THE EFFECTS OF CENTRAL INTERLEUKIN-1 SIGNALING ON PERIPHERAL IMMUNOMODULATION

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ABSTRACT

LEE WADE HUTSON: The Effects of Central Interleukin-1 Signaling on Peripheral Immunomodulation
(Under the direction of Donald T. Lysle)

Heroin administration suppresses the production of nitric oxide (NO), which is a molecule active in host defense against infection and disease. Previous research in our laboratory has demonstrated that the immunosuppressive effects of heroin can be conditioned by repeatedly pairing heroin administration with a unique environmental context. Re-exposure to the previously drug-paired context can illicit immunosuppressive effects similar to heroin administration alone. In addition, our laboratory has reported that the basolateral amygdala (BLA) and medial nucleus accumbens shell (mNAcS) are critical neural substrates that mediate this conditioned effect. The study presented in Chapter 2 revealed the presence of interleukin-1β (IL-1β) immunoreactivity in the BLA and mNAcS across various time points following re-exposure to a previously drug-paired environment; however, there were no differences in the level of IL-1β expression. Chapter 3 demonstrated that blockade of IL-1 signaling in the BLA, but not CPu or mNAcS, attenuates heroin-conditioned immunosuppression of NO production and inducible nitric oxide synthase (iNOS) mRNA expression in spleen tissue. Chapter 4 found that intra-BLA administration of various doses of IL-1β had no effect on NO production or iNOS mRNA expression following an immune challenge. Taken together, these findings suggest that

IL-1 signaling in the BLA is necessary for the expression of heroin-conditioned immunosuppression of NO and iNOS mRNA. In addition, these findings indicate that exogenous IL-1β administration into the BLA does not alter the peripheral induction of NO in blood plasma or iNOS mRNA expression in spleen tissue following an immune challenge.

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LIST OF ABBREVIATIONS

18S	18S ribosomal RNA
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
ATP	andenosine triphosphate
BLA	basolateral amygdala
C	celsius
$Ca2^{+}$	calcium
CNS	central nervous system
CPP	conditioned place preference
CS	conditioned stimulus
EPSP	excitatory post synaptic potential
GABA	gamma-aminobutyric acid
GPCR	G-protein coupled receptor
i.c.v.	intracerebroventricular
IL	interleukin
IL-1β	interleukin-1 beta
IL-1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IPSP	inhibitory post synaptic potential
LPS	lipopolysaccharide
LTP	long-term potentiation
Min	minute

mNAcS	medial nucleus accumbens shell
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NMDA	N-methyl-D-aspartate
NO	nitric oxide
RNA	ribonucleic acid
rrIL-1β	recombinant rat interleukin-1β
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
US	unconditioned stimulus
vol	volume
VTA	ventral tegmental area

CHAPTER 1

GENERAL INTRODUCTION

A Brief History of Opioids and Opioid Abuse

Heroin is derived from the sap of the opium poppy plant (*Papaver somniferum*), which is a major source of opium and poppy seeds. *Papaver somniferum* is just one of the 250 species of Papaveracea family, but it is the most cultivated of the poppy plants due to its pharmacotherapeutic and euphoric effects. Archaeological evidence suggests opium poppy was being used as long ago as 5000 BCE based on the discovery of Sumerian cuneiform tablets containing the words for opium and Assyrian reliefs illustrating opium. Sumerians referred to opium poppy as *hul gil*, which means the "plant of joy". Many believe the Sumerians were the first to cultivate opium poppy circa 3300 BCE, however, archaeological data collected from of opium poppy seeds discovered in Switzerland, Spain, the Netherlands, Greece, and Cyprus suggest that opium poppy had been cultivated and used in Western and Central Europe since 4600-3800 BCE (Baser and Arsan, 2014). In 1903, evidence of opium was found in ancient Egypt (ca 1400 BCE), where a sample was recovered from the tomb of the chief royal architect Kha; however, the reliability of the initially reported analyses of the contents of the tomb have been questioned (Bisset et al., 1994). During the prominence of the Egyptian Empire, the city of Thebes became so popular for its cultivation of opium poppy that Egyptian opium became

known as Thebic opium, which is how the alkaloid thebaine derived its name (Hagel et al., 2007).

References to opium use have been found among the writings of Greek authors and philosophers. Homer wrote of Helen, the daughter of Zeus, giving soldiers nepenthe, an opium-based drug, to "lull all pain and anger and bring forgetfulness of every sorrow." It has been suggested that opium poppy was used for various purposes, including religious rituals, pain relief, euthanasia, and even as a means to prevent excessive crying in children (Brownstein, 1993). In addition, Hippocrates advocated for the medicinal use of opium poppy. He prescribed it as a sleep aid, for pain relief, to seal wounds and stop bleeding, and for the alleviation of a negative affective state (Hagel et al., 2007, Fernandez and Libby, 2011).

By the 10th century, opium had spread from Asia Minor throughout Europe and China. Turkey had become a major cultivator of opium by the 16th century, with exports reaching India, China, and Indonesia (Baser and Arsan, 2014). After China banned tobacco smoking in the mid-17th century, smoking opium became the alternative. By the 18th century, the British run Eastern India Trading Company began trading India-grown opium to China. As a result, the increase in opium use caused a serious public health crisis, which contributed to China's 1729 decision to prohibit the sale and consumption of opium. Efforts to stop the East Indian Trading company's supply from entering China led to the 1st and 2nd Opium Wars, which China ultimately lost. China was the major consumer of opium at the time, accounting for 75% of the opium produced in India. In fact, Hong Kong was known as the Opium State (Baser and Arsan, 2014). Due to a 2nd Opium War and increased pressure from Britain and France, China eventually began to allow the growth of opium poppies, and, as a result, it is estimated that 27% (i.e. 13.5 million people) of China's population were opioid addicts by 1906 (Lu et al., 2008).

During this time, another opiate was synthesized. Morphine was isolated from opium sometime between 1803 and 1805 by the German scientist Friedrich Sertürner. He named this new drug after the god of dreams, Morpheus. Since that time, over 40 alkaloids – which account for the activity of opium - have been isolated from opium. The problem of opioid addiction first emerged as a widespread health concern in the United States during and following the Civil War. Morphine was most commonly used by doctors during surgical procedures and to treat pain. It was also widely used during this time as treatment for general discomfort and stress. However, it soon became apparent that the abuse potential and hazardous consequences associated with morphine use were equal to those of opium. Interestingly, following the Civil War, the majority of opioid addicts were not former soldiers. Two-thirds of opioid addicts were women because of the widespread prescription of opiates for menstrual and menopausal discomfort (SAMHSA, 2012). Due to their extensive medicinal use, efforts were made to produce a safer, more efficacious, nonaddicting opiate. In 1898, heroin was synthesized and believed to be a more potent and nonaddcitive replacement for morphine. Unfortunately, both of these claims were proven to be false (Brownstein, 1993). Heroin was initially introduced as a cough suppressant, but it soon began to be abused because of its euphoria-inducing effects. In addition, the spread of intravenous self-administration resulting from the advent of the hypodermic needle had a profound effect on opioid addiction.

In the early 20th century, the demographics of opioid abusers began to change. The arrival of young European immigrants led to overcrowded housing in ghettos and substandard neighborhoods where opiate use was common, resulting in many immigrants becoming addicted. As time went on, crime rates in these urban areas rose, as addicts were turning to illegal means to support their drug habit. Following World War II, many European immigrants relocated outside

of the crowded cities, which led to Hispanics and African-Americans moving into areas where opioid abuse was already a serious problem. As a result, many people from these groups also developed an addiction. Illicit opioid use and addiction-related crime continued to rise throughout the mid-20th century. By the 1980s, it is estimated that 500,000 Americans used illicit opioids, and by the end of the 1990s that number nearly doubled to 898,000 (Treatment, 2005).

In the early 20th century, the U.S. government began to pass legislation aiming to curtail the problem of opioid abuse. For example, in 1909, the Smoking Opium Exclusion Act was passed, banning the smoking and possession of opium, while continuing to allow its use for medicinal purposes. In order to tax the manufacture and distribution of narcotics, the Harrison Narcotic Act was enacted in 1914, requiring federal oversight in the production, distribution, and prescription of opioids. Furthermore, the Heroin Act was passed in 1924, which prohibited the manufacture, importation and possession of heroin, even for medicinal use. Ultimately, the Controlled Substance Act of 1970 was passed as a means to consolidate numerous laws regulating the manufacture and distribution of illicit and non-illicit drugs.

Over the past decade, the number of heroin users in the United States has steadily risen, with an estimated 681,000 users in 2013 (Lipari and Hughes, 2015). One factor that may have contributed to this steady rise in heroin use is increased prescribing of painkillers, such as hydrocodone and oxycodone. Recently, tighter restrictions have been placed on the prescribing of painkillers, thus reducing opioid analgesic availability (Dart et al., 2015). However, the reduction in prescription painkiller misuse has been accompanied by increases in heroin use, suggesting that opioid-dependent individuals are resorting to heroin as a substitute. As a result, heroin use will remain a significant threat to public health for the foreseeable future (Cicero and Kuehn, 2014).

Historical Background of the Effects of Opioids on Immunity

Long before scientific evidence of the immunomodulatory effects of opioids emerged, the deleterious effects of opiates on health had been observed. In the mid-16th century, Fallopious, the professor of anatomy at Pisa, investigated the effects of opium administration in a malaria infected prisoner. The prisoner died following the second dose of opium, suggesting that opium administration expedited the progression of infectious disease. Despite this observation, it wasn't until the 19th century that the notion of negative health consequences associated with opioid use began to be accepted. In fact, it was previously believed that opium could be used to treat infections. However, a change in thinking began in 1821 when Thomas De Quincey published "Confession of an English Opium-Eater", in which he provided an autobiographical account of his laudanum addiction and the impact it had on his life. In his book, he included a chapter discussing the "miseries of opium," which led to a long debate about the deleterious effects of chronic opiate use (Rishdal et al., 1996).

In 1866, Edmund Arnold suggested that morphine should not be used to treat diseases when inflammation and "constitutional irritation" have developed. In addition, Alonzo Calken argued against the commonly held belief that opium prolongs life, and that opium users are, in fact, "plagued with disease." Furthermore, Thomas Whipham (1875) concluded that the onset of acute pleuropneumonia occurred more rapidly and resulted in a higher mortality rate among opiate addicts, suggesting that opiates contributed to the development of the disease. As evidence for the detrimental health consequences associated with opioid use began to grow, researchers began investigating these effects in animal models. For example, Coronedi (1897) demonstrated that sub-lethal doses of morphine increased susceptibility to infection in dogs. In the early 20th century, many others noted similar disease complications associated with opioid use in clinical

and experimental settings (Rishdal et al., 1996). During a time when interest in this line of research was waning, Hussey and Katz (1950) published a seminal study reporting various medical and surgical complications resulting from addiction to opiates, including skin abscesses, thrombophlebitis, septicima, acute bacterial endocarditis and tetanus. Additional reports were subsequently published also reporting a high incidence of bacterial, fungal and viral infections as a result of opiate use (Louria et al., 1967, Luttgens, 1949). In the 1960s, there was a significant rise in drug use across American and among soldiers fighting in Vietnam. Thus, drug abuse was thrust back into the national consciousness. Despite this increased awareness, it was not until the 1980s and the emergence of the AIDS epidemic that the research community began to seek to understand the mechanisms mediating opiate abuse and increased susceptibility to infection.

Opioids and Immunity

The latter half of the 20th century produced a wealth of research investigating the effects of opiates on immune function. Clinical studies have revealed abnormalities in basic immune parameters in heroin users, including a decrease in circulating lymphocytes, natural killer (NK) cell activity, cytokine production, and antibody-dependent cellular cytotoxicity (Nair et al., 1986, Govitrapong et al., 1998, Yardeni et al., 2008). These finding have also been extended into preclinical rodent models of opiate-induced immunomodulation. Morphine has been shown to suppress NK cell activity (Friedman et al., 2003, Weber and Pert, 1989, Yokota et al., 2004) and lymphocyte proliferation (Friedman et al., 2003, Bayer et al., 1990b, Bayer et al., 1990a, Gomez-Flores and Weber, 2000, Hamra and Yaksh, 1996). In addition, opiate administration decreases phagocytosis (Tubaro et al., 1983, Rojavin et al., 1993, Pacifici et al., 1993) and chemotaxis (Grimm et al., 1998, Liu et al., 1992), which are important for pathogen clearance and transporting immune cells to sites of infection or injury, respectively. Furthermore, opioid

receptor agonists suppress nitric oxide (NO) production (Schneider and Lysle, 1998, Menzebach et al., 2004, Bhaskaran et al., 2007), the expression of inducible nitric oxide synthase (iNOS) (Lysle and How, 2000), and expression of the proinflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor – α (TNF-α) (Roy et al., 1998, Chao et al., 1993), and interleukin-1β (IL-1β) (Pacifici et al., 2000). Thus, these studies suggest that opioid administration results in an impaired ability to defend against infectious disease (Theodorou and Haber, 2005).

Studies from our own laboratory have demonstrated that morphine administration in rats suppresses splenic lymphocyte proliferation, splenic NK cell activity, blood lymphocyte proliferation, and the *in vitro* production of the cytokines interleukin-2 (IL-2) and interferon-γ (Fecho et al., 1993a, Fecho et al., 1993b, Fecho et al., 1996b, Fecho et al., 1996a, Lysle et al., 1993a, Saurer et al., 2006a, Saurer et al., 2006b). Importantly, we have demonstrated that heroin induces similar immunomodulatory effects (Fecho and Lysle, 2000, Fecho et al., 2000, Lysle and How, 2000, Saurer et al., 2009). Studies investigating opioid receptor involvement in the effects of heroin on immune status demonstrated that administration of the opioid receptor antagonist naltrexone attenuated lipopolysaccharide (LPS)-induced reductions of iNOS mRNA expression and NO production, indicating the immunosuppressive effect is mediated via the opioid receptor (Lysle and How, 2000). In addition, central monoamine signaling has been reported to alter peripheral immune parameters. Disruption of mesolimbic dopamine signaling impairs lymphocyte proliferation and NK cell activity (Deleplanque et al., 1994). Furthermore, pharmacological manipulation of dopamine in the nucleus accumbens has been shown to reduce splenic lymphocyte proliferation (Nistico et al., 1994). Studies from our laboratory identified the medial nucleus accumbens shell (mNAcS) as an important neural substrate mediating the effects of heroin on immune function. Intra-mNAcS administration of the D_1 antagonist SCH-23390

blocked heroin's immunosuppressive effects on NK cell activity, iNOS mRNA expression and NO production, indicating that D_1 signaling in the mNAcS is an important mediator of heroin-induced immune alterations (Saurer et al., 2009). Taken together, these findings clearly demonstrate that opioids alter host defense against infection.

