see Flagel et al. 2009). For this reason, the study of sign- versus goal-tracking can inform our understanding of mechanisms underlying individual differences in vulnerability to addictive behavior (Flagel et al. 2010). If we accept that the animals' interaction with the objects in our

experiments reflected sign-tracking behavior, it is interesting to note that this behavioral pattern was exhibited to some degree by almost all rats. A critical difference between COP and conditioning for food in an operant box is that in the former the opportunity to engage in goal-directed behavior is absent. This may suggest that rats are not destined to act solely as sign- or goal-trackers, but rather to exhibit sign-tracking behavior if there is no proximal source of the reward to which they can direct their responses.

Overall, these findings support COP as a valuable assay of drug-seeking behaviors in rodents capable of providing similar measures of acquisition, extinction, and reinstatement of cocaine preference as CPP. Despite minor discrepancies in level of significance, we found good agreement between object preference and raw investigation scores and both measures supported a robust preference for the cocaine-paired object across multiple tests. Furthermore, conditioned object preference provides opportunities to explore novel questions. As demonstrated in the present study, this task allows for the establishment of dose-response relationships by using multiple object cues and is also well suited for investigation of the effects of environment on conditioned preference. However, the COP is not without limitations. For example, object investigation is not as amenable to automated analysis as the locomotor behavior used to assess CPP. Furthermore, as addressed above, COP may be particularly sensitive to floor effects on object exploration. Given the particular strengths of the COP and CPP tests, these behavioral assays are ideally suited as complementary measures of reward-seeking behavior. Overall, given an inherently straightforward methodology that uses readily available materials, and the flexibility offered in terms of experimental design, COP

promises to become a customizable test for investigating mechanisms of reward processing and associated psychopathologies.

Overall Conclusions

Taken together, these findings help to further our understanding of the biological processes underlying opioid withdrawal and demonstrate the utility and applications of the novel behavioral task, conditioned object preference. In both cases there are potential broader implications and unanswered questions prompted by these findings that require further consideration.

The results of the experiments in Chapter 2 suggest that TLR4 is necessary for expression of the affective symptoms of opioid withdrawal. Given the substantial evidence indicating that microglia TLRs are involved in responses to other drugs of abuse, it would be of interest to determine whether the behavioral and molecular effects of TLR4 deletion are common to withdrawal from all substances, including ethanol or methamphetamines. Likewise, other TLRs such as TLR2 have also been associated with addictive-like behaviors and share common downstream targets as TLR4. It remains to be seen whether microglia signaling during opioid withdrawal recruits multiple TLRs and how they may interact to affect the overall microglia response.

The experiments described in Chapter 5 provided evidence that the conditioned object preference test reliably measures conditioned preference for a rewarding substance. Pilot studies following the same procedure in mice and/or using morphine have also been successful. Although promising, the testing conditions used thus far are still very limited. Additional work is needed to determine under what conditions the procedure can be used. The COP experiments also established the ability of objects to elicit conditioned approach behavior, which opens up the possibility of using other stimuli toward a similar end. Stimuli such as scents, textures, or tastes all have unique advantages and disadvantages within a conditioned preference task that could be utilized depending on the specific testing circumstances and experimental questions.

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