Nitric Oxide

In order to effectively defend against foreign invaders, the mammalian immune system has developed a wide range of intracellular defense mechanisms. One such mechanism of host defense is the production of NO. Its antimicrobial effects on a wide range of pathogens make it an effective defender against infections by bacteria, fungi, and parasites, viruses (James, 1995, Reiss and Komatsu, 1998, Kroncke et al., 1998, Bogdan et al., 2000). Studies have shown that NO is produced by many immune cells, particularly the macrophage, and its release results in several immunoprotective actions, including vasodilation, inhibition of platelet adhesion molecules, and mediating macrophage cytotoxicity (Suschek et al., 2004, Tuteja et al., 2004), as well as promoting pathogen elimination in non-NO producing bystander cells (Olekhnovitch et al., 2014).

NO is produced in a two-step oxidation of L-arginine by the three isozymes of nitric oxide synthases (NOS): constitutively expressed endothelial NOS and neuronal NOS, as well as iNOS (Cifone et al., 1995). NO is a gaseous molecule with a half-life of only 3-8 seconds in an aqueous solution, which presents a significant challenge when attempting to directly measure its production (Kelm, 1999). Alternatively, our laboratory utilizes two indirect methods of measuring NO production: we quantify the primary metabolites of NO, nitrate and nitrite, in blood plasma, as well as measure the level of iNOS expression in spleen tissue, which is an

enzyme responsible for NO production. Activation of iNOS results in high levels of NO being released for extended periods of time. Given that iNOS is not constitutively expressed, it requires induction via proinflammatory agents, such as LPS, IL-1β, or TNF-α. In order to induce iNOS expression and NO production, our laboratory utilizes LPS, which is a component of the outer cell wall of gram negative bacteria. LPS induces NO production and iNOS expression via activation of extracellular TLR4 receptors and subsequent activation of the intracellular NF-κB pathway. TLR4 receptor activation results in translocation of NF-κB into the nucleus and initiates transcription of several immunomodulatory genes, including iNOS.

Conditioned Immunomodulation

It was long believed that the central nervous and immune systems functioned independently of one another; however, this idea was challenged by Metalnikov and Chorine in 1926 when a Pavlovian associative learning model was used to demonstrate for the first time that a previously neutral stimulus can acquire immune altering effects after pairing with an immunomodulatory drug. In this study, guinea pigs were injected with the plant extract Tapioka (unconditioned stimulus, US), which stimulates peripheral leucocyte production. Immediately following Tapioka administration, the animals' skin was either heated or slightly slit (conditioned stimulus, CS). After repeated US-CS pairings, stimulation of the skin alone increased peripheral leucocyte production, indicating that skin stimulation had acquired immune altering effects. It wasn't until 1975 that Ader and Cohen continued this line of research with their seminal study demonstrating conditioned immunosuppression in a model of conditioned taste aversion. In this study, the immunosuppressive drug cyclophosphamide (US) was repeatedly paired with saccharin mixed in water (CS). Upon ingesting saccharin water alone, animals exhibited a deficient immune response following immune challenge, indicating that

saccharin had acquired immune altering properties similar to those of cyclophosphamide. Subsequent studies conducted by Ader and colleagues further demonstrated conditioned immunomodulatory effects on the autoimmune disease systemic lupus erythematosus (Ader and Cohen, 1982), humoral immunity (Ader et al., 1982, Ader et al., 1993), and cell-mediated immunity (Ader and Cohen, 1992), which established psychoneuroimmunology as a new field of study.

Building upon the work of Ader and Cohen, others began to investigate the neural circuitry involved in conditioned immunomodulation. Brain regions of interest were identified based on previous research that mapped out a basic neural circuit of the conditioned taste aversion model, which was used to model conditioned immunosuppression by pairing saccharin with the immunologic drug cyclosporine A. Excitotoxic lesions performed following the acquisition phase in the insular cortex and ventromedial hypothalamus, but not the amygdala, were found to prevent the expression of conditioned immunosuppression of lymphocyte proliferation, IL-2, and interferon-γ (Pacheco-Lopez et al., 2005).

Interestingly, opioid-induced immunosuppression was found to be mediated by the central nervous system (CNS) (Shavit et al., 1986, Fecho et al., 1996a, Hoffman et al., 1995). According to the previously described studies demonstrating conditioned immunomodulation, the immunosuppressive effects of exogenous opioid administration cannot exclusively be attributed to acute pharmacological effects on physiology. Early research from our laboratory reported that the immune altering effects of opioids, including those of morphine and heroin, can be conditioned to environmental stimuli by pairing opioid administration with exposure to a distinct environmental context. As a result, a morphine-paired context (CS) can acquire immune altering effects. For example, following conditioning sessions during which morphine injections

were paired with a CS, rats exhibited significant reductions in mitogenic responsiveness of lymphocytes, NK cell activity, and IL-2 production when re-exposed to the CS in a drug free state, demonstrating morphine-conditioned immunosuppression (Coussons et al., 1992, Coussons-Read et al., 1994a, Coussons-Read et al., 1994b). Moreover, our laboratory has shown that a heroin-associated context elicits a significant reduction of LPS-induced iNOS expression (Lysle and Ijames, 2002, Szczytkowski and Lysle, 2007, Szczytkowski and Lysle, 2008, Szczytkowski and Lysle, 2010, Szczytkowski et al., 2011, Szczytkowski et al., 2013, Hutson et al., 2014).

Studies investigating the role of neurotransmitters in conditioned immunomodulation found that antagonism of dopamine and glutamate receptors block this effect (Hsueh et al., 1999, Kuo et al., 2001), which guided studies in our laboratory investigating the contributions of specific neurotransmitters to morphine- and heroin-conditioned immunosuppression. The results of the studies demonstrated that dopamine receptor activity is necessary for the expression of morphine-conditioned immune alterations, as administration of a D_1 receptor antagonist prior to re-exposure to the conditioned stimulus prevented the suppression of NK cell activity (Saurer et al., 2008a). Our laboratory also demonstrated similar immunomodulatory effects with heroin (Fecho and Lysle, 2000, Lysle and Ijames, 2002, Szczytkowski and Lysle, 2010).

Emerging evidence suggests that a limbic neural circuit mediates the expression of heroin-conditioned immune alterations. In support of this, either GABA agonist-induced neural inactivation of, or dopamine D_1 receptor antagonism in, the basolateral amygdala (BLA) blocks heroin-conditioned immunosuppression (Szczytkowski and Lysle, 2008, Szczytkowski and Lysle, 2010). Moreover, unilateral dopamine D_1 receptor antagonism in the BLA coupled with contralateral N-methyl-D-aspartate (NMDA) glutamate receptor antagonism in the mNAcS

significantly attenuates the expression of heroin-conditioned immunosuppression. In contrast, ipsilateral manipulation of the same brain regions fails to disrupt heroin-conditioned immunomodulation (Szczytkowski et al., 2011). These findings suggest that activation of BLA D_1 receptors is necessary for intrahemispheric interactions between the BLA and the mNAc in the control of heroin-conditioned immune alterations. Furthermore, inactivation of the anterior, but not posterior, ventral tegmental area (VTA) attenuated heroin-conditioned immunosuppression of NO production in blood plasma, as well as the expression of iNOS, IL-6 and TNF-α in spleen tissue (Hutson et al., 2014). Taken together, these findings suggest heroin-conditioned immunomodulation is mediated by a mesolimbic circuit consisting of the anterior VTA, BLA, and mNAcS.

Centrally Active Interleukin-1

While our efforts to map the neural circuitry mediating heroin-conditioned immunosuppression have been informative, the cellular mechanisms contributing to this effect have yet to be thoroughly investigated. In recent history, there has been a conceptual shift regarding the functional significance of cytokines in the brain. Once viewed primarily as immune molecules that mediate inflammatory events during infection, cytokines are now recognized to be critical mediators of neuronal communication more akin to neurotransmitters and neuropeptides. Therefore, neuroimmune signaling within the brain may be integral to the impaired immune responses observed among drug abusers and animal models of drug abuse.

One mechanism of interest is the cytokine, interleukin-1 (IL-1). IL-1 is a critical proinflammatory cytokine active in many biological functions, but it is primarily known for its involvement in host defense against infection and disease. As part of the acute phase response of the innate immune system, IL-1 is immediately released from resident immune cells upon

detection of foreign pathogens, whereupon it works to limit the spread of infection and kill off foreign invaders. The IL-1 family is comprised of various subtypes functioning as agonists, antagonists or anti-inflammatory cytokines. Proinflammatory cytokines were often thought to be exclusively synthesized and secreted via immune cells of the peripheral nervous system, but it has been demonstrated that activated microglia and astrocytes are the primary source of cytokines in the brain (Rothwell et al., 1996, Hanisch, 2002, Davies et al., 1999, Toda et al., 2002).

The most widely studied cytokine is IL-1β. Due to its neurotoxic effects at high levels, IL-1β is expressed at a low concentration under normal conditions. However, upon release from microglia, IL-1β can further induce IL-1β release in an autocrine/paracrine fashion by binding to membrane bound IL-1 type 1 receptors (IL-1R1) (Rothwell and Luheshi, 2000, Toda et al., 2002, McMahan et al., 1991). Interestingly, dysfunctional IL-1 signaling has been observed in many pathological disease states, such as Alzheimer's disease, multiple sclerosis, and Parkinson's disease, in which IL-1β expression is exaggerated, thus glial activation and subsequent release of IL-1β could be important mediators of neurodegenerative diseases. Given its detrimental neuroinflammatory effects, it is clear that IL-1β signaling must be tightly regulated. To accomplish this, there are two primary biological mechanisms that regulate IL-1 activity: the endogenous antagonist interleukin-1 receptor antagonist (IL-1Ra) and the decoy receptor type 2 IL-1 (IL-1R2). In addition, anti-inflammatory cytokines such as IL-6 and IL-10 exist in part to further modulate IL-1 signaling. Further indicating the importance of controlling IL-1 signaling, IL-1Ra has a higher affinity for IL-1R1 than IL-1β, and IL-1β has a higher affinity for IL-1R2 (Dinarello, 2009). Taken together, these diverse regulatory mechanisms act in concert to main IL-1 homeostasis and preserve normal biologic function.

Central IL-1 signaling has been shown to be particularly important in neural plasticity, learning, and memory. For example, IL-1β expression in the rat hippocampus is increased following long-term potentiation (LTP) induction, and this effect is disrupted by administration of the IL-1R1 antagonist, IL-1Ra, following LTP induction (Schneider et al., 1998, Cunningham et al., 1996, Vereker et al., 2000a). Furthermore, IL-1R1 knockout mice display memory impairments and disrupted induction of LTP (Koo and Duman, 2009). Similarly, animals treated in two separate studies with IL-1Ra following repeated foot shock and exposure to a spatial learning task displayed impaired fear conditioning and performance in the Morris water maze, respectively (Pugh et al., 2001, Yirmiya et al., 2002). Finally, our laboratory has demonstrated that inhibition of IL-1β expression in the dorsal hippocampus attenuates the immunosuppressive effects of a previously heroin-paired context. Thus, there is substantial evidence for the role of IL-1 signaling in learning and memory. However, the effects of IL-1β expression seem to follow an inverted U-shaped pattern in regards to its role in learning and memory. It has been reported that peripheral induction of cytokine expression via central administration of IL-1β or systemic administration of endotoxin increases central cytokine expression, which can result in impaired cognitive performance (Eriksson et al., 2000). In addition, peripheral cytokines are able to induce cytokine release in glial cells via blood-borne and neuroimmune pathways (Nguyen et al., 1998, Pugh et al., 1998), indicating communication between the immune system and CNS. Moreover, progressive increase in IL-1β levels and microglial activation have been observed as a result of aging (Roubenoff et al., 1998, Wilson et al., 2002), which is often correlated with cognitive and memory impairments. Taken together, these findings indicate that exaggerated IL-1β expression is detrimental to cognitive performance and normal behaviors.

Based on the fact that both inhibition and overstimulation of IL-1 receptors produce various cognitive-behavioral deficits, it stands to reason that a physiologically active level of IL-1 signaling is involved in the facilitation of normal biological function. However, determining the range of IL-1β expression that facilitates homeostatic function is challenging because it likely varies depending on the physiological action. Despite this challenge, Yirmiya and colleagues (2002) demonstrated that intracerebroventricular (i.c.v.) administration of a low dose of IL-1β immediately following passive avoidance training resulted in enhanced memory 5-8 days later. Similar memory enhancing effects were also observed when i.c.v. IL-1β was administered prior to passive avoidance training, as well as before memory tests (Song et al., 2003). Furthermore, IL-1β has been found to facilitate spatial and contextual fear memories. For example, IL-1β administration enhanced the acquisition of spatial memory in a Morris water maze task (Gibertini, 1998). Moreover, administration of a low dose of IL-1β immediately following contextual fear conditioning improved fear learning (Goshen et al., 2007). In summary, these studies indicate that IL-1 is a tightly regulated and important neuromodulator of many physiological and behavioral processes.

Goals of Dissertation

The studies presented in this dissertation aimed to elucidate the peripheral immunomodulatory role of central IL-1 signaling in heroin-conditioned immunosuppression. In addition, I sought to determine the effects of central administration of IL-1β on peripheral immune function. Studies conducted in our laboratory have uncovered a neural circuit mediating heroin-conditioned immunosuppression. Thus, the current studies sought to identify a novel mechanism within this circuit mediating the conditioned effects of heroin on peripheral immune

parameters. Chapter 2 examines the effect of re-exposure to a heroin-paired context on IL-1β expression in the brain. Chapter 3 investigates the role of IL-1 signaling in the BLA, caudate putamen (CPu), and mNAcS in the expression of heroin-conditioned immunosuppression. Finally, Chapter 4 tested the effect of intra-BLA IL-1β administration on peripheral immune function. Together, these studies provided insights into the role of neuroimmune signaling in the modulation of peripheral immune function.

CHAPTER 2

TIME COURSE OF INTERLEUKIN-1β EXPRESSION IN THE BASOLATERAL AMYGDALA AND MEDIAL NUCLEUS ACCUMBENS SHELL FOLLOWING RE-EXPOSURE TO A PREVIOUSLY HEROIN-PAIRED CONTEXT

Introduction

A high incidence of infection has long been observed among opiate users (Luttgens, 1949, Hussey and Katz, 1950, Bussiere et al., 1993, Friedman and Eisenstein, 2004). While this was commonly thought to be due to non-sterile intravenous drug practices, such as needle sharing, it has been demonstrated that opiates directly alter the ability of the immune system to eliminate infection. Clinical studies have revealed abnormalities in many defensive immune parameters among heroin users, including decreased circulating lymphocytes, NK cell activity, cytokine production, and antibody-dependent cellular cytotoxicity (Nair et al., 1986, Govitrapong et al., 1998, Yardeni et al., 2008). In addition, our laboratory has found similar opioid-induced immune alterations in animals. For example, animals treated with morphine, which is a major metabolite of heroin, exhibited decreased splenic lymphocyte proliferation, splenic NK cell activity, blood lymphocyte proliferation, and proinflammatory cytokine production (Fecho et al., 1993a, Fecho et al., 1993b, Fecho et al., 1996a, Fecho et al., 1996b, Lysle et al., 1993b, Saurer et al., 2006a, Saurer et al., 2006b).

Interestingly, the immunosuppressive effects of opiates have been shown to be conditioned to environmental stimuli. Our laboratory has demonstrated that repeatedly pairing morphine or heroin with a unique environmental context causes the previously neutral context to

elicit an immunosuppressive response when an animal is re-exposed to that context in a drug free state (Coussons et al., 1992, Coussons-Read et al., 1994a, Coussons-Read et al., 1994b, Lysle and Ijames, 2002, Szczytkowski and Lysle, 2007). Recently, we identified a mesolimbic neural circuit that mediates these conditioned immunosuppressive effects. Investigations in our laboratory have shown that the functional integrity of the BLA, mNAcS and VTA is necessary for the expression of heroin-conditioned immunomodulation (Hutson et al., 2014, Szczytkowski and Lysle, 2008, Szczytkowski and Lysle, 2010, Szczytkowski et al., 2011, Saurer et al., 2008a). Despite the insights gained from these studies, there is still little known about the mechanisms mediating heroin-conditioned immunosuppression within these brain regions.

One potential mechanism is IL-1, which is a potent proinflammatory cytokine that is an essential component of the innate immune response to infection and injury. Induction of IL-1 in the peripheral nervous system in response to infection leads to production of other proinflammatory cytokines in order to rid the body of foreign pathogens (Maier et al., 1998, Quan et al., 1999, Konsman et al., 2000). Interestingly, IL-1 synthesis and secretion is not limited to peripheral immune cells. In fact, IL-1 has been shown to be produced and released by glia within the CNS (Rothwell et al., 1996, Hanisch, 2002).

Of particular interest to the current studies, the BLA contains a high density of IL-1 receptors (Konsman et al., 2000, Yabuuchi et al., 1994a). Over the past 20 years, evidence has emerged in support of IL-1 serving as a neuromodulator in the brain. For example, Yu and Shinnick-Gallagher (1994) demonstrated that i.c.v. IL-1β administration modulates neuronal firing in the BLA. In addition, studies have demonstrated that both blockade of IL-1 receptors and inhibition of IL-1 synthesis prevent IL-1-induced memory impairments (Pugh et al., 1998, Pugh et al., 1999, Gemma et al., 2005). Furthermore, IL-1β administration directly into the

dorsal hippocampus has been shown to impair memory (Barrientos et al., 2002) and inhibit LTP (Cunningham et al., 1996, Vereker et al., 2000a, Vereker et al., 2000b, Murray and Lynch, 1998). While it is not surprising that complete inhibition or gross over expression of a potential neuromodulator is detrimental to biological function, it is, however, interesting that central administration of low doses of IL-1β have been shown to facilitate learning in various learning and memory paradigms (Yirmiya et al., 2002, Song et al., 2003, Goshen et al., 2007). Based on these findings, it is clear that IL-1 is an important mediator of learning and memory. To date, the overwhelming majority of published research investigating the role of central IL-1 in learning and memory has focused on the hippocampus. Therefore, there is a need to explore the contribution of IL-1 in neural processing within other brain regions, such as the BLA and mNAcS, as these brain regions have been reported to be important for the expression of conditioned learning.

Recently, our laboratory has worked to identify the neural substrates that mediate heroin-conditioned immunosuppression. As previously mentioned, we have determined that activation of the VTA, BLA, and mNAcS are necessary for the expression of heroin-conditioned immunosuppression, further supporting research indicating that these brain regions are important for the expression of associative learning (See et al., 2003) Given the importance of IL-1 signaling in learning and memory, the current study sought to investigate the importance of IL-1 signaling in our model of heroin-conditioned immunomodulation. The goal of the study described in this chapter was to determine the time course of IL-1β expression in the BLA and mNAcS following re-exposure to the previously heroin-paired environment using immunohistochemical techniques to assess IL-1β immunoreactivity.

Materials and Methods

Animals

Male Lewis rats, weighing 225–250 g, were purchased from Charles River Laboratories (Raleigh, NC, USA). Upon arrival, animals were housed individually in plastic cages in a colony room with a reversed light-dark (12-h) cycle maintained through artificial illumination. Animals were allowed access to food and water *ad libitum* throughout the experiment except for the time spent in the conditioning chambers when food and water were not available. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the University of North Carolina at Chapel Hill and conformed to NIH guidelines on the care and use of laboratory animals.

Drug Administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD, USA) and dissolved in 0.9% sterile saline. Heroin was administered subcutaneously at a dose of 1 mg/kg. This dose was selected based on prior experiments in our laboratory showing that it induces conditioning and alters LPS-induced iNOS expression in spleen tissue and NO production in blood plasma (Lysle and How, 2000, Lysle and Ijames, 2002, Szczytkowski and Lysle, 2007).

Conditioning Procedure

All animals received five conditioning sessions in standard conditioning chambers (BRS/LVE, Laurel, MD, USA). Chambers were fitted with a metal grid floor design and cedar bedding to create an environment distinct from that of the home cage and to provide both olfactory and tactile cues for conditioning. Artificial noise machines were used to minimize

background noise. All conditioning took place during the dark phase of the light cycle in a room separate from the animal colony and the conditioning chambers were kept dark to minimize effects on circadian rhythms. On each conditioning day, a subcutaneous injection of heroin (1 mg/kg) was administered immediately prior to placement into the chamber for 60-min. Training sessions were separated by 48 h.

Test of Heroin-Conditioned Immunomodulation

Six days after the final conditioning session, rats were sacrificed following a 0, 15, 60 or 120 min (120 min animals were re-exposed for 60 minutes then returned to home cage for 60 min) exposure to the heroin-paired context.

Immunohistochemistry

Rats were overdosed with anesthetic (Ketamine/xylazine 100mg/kg; i.p.) and transcardially perfused with 0.1M phosphate buffered saline (PBS, pH = 7.4) followed by 4% paraformaldehyde in PBS. Brains were extracted, postfixed in paraformaldehyde for 24 hours and sectioned coronally at 40μm using a vibrating microtome (Leica VT1000S; Wetzlar, Germany). Sections were collected in a 1:12 series and stored in cryoprotectant at -20˚C until processing so that every twelfth section was stained for IL-1β immunoreactivity. Tissue was washed in 0.01M TBS (pH=7.4) and pre-incubated for 1 hr at room temperature in 3% goat serum block. Following additional washes, sections were blocked for nonspecific binding (TBS, 0.1% triton X-100, and 3% goat serum) for 1 hr, and then incubated overnight in rabbit anti-IL-1β (1:1000, Abcam; Cambridge, MA) primary antibody at 4˚C. The next day, tissue underwent several washes in 0.01M TBS followed by a wash with 3% H_2O_2 in TBS to quench endogenous peroxidases. Sections were again blocked for nonspecific binding and incubated in biotinylated goat anti-rabbit secondary antibody (Vector

Laboratories, Burlingame, CA) and washed in TBS. Staining of antibody binding was achieved with an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) and chromagen, nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Warrington, PA) to detect IL-1β binding. Sections were dried then mounted to glass slides and coverslipped with SHUR/Mount™ (Triangle Biomedical Sciences; Durham, NC).

Quantification and Statistical Analysis

Slides were coded to ensure experimenter blindness to treatment to conditions for quantification. Images were taken of the regions of interest at 20x magnification with a digital camera (Roper Scientific) mounted on an optical microscope. IL-1β immunoreactivity was determined by optical density using an autothreshold in the ImageJ software, with percent area of staining reported as percent control. A one-way ANOVA was used to determine differences in percent area of total IL-1β staining across all groups in the BLA and mNAcS. All analyses were performed with the alpha level of significance set at $p < 0.05$.

Results

IL-1β immunoreactivity in the basolateral amygdala and medial nucleus accumbens shell following re-exposure to a heroin-paired context

As you can see in Figure 2.1, there are IL-1β immunoreactive cells in the BLA at all time points (A, 0 min; B, 15 min; C, 60 min; D, 120 min); however, ANOVA revealed no differences in IL-1β immunoreactivity (E) [$F(3,16) = 0.18$, $p > 0.05$] across all groups, indicating that re-exposure to the previously heroin-paired environment does not alter IL-1β immunoreactivity in a time-dependent manner. Similarly, Figure 2.2 confirms the presence of IL-1β immunoreactivity

in the mNAcS at all time points (A, 0 min; B, 15 min; C, 60 min; D, 120 min). Furthermore, ANOVA revealed no differences in IL-1β immunoreactivity (E) [$F(3,16) = 1.48$, $p > 0.05$].

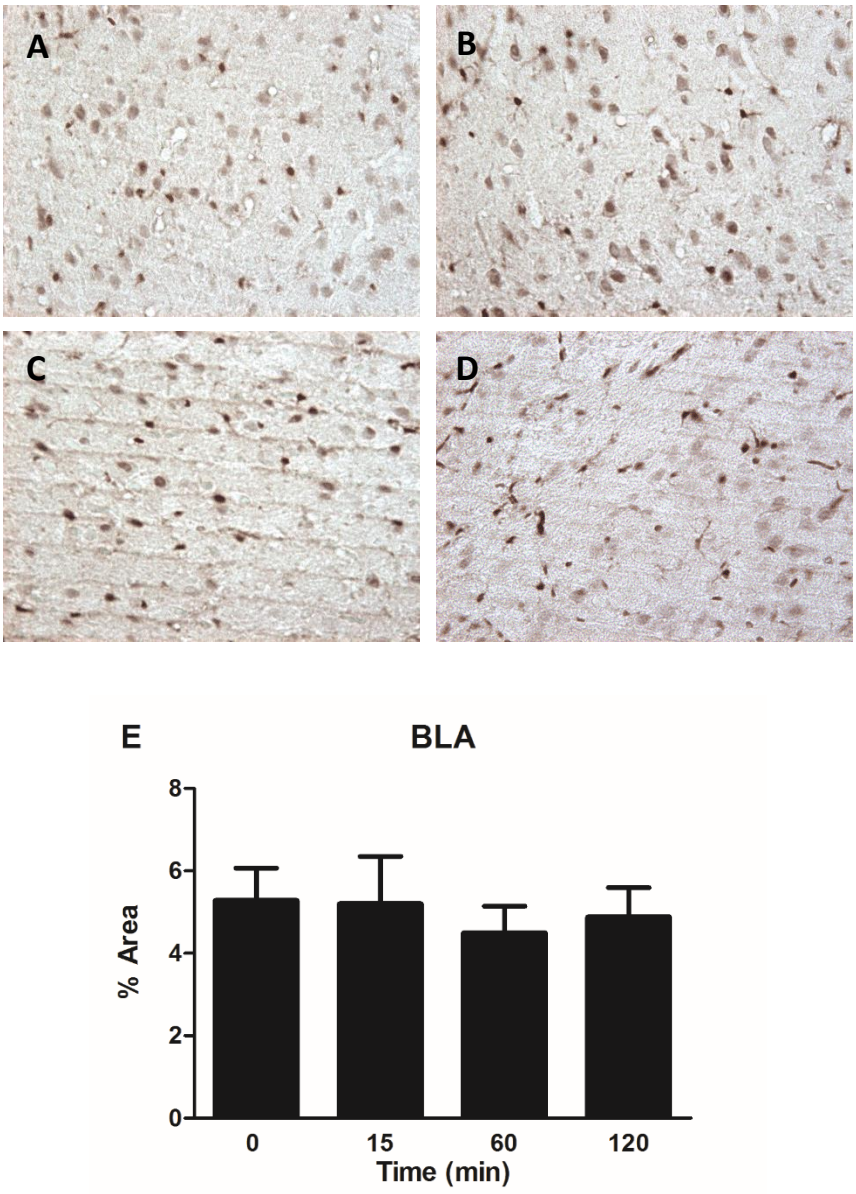

Figure 2.1: IL-1β immunoreactivity in the BLA following 5 days of heroin conditioning. Animals were re-exposed to the CS for (A) 0 minutes, (B) 15 minutes, (C) 60 minutes, or (D) 60 minutes plus 60 minutes in home cage (120 minutes). Representative photomicrographs show similar IL-1β immunoreactivity at all time points (A-D) and no statistically significant differences across groups (E). All data presented as mean ± SEM.

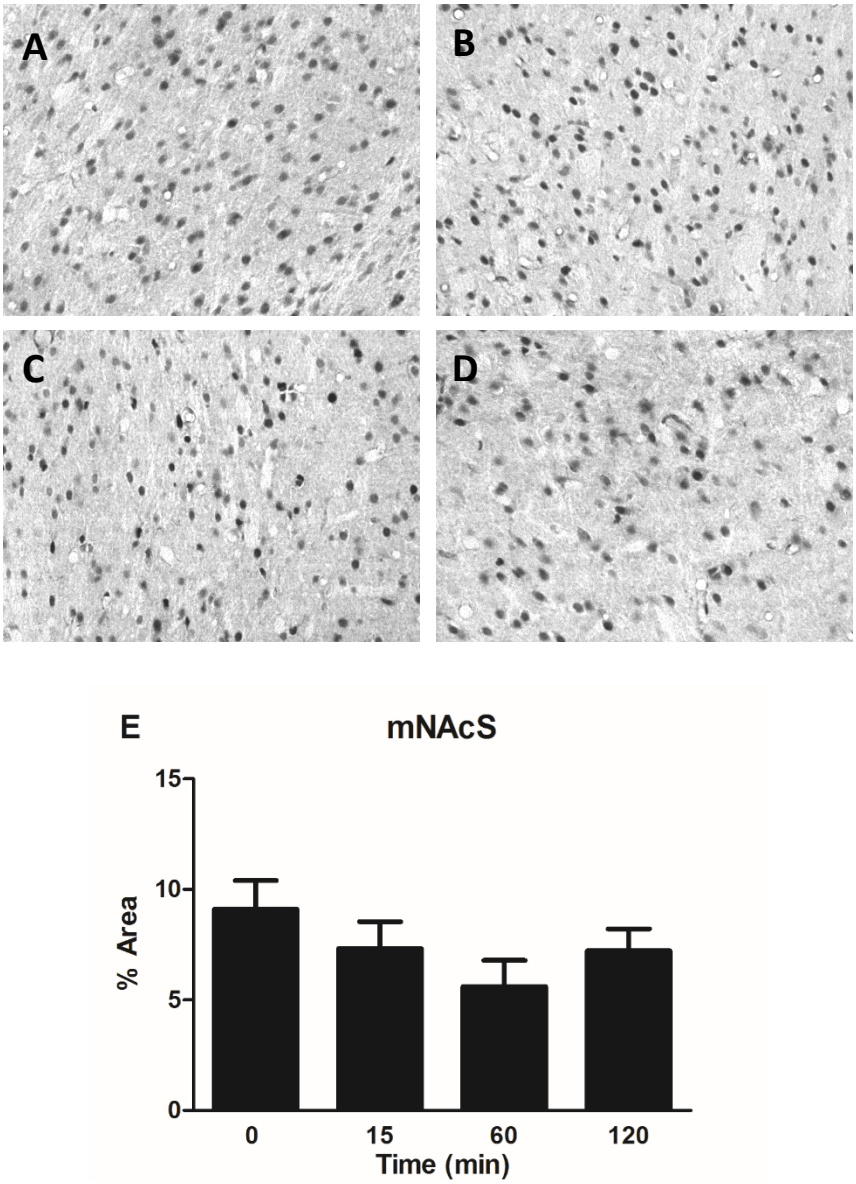

Figure 2.2: IL-1β immunoreactivity in the mNAcS following 5 days of heroin conditioning. Animals were re-exposed to the CS for (A) 0 minutes, (B) 15 minutes, (C) 60 minutes, or (D) 60 minutes plus 60 minutes in home cage (120 minutes). Representative photomicrographs show similar IL-1β immunoreactivity at all time points (A-D) and no statistically significant differences across groups (E). All data presented as mean ± SEM.

Discussion

The findings presented in the current chapter demonstrate that IL-1β is expressed in the BLA and mNAcS prior to and during re-exposure to a previously heroin-paired context. The

presence of BLA IL-1β expression is supported by previous studies indicating dense IL-1 receptor expression in the BLA (Konsman et al., 2000, Yabuuchi et al., 1994b), which suggests that IL-1β signaling may be important for BLA neural processing. I hypothesized that IL-1β would be elevated as a result of re-exposure to a heroin-paired context based on previous research indicating that IL-1β plays an important role in associative learning (Goshen et al., 2007, Yirmiya et al., 2002).

The primary source of IL-1β synthesis and release is believed to be glial cells. Interestingly, increased microglial activation has been observed following re-exposure to a morphine-paired context in a model of CPP (Schwarz et al., 2011). Therefore, it is possible that re-exposure to a heroin-paired context stimulates microglial activation and results in subsequent increased IL-1β expression. However, based on our findings, it does not appear that BLA or mNAcS IL-1β levels were increased following re-exposure to the heroin-paired context. One possibility is that heroin administration induced persistent IL-1β expression, as it is well documented that opiate administration activates microglial cells (Milligan and Watkins, 2009). For example, increases in microglial activation markers have been observed following chronic codeine (Johnson et al., 2014) and morphine administration (Raghavendra et al., 2002, Hutchinson et al., 2009, Song and Zhao, 2001). Thus, it is possible that this activated state could persist long after morphine exposure. In fact, Zhang and colleagues (2012) found microglial activation five days following morphine administration in a model CPP, indicating that microglial activation on test day in the studies discussed in this dissertation is possible, as brain tissue was collected at a similar time point. Under normal physiologic conditions, IL-1β is expressed at low levels and often hard to detect. Thus, the high levels of IL-1β immunoreactivity observed in the current study could be due to sustained increases in IL-1β expression as a result

of persistent microglia activity following heroin conditioning. There is also evidence that conditioning in the absence of drug induces IL-1β release. Increases in IL-1β mRNA expression have been observed at 24 hours (Goshen et al., 2007) and up to 72 hours following fear conditioning (Jones et al., 2015), suggesting that IL-1β may play a role in memory formation. Indeed, inhibition of IL-1 receptor signaling following fear conditioning has resulted in impaired memory upon testing (Jones et al., 2015). Collectively, these data suggest that heroin-induced associative learning could induce IL-1β expression well beyond exposure to the CS. In addition, opioid administration has been shown to increase the expression of IL-1β in CNS endothelial cells and spinal cord tissue (Wang et al., 2012, Liu et al., 2011b, Liu et al., 2011a, Fukagawa et al., 2013); however, the time course of IL-1β expression following opioid administration has proven to be complex. Some studies did not observe increases in IL-1β mRNA expression beyond 2 hours following opiate administration (Wang et al., 2012), whereas others have reported increases occurring 3-7days following the cessation of opioid administration (Berta et al., 2013). These discrepancies are likely due to varying doses, schedules of drug administration, time points at which IL-1β was measured, as well as tissues analyzed. Nevertheless, it is plausible that heroin administration produced the high levels of IL-1β expression observed in the current study.

Taken together, these data indicate dense IL-1β expression in the BLA and mNAcS following heroin conditioning; however, additional control studies are needed to determine if repeated administration of heroin and/or exposure to our conditioned stimulus could result in long-term microglial activity and IL-1β release compared to naïve animals. These findings suggest that IL-1 signaling may play an important role in heroin-conditioned immune alterations,

thus, Chapter 3 continues to explore the role IL-1 in the BLA and mNAcS in the expression of heroin-conditioned immunosuppression.

CHAPTER 3

THE ROLE OF INTERLEUKIN-1 SIGNALING IN THE EXPERSSION HEROIN-CONDITONED IMMUNOMODULATION

Introduction

The results presented in Chapter 2 demonstrate that IL-1β immunoreactivity is present in the BLA and mNAcS of animals that were re-exposed to the heroin-paired CS and in animals that were not re-exposed to the CS. Based on the presence of IL-1β in these brain regions following heroin conditioning, the goals of the studies conducted in Chapter 3 were to determine whether IL-1 signaling within the BLA and mNAcS is necessary for the expression of heroin-conditioned immunomodulation.

It is well established that the BLA is important mediator of associative learning. In 1956, Weisenkrantz observed a marked decrease of fear responses to previously aversive stimuli in amygdala lesioned monkeys. More recently, studies have revealed that the functional integrity of the BLA is required for associative learning. For example, lesioning the BLA has been shown to impair classical eye blink conditioning (Blankenship et al., 2005). In addition, inactivation of the BLA via GABAergic stimulation prevents the development of fear conditioning (Muller et al., 1997). The BLA has also been reported to be critical in models of drug-induced conditioning, as inactivation of the BLA has been shown to prevent the reinstatement of heroin-CPP (Cummins et al., 2014). Similarly, disruption of BLA signaling prevents context-induced reinstatement of

heroin-(Fuchs and See, 2002) and cocaine-seeking behavior (Meil and See, 1997, Grimm and See, 2000, Kruzich and See, 2001, See et al., 2001).

Recently, our laboratory demonstrated the importance of the BLA and mNAcS in heroin-conditioned immunomodulation. Inactivation of the BLA with the $GABA_{A/B}$ agonist cocktail muscimol/baclofen attenuated the immunosuppressive effect of the heron-paired context on several proinflammatory mediators (Szczytkowski and Lysle, 2008). Similarly, antagonism of D_1 receptors in the BLA (Szczytkowski and Lysle, 2010) and mNAcS (Saurer et al., 2008b) prior to re-exposure to a previously heroin-paired context significantly attenuated the expression of proinflammatory mediators and NK cell activity, respectively. Furthermore, activation of NMDA/AMPA glutamate receptors in the mNAcS was found to be necessary for the expression of these conditioned effects (Szczytkowski et al., 2011). Taken together, these studies demonstrate that the BLA and mNAcS are important neural substrates mediating the expression of heroin's conditioned effects on immune function.

As previously discussed in Chapter 2, IL-1 signaling is a critical mediator of neural plasticity, learning and memory, as disruption of IL-1 signaling has been shown to impair fear conditioning, spatial memory and LTP. In addition, a recent study from our laboratory demonstrated that IL-1β expression mediates conditioned immunomodulation. Genetic inhibition of IL-1β expression in the dorsal hippocampus prior to re-exposure to a previously heroin-paired context attenuated heroin-conditioned immunosuppression of iNOS expression and NO production. (Szczytkowski et al., 2013). Given the level of IL-1β immunoreactivity found in the BLA and mNAcS and the importance of these brain regions in heroin-conditioned immunosuppression, it's possible that IL-1could mediate these effects.

The studies conducted in this chapter aimed to determine the role of IL-1 signaling in the BLA and mNAcS in the expression of heroin-conditioned immunomodulation. Rats received five conditioning sessions, with each session separated by 48-hrs, where 1 mg/kg heroin was administered immediately prior to placement in to a distinct context. At least six days following the final conditioning session, animals received bilateral microinfusions of saline vehicle or IL-1Ra into the BLA, CPu, or mNAcS and then returned to their home cage or re-exposed to the previously heroin-paired context for 60 minutes. Animals were then administered a subcutaneous injection of LPS to induce an immune response. Six hours later animals were sacrificed and brain, blood plasma and spleen tissue were collected from each subject. Data collected from these studies indicate that IL-1 signaling in the BLA, but not CPu or mNAcS, is important for the expression of heroin-conditioned immunosuppression. This is the first time IL-1 signaling in the BLA has been shown to mediate any form of associative learning, including conditioned immunosuppression.

Materials and Methods

Animals

Male Lewis rats, weighing 225–250 g, were purchased from Charles River Laboratories (Raleigh, NC, USA). Upon arrival, animals were housed individually in plastic cages in a colony room with a reversed light-dark (12-h) cycle maintained through artificial illumination. Animals were allowed access to food and water *ad libitum* throughout the experiment except for the time spent in the conditioning chambers when food and water were not available. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the

University of North Carolina at Chapel Hill and conformed to NIH guidelines on the care and use of laboratory animals.

Drug Administration

Heroin (diacetylmorphine) was obtained from NIDA (Bethesda, MD, USA) and dissolved in 0.9% sterile saline. Heroin was administered subcutaneously at a dose of 1 mg/kg. This dose was selected based on prior experiments in our laboratory showing that it induces conditioning and alters LPS-induced iNOS and TNF-α mRNA expression in spleen tissue (Lysle and How, 2000, Lysle and Ijames, 2002, Szczytkowski and Lysle, 2007).

Surgical Procedures

Animals were fully anesthetized with 0.35 mL intramuscular injections of 1:1 (vol:vol) ketamine hydrochloride (100 mg/mL) mixed with xylazine (20 mg/mL) and placed into the stereotaxic apparatus. Animals were implanted bilaterally with 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA). The cannulae were directed towards BLA (AP −2.5, ML ± 5.0, DV −6.6), mNAcS (AP −1.7, ML ± 0.8, DV −5.4), or CPu (AP −2.5, ML ± 5.0, DV −4.0). Coordinates are mm relative to bregma. Animals were given a 2-week post-surgical recovery period before the start of conditioning trials.

Conditioning Procedure

All animals received five conditioning sessions in standard conditioning chambers (BRS/LVE, Laurel, MD, USA). Chambers were fitted with a metal grid floor design and cedar bedding to create an environment distinct from that of the home cage and to provide both olfactory and tactile cues for conditioning. Artificial noise machines were used to minimize background noise. All conditioning took place during the dark phase of the light cycle in a room

separate from the animal colony and the conditioning chambers were kept dark to minimize effects on circadian rhythms. On each conditioning day, a subcutaneous injection of heroin (1 mg/kg) was administered immediately prior to placement into the chamber for 60-min. Training sessions were separated by 48 h.

Test of Heroin-Conditioned Immunomodulation

Six days following the final conditioning session, animals received bilateral microinfusions of saline vehicle (0.5 µl per hemisphere) or interleukin-1 receptor antagonist (IL-1Ra) (1.25 µg per 0.5 µl per hemisphere) into the BLA or mNAcS. Injectors extended 2 mm beyond the tip of the guide cannula. Injections were delivered over 2 min, and the injectors were left in place for 1 min after the injection to allow for proper diffusion of fluid away from the infusion site. Thirty minutes later, the rats were re-exposed to the previously heroin-paired conditioning chamber or returned to their home cage for 60 min. Heroin was not administered on test day in order to isolate the effect of the CS on immune responses. After the 60-min time period, all rats received a subcutaneous injection of LPS (1,000 µg/kg) and were immediately returned to their home cages. LPS, a component of the cell wall of Gram negative bacteria, was used to induce iNOS expression and NO production. Six hours following LPS administration, all animals were euthanized. The 6-h time point was selected based on previous research in our laboratory showing maximal iNOS induction 6 h following LPS administration (Lysle and How, 2000).

Histology

Samples of spleen and blood were collected for analysis. Spleen samples were either stored in an Ambion® RNA Later solution or Roche complete protease inhibitor cocktail

solution. To confirm proper cannula placement, Alcian blue dye was infused via the cannula. Brains were then extracted and flash frozen and at − 80 °C until further analysis. Coronal sections (40 μm) were taken and stained with cresyl violet for verification of cannula placement. The data of animals with cannula placement outside of the targeted region were removed from subsequent data analyses.

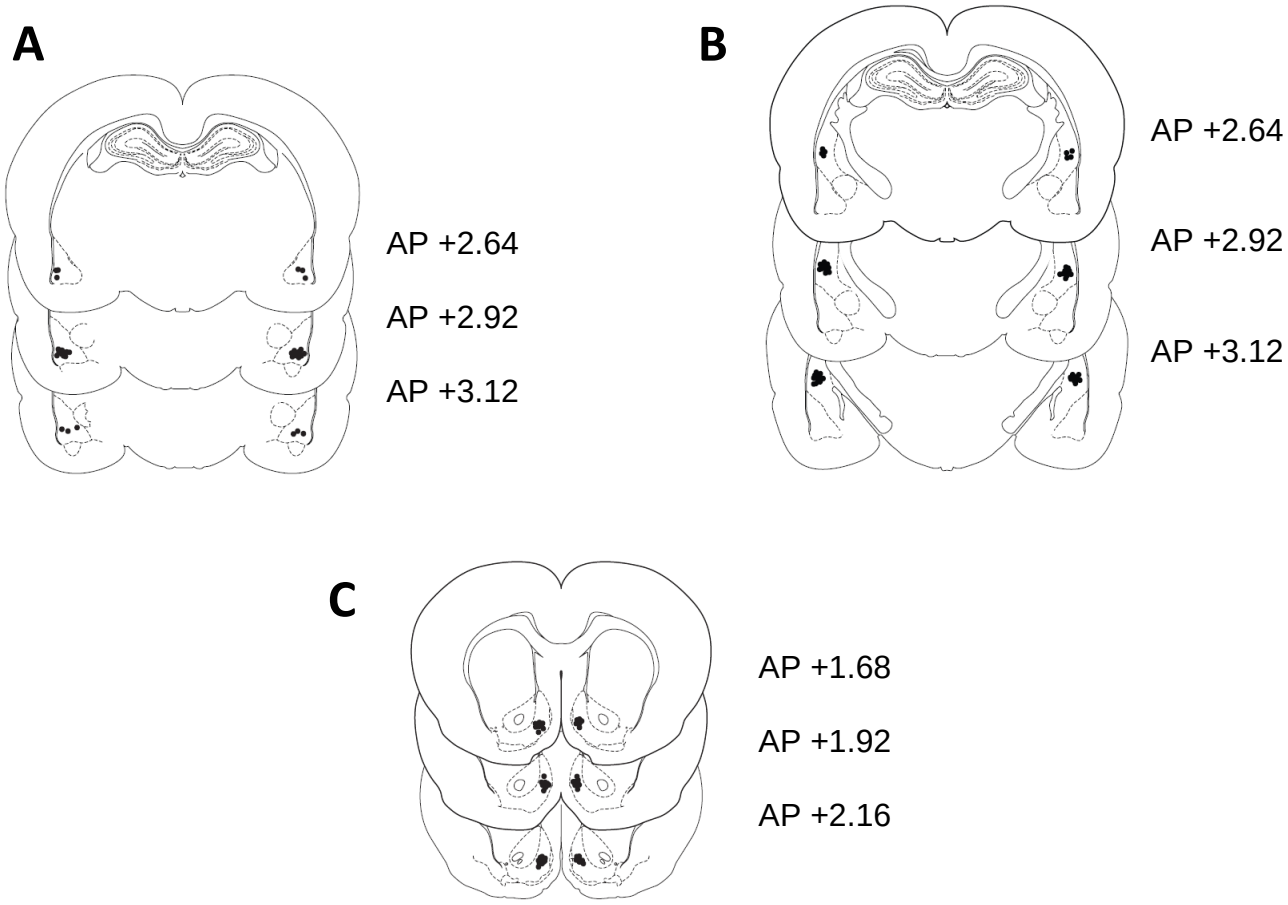

Figure 3.1: Illustration of verified cannula placement. Black dots represent the ventral most point of injector in the (A) BLA, (B) CPu, and (C) mNAcS.

Real-Time RT-PCR

Tissue was processed by the UNC Animal Clinical Chemistry and Gene Expression Laboratories according to protocols previously reported (Kim et al, 2002). In brief, spleen tissue

(50-100 mg) was homogenized in RNA lysis buffer (PE Biosystems, Foster City, CA) with Ca2and Mg2- free PB using a Fast Prep 120 mixer (QBIOgene, Vista, CA). RNA isolations were purified using the ABI Prism 6700 automated nucleic acid workstation (PE Biosystems) according to the manufacturer's protocol. Real-time RT-PCR amplifications were performed in the ABI Prism 7700 sequence detector (PE Biosystems) in a total volume of 30 µl (10 µl RNA plus 20 µl reaction mixture). RT-PCR amplification was performed in duplicate: 30 min at 48°C for the RT reaction and 10 min at 94°C followed by 40 temperature cycles (15 s at 94°C and 1 min at 60°C). Copy numbers were measured using the Sequence Detector Software in the ABI Prism 770 Sequence Detector System. Signal intensity was normalized to 18S as an endogenous control. The nucleotide sequences of the PCR primers and fluorogenic probes used for the iNOS and 18S genes were as follows: iNOS forward: 5'-AGCGGC TCC ATGACTCTC A-3', reverse: 5'-TGC CTGCACCCAAACACCAA-3', probe: 5'-FTCATGCGGCCTCCTTTGAGCCCTCQ-3'; 18S: forward: 5'-AGAAACGGCTACCACATCCA-3', reverse: 5'-CTCGAAAGAGTCCTGTATTGT-3', probe: 5'-FAGGCAGCAGGCGCGCAAATTACQ.

Nitrite/Nitrate Assay

The nitrite/nitrate concentration in plasma samples was assessed using the Greiss reagent assay described previously (Szczytkowski and Lysle, 2007). Briefly, 6 µl of plasma was diluted in 44 µl of dH2O, and the sample was incubated in the dark for 90 min with 10 µl of nitrate reductase (1.0 U/ml), 20 µl of 0.31M phosphate buffer (pH 7.5), 10 µl of 0.86mM NADPH (Sigma), and 10 µl of 0.11mM flavin adenine dinucleotide in individual wells of a 96-well plate. Next, 200 ml of Greiss reagent, consisting of a 1:1 (v/v) solution 1% sulfanilamide in 5.0% phosphoric acid and 0.1% N-(1-napthyl) ethyl-enedamine dihydrochloride in distilled water, was added to the samples. The color was allowed to develop for 10 min at room temperature, after

which, the absorbance was determined using a spectrophotometer set at 550 nm. All reactions were carried out in triplicate. The total micromolar concentration of nitrite was determined for each sample based on a standard curve. Recovery of nitrate is greater than 95% using this assay.

Statistical Analysis

Two-way analysis of variance was performed on all data sets. For all analyses, planned comparisons were performed in accordance with *a priori* hypotheses that IL-1Ra would attenuate the effect of the immunosuppressive effect of the CS on iNOS expression and NO production. All analyses were performed with the alpha level of significance set at $p < 0.05$.

Results

Effects of basolateral amygdala IL-1 antagonism on heroin-conditioned immunomodulaiton

Experiment 1 assessed the role of IL-1 signaling in the BLA in the expression of heroin-conditioned peripheral immune alterations. Figure 3.2a shows the effect of intra-BLA IL-1 antagonism on LPS-induced iNOS mRNA expression. ANOVA revealed a nearly significant context by treatment interaction in the BLA [$F(2, 16) = 4.21$, $p = .057$]. In addition, ANOVA revealed a significant main effect of re-exposure to the CS [$F(1, 16) = 8.37$, $p < .05$] on iNOS mRNA expression, demonstrating that re-exposure to the CS resulted in the suppression of iNOS and NO. Planned comparisons revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in iNOS expression compared to animals placed in home cages ($p < 0.005$), indicating that heroin-conditioned immunosuppression did occur in our saline treated groups. Furthermore, there was not a significant difference between IL-1Ra treated animals that were re-exposed to the CS and IL-1Ra treated animals that were returned to their home cage ($p >$

0.05), indicating that blockade of IL-1 receptor signaling attenuates heroin-conditioned immunosuppression of iNOS mRNA.

Figure 3.2b demonstrates the effect of intra-BLA IL-1 antagonism on LPS-induced nitrate/nitrite levels in blood plasma. ANOVA revealed a significant context by treatment interaction in the BLA [$F(2, 16) = 5.97, p < .05$]. In addition, ANOVA revealed a significant main effect of re-exposure to the CS [$F(1, 16) = 11.09, p < .005$] on nitrate/nitrite levels. Planned comparisons revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in nitrate/nitrite levels compared to animals placed in home cages ($p < 0.005$). Again, there was not a significant difference between IL-1Ra treated animals that were re-exposed to the CS and IL-1Ra treated animals that were returned to their home cage ($p > 0.05$), indicating that antagonism of IL-1 receptor signaling attenuates heroin-conditioned immunosuppression of nitrate/nitrite production.

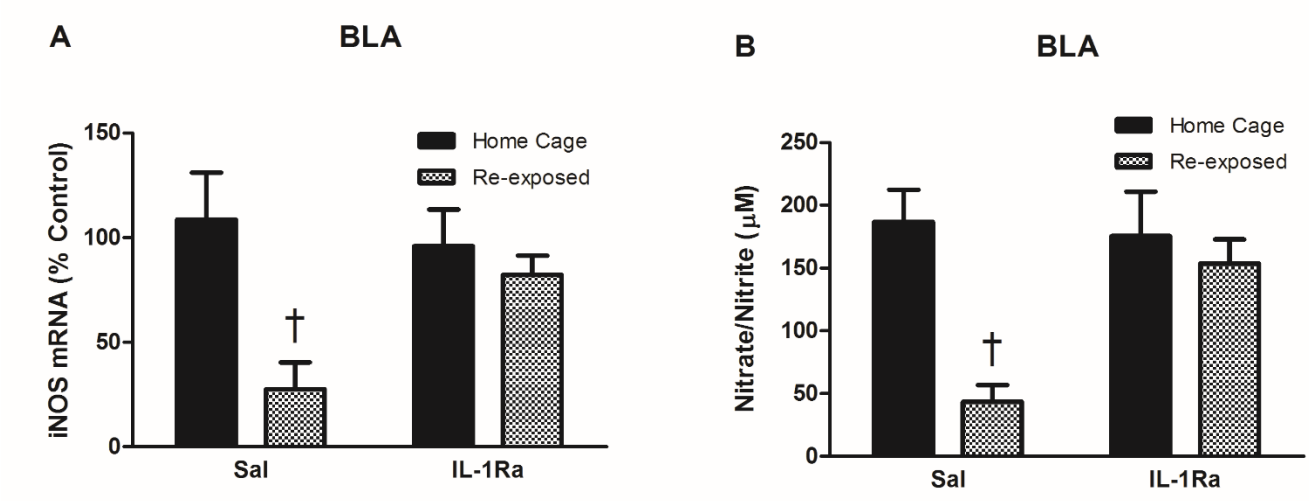

Figure 3.2: (A) Effects of BLA IL-1 antagonism on LPS-induced expression of iNOS mRNA as determined by real-time RT-PCR. (B) Effects of BLA IL-1 antagonism on LPS-induced nitrate/nitrite serum levels as determined by Greiss Reagent Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent SEM. † $p < 0.05$ compared with Home Cage-Saline group.

Effects of caudate putamen IL-1 antagonism on heroin-conditioned immunomodulaiton

Following the discovery that BLA IL-1 signaling mediates heroin-conditioned immunosuppression, experiment 2 was conducted to verify that these effects were specific to the BLA and not a result of diffusion of IL-1Ra into the CPu, which is located dorsal to the BLA. Figure 3.3a shows the effect of CPu IL-1 receptor blockade on LPS-induced iNOS mRNA expression. ANOVA revealed no significant context by treatment interaction in the mNAcS [$F(2,24) = .02$, $p > 0.05$]; however, the ANOVA revealed a significant main effect of re-exposure to the CS on iNOS mRNA expression [$F(1,24) = 16.89$, $p < 0.001$]. Planned comparisons revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in iNOS mRNA expression compared to animals placed in home cages ($p < 0.01$), confirming that our model produced conditioned immunosuppression in saline treated animals. Furthermore, IL-1Ra infusion had no effect on iNOS levels in animals re-exposed to the CS compared to saline treated animals re-exposed to the CS ($p < 0.05$), demonstrating that blockade of IL-1 receptor signaling does not attenuate heroin-conditioned immunosuppression of iNOS mRNA.

Figure 3.3b illustrates the effect of CPu IL-1 receptor antagonism on LPS-induced levels of nitrate/nitrite in blood plasma. One animal was excluded from the following analyses due to qualifying as an outlier as determined by Grubbs test. The ANOVA revealed a significant main effect of re-exposure to the CS on nitrate/nitrite plasma levels [$F(1,23) = 22.98$, $p < 0.0001$] and no significant context by treatment interaction [$F(2,23) = .02$, $p > 0.05$]. Planned comparisons revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in nitrate/nitrite levels compared to animals placed in home cages ($p < 0.005$). Importantly, there was a significant difference in nitrate/nitrite levels between in IL-1Ra treated animals that were

re-exposed to the CS compared to home cage ($p < 0.05$), indicating that CPu IL-1 does not mediate heroin-conditioned immunosuppression of NO production. Thus these data confirm that our findings following BLA IL-1Ra administration were not due to diffusion into the CPu.

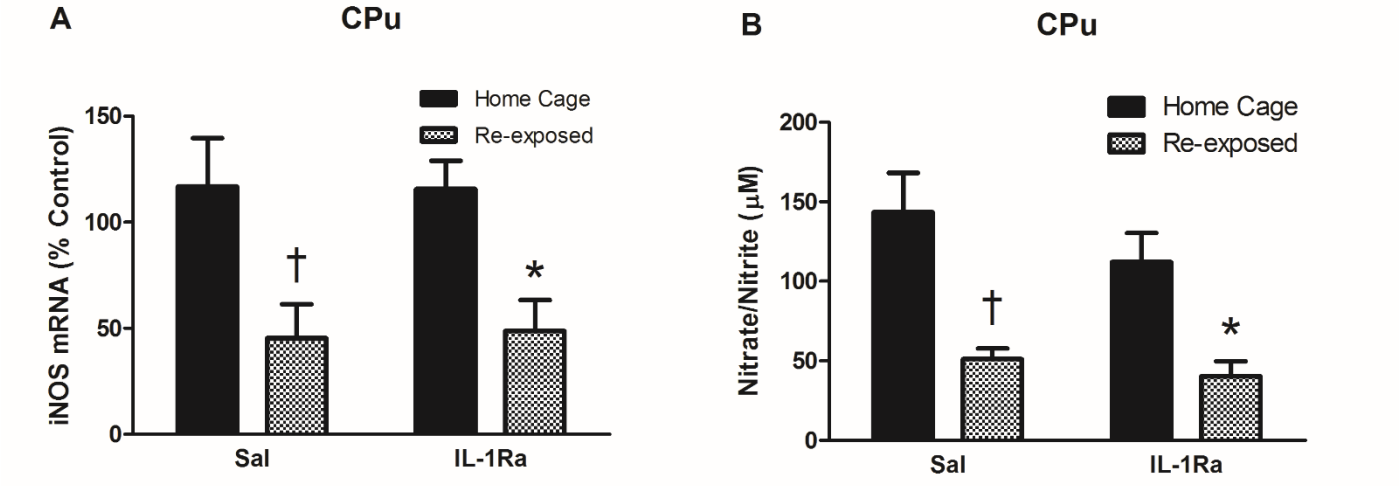

Figure 3.3: (A) Effects of CPu IL-1 antagonism on LPS-induced expression of iNOS mRNA as determined by real-time RT-PCR. (B) Effects of CPu IL-1 antagonism on LPS-induced nitrate/nitrite serum levels as determined by Greiss Reagent Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent SEM. † $p < 0.05$; *$p < 0.05$ compared with appropriate Home Cage control.

Effects of medial nucleus accumbens shell IL-1 antagonism on heroin-conditioned immunomodulation

Figure 3.4a depicts the effect of mNAcS IL-1 receptor blockade on LPS-induced iNOS mRNA expression. ANOVA revealed no significant context by treatment interaction in the mNAcS [$F(2,22) = 0.20$, $p > 0.05$]; however, ANOVA revealed a significant main effect of re-exposure to the CS on iNOS mRNA expression [$F(1,22) = 31.93$, $p < 0.0001$]. Planned comparisons revealed that saline-treated rats exposed to the heroin-paired context exhibited a reduction in iNOS expression compared to animals placed in home cages ($p < 0.005$). Furthermore, IL-1Ra treated animals re-exposed to the CS had significantly lower iNOS levels

compared to IL-1Ra treated home cage animals ($p < 0.05$), indicating that blockade of IL-1 receptor signaling had no effect on heroin-conditioned immunosuppression of iNOS mRNA.

Figure 3.4b represents the effect of mNAcS IL-1 receptor antagonism on LPS-induced levels of nitrate/nitrite in blood plasma. The ANOVA revealed a significant main effect of re-exposure to the CS on nitrate/nitrite plasma levels [$F(1,22) = 6.80$, $p < 0.05$] and no significant context by treatment interaction [$F(2, 22) = 2.09$, $p > 0.05$]. Planned comparisons revealed that IL-1Ra did not alter the immunosuppressive effect of the CS compared to home cage treated animals ($p < 0.05$), demonstrating that mNAcS IL-1 does not mediate heroin-conditioned immunosuppression of iNOS mRNA expression and NO production.

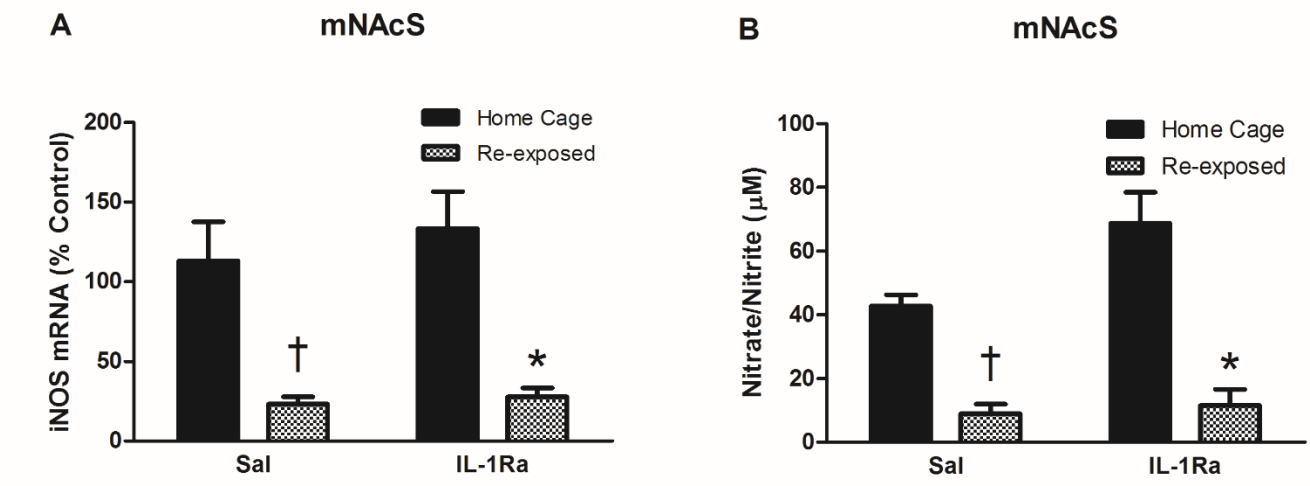

Figure 3.4: (A) Effects of mNAcS IL-1 antagonism on LPS-induced expression of iNOS mRNA as determined by real-time RT-PCR. (B) Effects of mNAcSIL-1 antagonism on LPS-induced nitrate/nitrite serum levels as determined by Greiss Reagent Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent SEM. † $p < 0.05$; *$p < 0.05$ compared with appropriate Home Cage control.

Discussion

It is well established that re-exposure to a previously heroin-paired context results in immunosuppressive effects that are similar to heroin administration alone. The studies presented in the current chapter indicate that blockade of IL-1 receptor signaling in the BLA, but not the mNAcS or CPu, mediates the immunosuppressive effects of a heroin-paired context on iNOS mRNA expression and NO production. These studies are the first to identify BLA IL-1 as an important mediator in the expression of learned associations and, specifically, heroin-conditioned immunosuppression.

The mechanisms by which IL-1 mediates the expression of heroin-conditioned immunosuppression are unknown. One potential contributor to this effect is microglial cell activation. It is understood that the primary source of IL-1β in the brain is glial cells. Microglia indirectly modulate neuronal transmission via the release of astroglial modulators, which subsequently act upon neighboring astrocytes. A wealth of evidence has accumulated indicating that microglia synthesize and release neuro- and glialmodulators, such as IL-1, which has been shown to be critical mediators of neurotransmission. For instance, *in vitro* IL-1β application produces electrophysiological changes in various brain regions, such as modulation of inhibitory transmission in the hippocampus (Zeise et al., 1992, Zeise et al., 1997, Wang et al., 2000), amygdala (Yu and Shinnick-Gallagher, 1994, Bajo et al., 2015), cortex (Miller and Fahey, 1994, Miller et al., 1991, Pringle et al., 1996), and hypothalamus (Feleder et al., 1998). In addition, IL-1β affects excitatory transmission in the cortex (Miller and Fahey, 1994), hippocampus (Bellinger et al., 1993, Cunningham et al., 1996, Coogan and O'Connor, 1997, Luk et al., 1999), and amygdala (Yu and Shinnick-Gallagher, 1994). Based on the regions of interest and experimental procedures used in these studies, it appears that IL-1 receptor activation modulates

neurotransmission in a site-specific and concentration-dependent manner. Thus, it is clear that the role of IL-1 signaling is complex and requires further study to elucidate the mechanisms and conditions under which IL-1 exerts its physiologic and pathophysiologic effects.

The observation that BLA IL-1 is important for the expression of heroin-conditioned immunosuppression further supports the significance of IL-1 signaling in models of learning and memory. Surprisingly, there is a dearth of studies investigating the effect of BLA IL-1 in these models. However, there has been extensive research conducted investigating IL-1 within the hippocampus and its role in cognitive function. Findings indicate that IL-1 is important for memory consolidation and the induction of LTP (Schneider et al., 1998, O'Connor and Coogan, 1999, Coogan et al., 1999). In addition, inhibition of IL-1 signaling by genetic or pharmacological means results in learning and/or memory impairments. For example, a study conducted in our laboratory found that inhibition of IL-1β mRNA expression in the dorsal hippocampus prior to re-exposure to a previously heroin-paired context attenuated the conditioned immunosuppressive effect on NO production and iNOS mRNA expression, indicating that IL-1β is an important mediator in heroin-conditioned immunosuppression (Szczytkowski et al., 2013).

The lack of an effect of mNAcS IL-1 receptor antagonism is somewhat surprising. The dense IL-1β immunoreactivity in the mNAcS reported in Chapter 1 is indicative of microglia activity, which induces IL-1β expression. In addition, a previous study reported significant microglial activation following morphine CPP (Zhang et al., 2012), suggesting that glialmodulators could play a role in drug-context associative learning. Furthermore, a study in our laboratory demonstrated that antagonism of dopamine in the mNAcS blocks heroin-conditioned immunosuppression. Interestingly, Song and colleagues (1998) suggested that IL-1β

may modulate dopamine activity in the NAc. Following IL-1β administration, the typically observed decline of the dopamine metabolite, dihydroxyphenylacetic acid, in saline treated animals was prevented, suggesting an increase in dopamine release. However, it's unknown whether this effect was due to inefficient metabolite clearance or increased dopamine levels. Nevertheless, BLA IL-1 signaling could potentially modulate NAc dopamine, as stimulation of BLA glutamatergic efferents to the NAc have been shown to result in dopamine release (Floresco et al., 1998). Further studies are needed in order to elucidate the cell types and mechanisms by which IL-1 mediates these conditioned effects. In addition, a larger dose of IL-1Ra may prove to result in the suppression of other proinflammatory mediators in addition to NO production and iNOS mRNA expression.

CHAPTER 4

THE ROLE OF BASOLATERAL AMYGDALA INTERLEUKIN-1β SIGNALING IN THE INDUCTION OF PERIPHERAL IMMUNOSUPPRESSION

Introduction

The studies discussed in Chapter 3 indicate that IL-1β signaling in the BLA is necessary for the expression of heroin-conditioned immunosuppression of NO production and iNOS expression. While I demonstrated that these effects are due to re-exposure to the heroin-paired context, it is unknown whether IL-1β signaling alone in the BLA can induce peripheral immune alterations. Therefore, the current study sought to determine the effects of BLA recombinant rat IL-1β (rrIL-1β) administration on the production of NO and expression of iNOS.

Interestingly, centrally administered IL-1β has been shown to modulate peripheral immune function. For instance, i.c.v. IL-1β administration produced a reduction in NK cell activity and IL-2 production in blood and spleen tissue. These effects were not observed following peripheral IL-1β administration, suggesting centrally active IL-1β is important for the induction of peripheral immunosuppression (Sundar et al., 1989). These findings indicate that IL-b mediates peripheral immunosuppression, but the mechanisms mediating this effect have not been well characterized.

As discussed in Chapter 3, IL-1β has been shown to produce many electrophysiological changes *in vitro*. In addition, IL-1β modulates neurotransmitter release in various brain regions *in vivo*. For example, i.c.v. infusions of IL-1β decrease norepinephrine in the amygdala and

hippocampus, increase serotonin in the amygdala, and increase dopamine in the midbrain, hippocampus and amygdala (Song et al., 2006). Furthermore, IL-1β potentiates the release of dopamine, norepinephrine and serotonin in the hypothalamus (Mohankumar et al., 1991, Shintani et al., 1993, Deleplanque et al., 1994). These data indicate that IL-1β signaling has widespread effects on neurobiolgoical function.

IL-1β's effects on dopamine are of particular interest to the current line of research. Dopamine signaling has been previously shown to mediate peripheral immunosuppression. Lesioning of nigrostriatal and mesolimbic dopaminergic neurons impairs lymphocyte proliferation and NK cell activity (Deleplanque et al., 1994). Moreover, stimulation of dopamine D_1 receptors in the nucleus accumbens and hippocampus has been shown to reduce splenic lymphocyte proliferation (Nistico et al., 1994). Furthermore, previous studies in our laboratory have demonstrated that morphine and heroin's immunosuppressive effects are mediated by central dopamine signaling. Pharmacological blockade of dopamine D_1 receptors in the mNAcS attenuated the immunosuppressive effect of heroin and morphine on NK cell activity and iNOS expression, whereas stimulation of mNAcS D_1 receptors produced reductions in NK cell activity similar to opioid administration alone (Saurer et al., 2004). Evidence demonstrating that stimulation of BLA glutamatergic efferents increases dopamine release in the NAc suggests that BLA neurotransmission can modulate NAc dopamine (Floresco et al., 1998). Thus, given IL-1β's ability to modulate amygdalar dopamine (Song et al., 2006), astrocytes (Jing et al., 2010), it is possible that IL-1β modulates BLA efferent synaptic transmission of dopamine. In theory, BLA IL-1β could produce an immunosuppressive effect similar to opiate administration.

Little is known about the effect IL-1β signaling in the BLA. Exogenous application of IL-1β to brain slices has been shown to modulate excitatory and inhibitory BLA neurotransmission.

These effects appear to mediated by presynaptic mechanisms, as IL-1β application inhibited both excitatory and inhibitory postsynaptic potentials (EPSPs/IPSPs) induced by stimulation of stria terminalis or lateral amygdala afferents but not GABA or glutamate receptor postsynaptic actions within the BLA (Yu and Shinnick-Gallagher, 1994). It is unknown how these effects influence BLA efferent signaling mediated by other projections to the BLA, such as dopaminergic projections from the VTA. These BLA dopaminergic afferents could produce inhibitory or excitatory signaling or both depending on the region receiving BLA projections and cell type being modulated by IL-1β. This possibility is supported by findings from Yu and colleagues (1994) based on the observation that EPSP amplitude was increased in some neurons despite IL-1β-induced inhibition, suggesting that disinhibition may result in certain neuronal populations. Disinhibition of BLA neurons, such as glutamatergic efferents projecting to the mNAcS, could affect neurotransmitters known to mediate peripheral immunosuppression, such as dopamine (Saurer et al., 2004). In order to test this hypothesis, NO production and iNOS mRNA expression were assessed in animals receiving intra-BLA infusions of various doses of rrIL-1β.

Materials and Methods

Animals

Male Lewis rats, weighing 225–250 g, were purchased from Charles River Laboratories (Raleigh, NC, USA). Upon arrival, animals were housed individually in plastic cages in a colony room with a reversed light-dark (12-h) cycle maintained through artificial illumination. Animals were allowed access to food and water *ad libitum* throughout the experiment except for the time spent in the conditioning chambers when food and water were not available. All animals were given a 2-week habituation period before the start of experimental manipulations and were handled regularly during this time. All procedures described were approved by the IACUC of the

University of North Carolina at Chapel Hill and conformed to NIH guidelines on the care and use of laboratory animals.

Surgical Procedures

Animals were fully anesthetized with 0.35 mL intramuscular injections of 1:1 (vol:vol) ketamine hydrochloride (100 mg/mL) mixed with xylazine (20 mg/mL) and placed into the stereotaxic apparatus. Animals were implanted bilaterally with 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA). The cannulae were directed towards the BLA (AP −2.5, ML ± 5.0, DV −6.6 mm, relative to bregma). Animals were given a 2-week post-surgical recovery period before experimentation began.

Test of Recombinant IL-1β-induced Peripheral Immunosuppression

One week following surgery animals received bilateral site-specific microinfusions of 0, 1, 10, or 100ng of recombinant rat IL-1β (0.5 μl per hemisphere). Injectors extended 2 mm beyond the tip of the guide cannula. Injections were delivered over 2 min, and the injectors were left in place for 1 min after the injection to allow for proper diffusion of fluid away from the infusion site. Thirty minutes following microinfusions all rats received a subcutaneous injection of LPS (1,000 μg/kg) and were immediately returned to their home cages. LPS, a component of the cell wall of Gram negative bacteria, was used to induce iNOS production. Six hours after LPS administration, all animals were euthanized. The 6-h time point was selected based on previous research in our laboratory showing maximal iNOS induction 6 h following LPS administration (Lysle and How, 2000).

Histology

Samples of spleen and blood were collected for analysis. Spleen samples were either stored in an Ambion® RNA Later solution or Roche complete protease inhibitor cocktail solution. To confirm proper cannula placement, Alcian blue dye was infused via the cannula. Brains were then extracted and flash frozen and at − 80 °C until further analysis. Coronal sections (40 μm) were taken and stained with cresyl violet for verification of cannula placement. The data of animals with cannula placement outside of the targeted region were removed from subsequent data analyses.

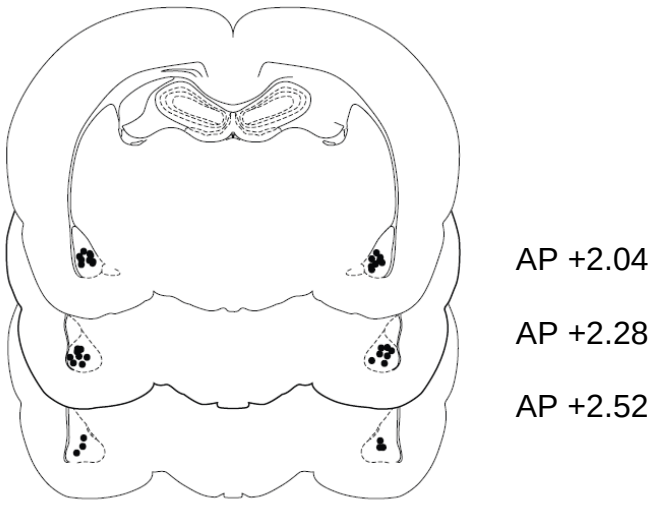

Figure 4.1: Illustration of verified cannula placement. Black dots represent the ventral most point of injector in the BLA.

Real-Time RT-PCR

Tissue was processed by the UNC Animal Clinical Chemistry and Gene Expression Laboratories according to protocols previously reported (Kim et al, 2002). In brief, spleen tissue (50-100 mg) was homogenized in RNA lysis buffer (PE Biosystems, Foster City, CA) with Ca2 and Mg2- free PB using a Fast Prep 120 mixer (QBIOgene, Vista, CA). RNA isolations were

purified using the ABI Prism 6700 automated nucleic acid workstation (PE Biosystems) according to the manufacturer's protocol. Real-time RT-PCR amplifications were performed in the ABI Prism 7700 sequence detector (PE Biosystems) in a total volume of 30 µl (10 µl RNA plus 20 µl reaction mixture). Each RT-PCR amplification was performed in duplicate: 30 min at 48°C for the RT reaction and 10 min at 94°C followed by 40 temperature cycles (15 s at 94°C and 1 min at 60°C). Copy numbers were measured using the Sequence Detector Software in the ABI Prism 770 Sequence Detector System. Signal intensity was normalized to 18S as an endogenous control. The nucleotide sequences of the PCR primers and fluorogenic probes used for the iNOS and 18S genes were as follows: iNOS forward: 5'-AGCGGC TCC ATGACTCTC A-3', reverse: 5'-TGC CTGCACCCAAACACCAA-3', probe: 5'-FTCATGCGGCCTCCTTTGAGCCCTCQ-3'; 18S: forward: 5'-AGAAACGGCTACCACATCCA-3', reverse: 5'-CTCGAAAGAGTCCTGTATTGT-3', probe: 5'-FAGGCAGCAGGCGCGCAAATTACQ.

Nitrite/Nitrate Assay

The nitrite/nitrate concentration in plasma samples was assessed using the Greiss reagent assay described previously (Szczytkowski and Lysle, 2007). Briefly, 6 µl of plasma was diluted in 44 µl of dH2O, and the sample was incubated in the dark for 90 min with 10 µl of nitrate reductase (1.0 U/ml), 20 µl of 0.31M phosphate buffer (pH 7.5), 10 µl of 0.86mM NADPH (Sigma), and 10 µl of 0.11mM flavin adenine dinucleotide in individual wells of a 96-well plate. Next, 200 ml of Greiss reagent, consisting of a 1:1 (v/v) solution 1% sulfanilamide in 5.0% phosphoric acid and 0.1% N-(1-napthyl) ethyl-enedamine dihydrochloride in distilled water, was added to the samples. The color was allowed to develop for 10 min at room temperature, after which, the absorbance was determined using a spectrophotometer set at 550 nm. All reactions

were carried out in triplicate. The total micromolar concentration of nitrite was determined for each sample based on a standard curve. Recovery of nitrate is greater than 95% using this assay.

Statistical Analysis

One-way analysis of variance was performed on iNOS mRNA expression and nitrate/nitrite levels. All analyses were performed with statistical significance set at $p < .05$.

Results

Effects of intra-basolateral amygdala recombinant rat IL-1β administration

Figure 4.2 shows the effect of intra-BLA administration of rrIL-1β on LPS-induced iNOS mRNA expression following re-exposure to the heroin-paired context or home cage. ANOVA revealed no significant differences between groups receiving different doses or rrIL-1β [$F(3,21) = 2.47$, $p > 0.05$]. Similarly, figure 4.3 illustrates the effect of intra-BLA administration of rrIL-1β on LPS-induced nitrate/nitrite production in blood plasma. Again, ANOVA revealed no significant differences between groups across various doses [$F(3,21) = 2.03$, $p > 0.05$]. Taken together, these data suggest that intra-BLA rrIL-1β administration does not alter peripheral immune function at the doses tested.

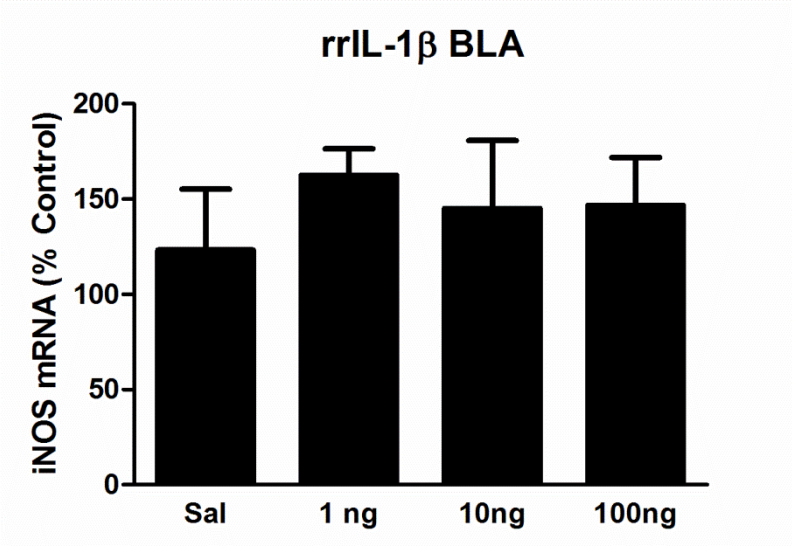

Figure 4.2: Effects of BLA recombinant rat IL-1β on LPS-induced iNOS mRNA as determined by Greiss Reagent Assay. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent SEM.

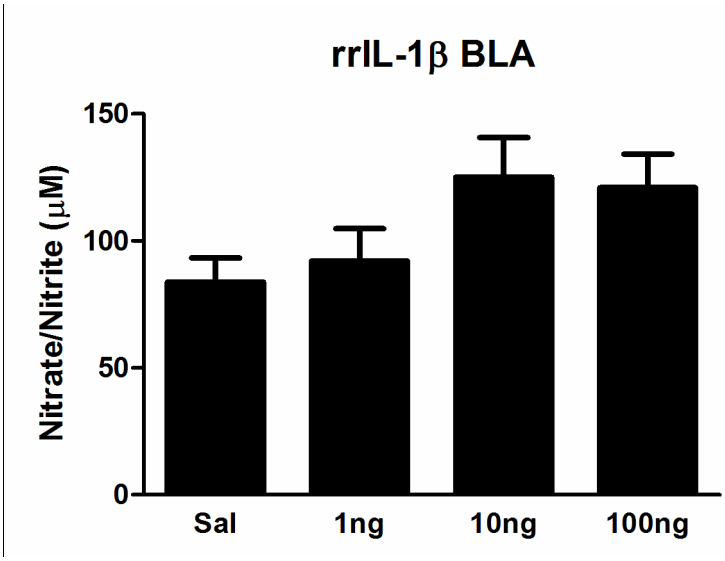

Figure 4.3: Effects of BLA recombinant rat IL-1β on LPS-induced expression of nitrate/nitrite serum levels as determined by real-time RT-PCR. The data are expressed as the mean micromolar concentration of nitrite/nitrate. The error bars represent SEM.

Discussion

The study presented in the current chapter investigated the role of BLA IL-1β signaling in the modulation of peripheral immune alterations. Animals received intra-BLA administration of various doses of rrIL-1β in order to determine if IL-1β expression in the BLA is capable of

modulating NO production and iNOS expression following an immunologic challenge. Our findings indicate that intra-BLA administration of IL-1β has no effect on iNOS mRNA expression or NO production.

It is well established that IL-1β plays a role in learning and memory (Schneider et al., 1998, Goshen et al., 2007, Yirmiya et al., 2002). However, the role of IL-1β signaling is complex, producing diverse and even opposing effects on synaptic transmission in different CNS tissue. For example, IL-1β has been shown to enhance the frequency and amplitude of excitatory post synaptic currents in the spinal cord, indicating that it can potentiate synaptic transmission (Kawasaki et al., 2008), whereas certain levels of IL-1β expression blocks LTP in the hippocampus *in vitro* (Katsuki et al., 1990, Bellinger et al., 1993, Cunningham et al., 1996) and *in vivo* (Murray and Lynch, 1998, O'Connor and Coogan, 1999) rather than facilitating glutamatergic synaptic transmission. Furthermore, *in vitro* evidence suggests that IL-1β has vast effects on mechanisms that modulate LTP, such as inhibition of hippocampal calcium ($Ca2^{+)}$ influx and glutamate release (Murray and Lynch, 1998) and increased chloride transport and inhibition of $Ca2^{+}$ increases in cortical synaptosomes (Miller and Fahey, 1994). These contradictory and region-specific findings remain to be understood in regards to how IL-1β modulates synaptic transmission in CNS tissue. Thus, it is difficult to speculate on how IL-1β may be acting in the BLA. However, based on these previous studies, it's possible that exogenous intra-BLA administration acts to inhibit BLA excitatory signaling, thus inhibiting, instead of stimulating, neurotransmitter release in BLA-projecting regions, such as the mNAcS. If this is, in fact, what is taking place, it would be interesting to investigate the role of intra-BLA IL-1β administration in the expression of heroin-conditioned immunosuppression. Functional inactivation of the BLA using a GABA agonist cocktail has been shown to block heroin-

conditioned immunosuppression (Szczytkowski and Lysle, 2008); therefore, I would expect to see a similar effect following exogenous IL-1β administration.

It is also possible that intra-BLA IL-1β administration did produce suppression of the peripheral immune response, but due to the doses of IL-1β used this effect could have been washed out. IL-1β has been shown to induce the expression of iNOS and production of nitric oxide. In addition, IL-1β has been reported to damage the blood-brain barrier, which could cause IL-1β to leak into the bloodstream and migrate to peripheral organs, such as the spleen. This leakage into the periphery could then stimulate the expression of iNOS and production of NO, causing levels to return to those normally observed in control animals. Further studies are needed to investigate whether these doses of IL-1β do indeed affect blood-brain barrier permeability in the BLA. If IL-1β is affecting blood-brain barrier integrity, a lower dose range of IL-1β would be needed to explore the effects of IL-1β-induced peripheral immunosuppression. Finally, it would be interesting to characterize the role IL-1β in the VTA and mNAcS, as IL-1β's inhibitory effects could result in disinhibition of VTA dopaminergic projections and modulate dopamine release in the mNAcS.

CHAPTER 5

GENERAL DISCUSSION

Experimental Findings

The studies presented in this dissertation offer novel insights into a neurobiological mechanism mediating the conditioned immunosuppressive effects of heroin. Over the past two decades research has discovered the involvement of many traditional neuromodulators responsible for the immunosuppressive effects of opiates, with some these neuromodulators also being necessary for the expression of heroin-conditioned immunosuppression. However, the current studies investigated the importance of a non-traditional neuromodulator, IL-1, in our established model of heroin-conditioned immunosuppression. Several lines of research have begun to characterize the role of IL-1 signaling in normal and pathological biological function, including learning and memory, but the mechanisms mediating IL-1-induced memory impairments are poorly understood. Thus, the studies presented in this dissertation sought to offer insight into a novel mechanism mediating heroin-conditioned immunomodulation.

Chapters 2 and 3 discuss experiments exploring the role of IL-1 in a model of heroin-conditioned immunosuppression. Our laboratory has demonstrated that repeatedly pairing an immune altering stimulus (e.g. heroin) with a neutral stimulus causes the previously neutral stimulus to elicit immune altering effects *per se*. Subsequently, numerous studies were conducted investigating the neural circuit mediating this phenomenon. For example, the functional integrity of the VTA, BLA, mNAcS and dorsal hippocampus have been demonstrated to be necessary for

the expression of heroin-conditioned immunosuppression (Szczytkowski et al., 2011, Szczytkowski et al., 2013, Szczytkowski and Lysle, 2008, Saurer et al., 2008a, Hutson et al., 2014). In addition, our laboratory demonstrated that blockade of dopamine D_1 receptors in the BLA and mNAcS prior to re-exposure to a previously drug-paired context results in the attenuation of heroin-conditioned immune suppression of various immune parameters (Saurer et al., 2008a, Szczytkowski and Lysle, 2010). Thus, these studies identified several mesolimbic neural substrates important in the expression of our conditioned effects.

Previous research into the neurobiological mechanisms of IL-1 signaling has offered valuable insights into its potential effects on neural processing. Interestingly, i.c.v. IL-1β administration altered dopamine concentrations in the amygdala (Song et al., 2006), suggesting that IL-1β signaling could play a role in dopamine transmission. In addition, IL-1 has been shown to have a critical role in neural plasticity, learning and memory. For instance, IL-1β mRNA expression is elevated following fear conditioning (Goshen et al., 2007) and is necessary for the induction and maintenance of LTP, as blockade of IL-1 signaling attenuated LTP (Schmid et al., 2009). Moreover, blockade of IL-1 signaling impaired memory of passive avoidance behavior and contextual fear conditioning (Yirmiya et al., 2002, Goshen et al., 2007). Lastly, genetic deletion of IL-1 receptors and IL-1Ra disrupted learning in a spatial memory paradigm (Avital et al., 2003). In contrast to these findings, excessive IL-1β expression also results in memory impairments. Bath application of IL-1β has been shown to inhibit LTP in hippocampal slices. In addition, IL-1β administration prior to exposure to a spatial water maze task impaired memory (Oitzl et al., 1993). Furthermore, IL-1β infused into the ventricles or dorsal hippocampus following fear conditioning resulted in impaired contextual memory (Goshen et al., 2007, Jones et al., 2015). Taken together, it is evident that basal levels of IL-1 are

necessary for normal physiologic function and deviation from these levels results in deficits in learning and memory. Based on these findings, the current studies sought to determine whether BLA and mNAcS IL-1 signaling was necessary for the expression of heroin-conditioned immunosuppression.

As discussed in Chapter 2, prior to studying the role of IL-1 in heroin-conditioned immunomodulation, I performed an experiment to verify the presence of IL-1β in the BLA and mNAcS. In addition, I aimed to determine the time course of IL-1β expression following re-exposure to a heroin-paired context. Animals received five conditioning sessions, with each session separated by 48-hrs, where 1 mg/kg heroin was administered immediately prior to placement into a distinct context. Six days after the final conditioning session, rats were sacrificed following a 0, 15, 60 or 120 min (re-exposure for 60 minutes then returned to home cage for 60 min) re-exposure to the heroin-paired context. Immunohistochemical analysis of IL-1β immunoreactivity in the BLA and mNAcS revealed that IL-1β is expressed at all time points and at similar levels, indicating that IL-1 signaling is active within these brain regions six days following heroin conditioning. These findings are in line with previous reports indicating dense IL-1 receptor expression in the BLA (Konsman et al., 2000, Yabuuchi et al., 1994b). I hypothesized that IL-1β expression would be elevated as a result of re-exposure to a heroin-paired context based on findings demonstrating increased microglia activation following re-exposure to a morphine-paired context in a model of CPP (Schwarz et al., 2011). Therefore, it is possible that re-exposure to a heroin-paired context stimulates microglial activation and results in subsequent increased IL-1β expression. However, based on our findings, it does not appear that BLA or mNAcS IL-1β levels were increased following re-exposure to the heroin-paired context.

It is well documented that opiate administration activates microglial cells (Milligan and Watkins, 2009). Thus, it is possible that this activated state could persist long after morphine exposure. In fact, persistent microglial activation was found five days following morphine administration in a model of CPP (Zhang et al., 2012), indicating that microglial activation on test day in the studies discussed in this dissertation is possible, as brain tissue was collected at a similar time point. Consequently, the lack of differences in IL-1β levels in the BLA and mNAcS could be due to persistent microglia activity resulting from heroin conditioning. In order to determine whether IL-1β levels were elevated above those of naïve animals, control studies are needed to determine if repeated administration of heroin and/or exposure to the conditioned stimulus alone results in on long-term microglial activity.

The series of experiments conducted in chapter 3 investigated the role of IL-1 signaling in heroin-conditioned immunomodulation. As previously discussed, our laboratory has identified the BLA and mNAcS as important neural substrates mediating the expression of this conditioned effect. Interestingly, morphine-associated contexts have been shown to induce microglial activation upon re-exposure to the drug-paired context (Schwarz et al., 2011). Moreover, NFκ-b expression, which is a pathway responsible for IL-1β production and release in glial cells, is necessary for the acquisition of morphine CPP (Zhang et al., 2011). Taken together, these findings demonstrate that mechanisms involved in IL-1β release are important for associative learning. Therefore, to test the hypothesis that IL-1 signaling in the BLA and mNAcS mediates heroin-conditioned immunosuppression, animals underwent the same conditioning regimen discussed above. At least six days following the final conditioning session, animals received bilateral microinfusions of saline vehicle or IL-1Ra into the BLA or mNAcS and then returned to their home cage or re-exposed to the previously heroin-paired context for 60 minutes. Following

re-exposure, animals were then administered a subcutaneous injection of LPS to induce the immune response. Six hours later brain, blood plasma and spleen tissue were collected from each subject. Interestingly, blockade of IL-1signaling with IL-1Ra in the BLA, but not mNAcS or CPu, attenuated the immunosuppressive effects of the heroin-paired context on NO production and iNOS expression. These findings demonstrate for the first time that IL-1 signaling in the BLA is necessary for the expression of heroin-conditioned immunosuppression. In fact, to my knowledge, this is the first study to demonstrate that BLA IL-1 is an important mechanism mediating the expression of any conditioned response. Our laboratory previously found similar results by inhibiting IL-1β mRNA expression in the dorsal hippocampus, indicating that IL-1-mediated expression of heroin-conditioned immunosuppression is not limited to the BLA.

Based on the findings presented in Chapter 3 demonstrating that blockade of BLA IL-1 receptors attenuated the expression of heroin-conditioned immunomodulation, I wanted to determine if BLA IL-1β signaling could modulate the induction of peripheral immunosuppression. Interestingly, i.c.v. IL-1β administration has been previously shown to suppress the peripheral immune response (Sundar et al., 1989). Therefore, I investigated the immunomodulatory role of BLA IL-1β signaling alone in response to an immune challenge. Our findings show that various doses of rrIL-1β administration do not suppress peripheral NO production or iNOS mRNA expression. These findings oppose what Sundar and colleagues (1989) observed, although different proinflammatory mediators were measured. This difference could be due to several factors. First, it is unknown which brain region(s) mediate the peripheral immunosuppessive effect of i.c.v. IL-1β. One possibility is the hypothalamus, which has been shown to increase IL-1 receptor expression following immune challenge (Ilyin et al., 1998) and modulate NK cell activity and lymphocyte proliferation via vagal nerve stimulation (Okamoto et

al., 1996). In addition, IL-1β stimulates the release of corticotropin releasing hormone (Berkenbosch et al., 1987, Sapolsky et al., 1987), which is a critical mediator of the stress response. Our laboratory has demonstrated that stimulation of the stress response via the hypothalamic-adrenal-pituitary axis suppresses NK cell activity, indicating that activation of the stress pathway can result in peripheral immunosuppression (Perez and Lysle, 1995). Lastly, excessive IL-1β expression can cause damage to the blood-brain barrier, which could have provided a means for BLA-infused rrIL-1β to leak into the bloodstream and migrate to peripheral organs (Quagliarello et al., 1991). Thus, if intra-BLA IL-1β infusion reduced NO production or iNOS mRNA expression, these levels could have returned to those of control animals due to the fact that IL-1β stimulates the production of NO in the periphery.

Potential Mechanisms

Neuro-Glial Communication

While much is unknown about the mechanisms by which central IL-1 modulates neurotransmission, recent studies have offered a great deal of insight into the complex signaling processes that occur during synaptic transmission. Since IL-1β is synthesized and released primarily from glial cells, there is an ongoing effort to characterize the role that glia play in IL-1 signaling. Interestingly, there appears to be little evidence of direct effects of microglia on neurotransmission. Thus, it is likely that the neuromodulatory role of microglia-released molecules is mediated via astrocytic activation.

Gliotransmission is defined as the release of gliotransmitters by astrocytes, which include D-serine, IL-1β, TNF-α, glutamate, adenosine triphosphate (ATP), lactate, among others. These gliotransmitters act to modulate neuronal transmission and plasticity via activation of G_q GPCRs

and subsequent increases in $Ca2^{+}$ levels. However, it is unclear whether $Ca2^{+}$ release alone is sufficient to modulate neural processing, as studies have reported that inhibition of $Ca2^{+}$ in astrocytes has no effect on synaptic activity (Fiacco et al., 2007, Petravicz et al., 2008, Agulhon et al., 2010). Recently, proinflammatory mediators have been implicated as playing a critical role in astrocyte-mediated neuronal activation. Evidence suggests that activated microglia release proinflammatory cytokines, which then activate astrocytes and induce glutamate release (Bezzi et al., 2001). Thus, proinflammatory cytokines may act in concert with basal Ca2+ signaling to modulate neuronal excitability.

IL-1 has been identified as a critical mediator of microglia-induced astrocyte activation, as central IL-1 administration causes an upregulation of glial fibrillary acidic protein (GFAP), which is a marker of astrocyte activation (Balasingam et al., 1994; Lee et al., 2010). IL-1 has also been reported to upregulate the proinflammatory cytokines IL-6 and TNF-α (John et al., 2004). Conversely, blockade of IL-1 signaling inhibits microglia-induced TNF-α release (Basu et al., 2002), indicating that IL-1 modulates microglia-derived proinflammatory cytokine release. In addition, IL-1 dose-dependently attenuates astrocytic glutamate uptake, which results in excessive synaptic activity (Jing et al., 2010). These molecules subsequently induce astrocytic activation and, in turn, further release proinflammatory cytokines in an autocrine/paracrine fashion, which directly modulate neuronal activity.

One study recently demonstrated that LPS-induced microglial activation increased hippocampal excitatory synaptic transmission. This effect was found to involve microglia-initiated activation of metabotropic P2Y purinoceptor 1(P2Y1) receptors on astrocytes, which then stimulated the release of glutamate from astrocytes to modulate synaptic metabotropic glutamate receptors (Pascual et al., 2012). Interestingly, ATP-induced P2Y1 receptor activation

of astrocytes can also increase TNF-α, which has been reported to play a role in neural plasticity and learning (Beattie et al., 2002). These increases in TNF-α subsequently produced elevated glutamate release (Domercq et al., 2006, Santello et al., 2011). Taken together, evidence suggests that IL-1 activates microglial cells and may initiate microglia-astrocyte communication via autocrine/paracrine signaling pathways, resulting in the initiation and enhancement of intercellular astrocytic $Ca2^{+}$ signaling and subsequent facilitation of increased neuronal excitation.

TLR4-Dependent Activation of Microglia

A potential mechanism mediating BLA IL-1 signaling is the TLR4 receptor. Of the 12 members of the TLR family, TLR4 is the most studied subtype. It is activated by LPS and endogenous danger signals, such as residual fragments released from stressed or damaged cells (Buchanan et al., 2010). TLR4 is composed of accessory proteins (myeloid differentiation protein 2 (MD-2) and cluster of differentiation 14 (CD14)) that play an important role in ligand recognition and subsequent intracellular events. Activation of TLRs and classical opioid receptors are thought to be, at least in part, responsible for microglial activation and subsequent IL-1β release (Hutchinson et al., 2010). However, the contribution of classical opioid receptors to neuroglial signaling has recently come into question.

Interestingly, LPS and opioids bind to similar components of the TLR4/CD14/MD-2 complex. For example, morphine and LPS preferentially bind to the MD-2 accessory protein and subsequently induces oligomerization of the TLR4/MD-2 complex thereby inducing a proinflammatory response. Blocking MD-2 eliminates morphine-induced increases in proinflammatory markers (Wang et al., 2012), whereas upregulation of TLR4 potentiates proinflammatory signaling (Holdridge et al., 2007). This effect, however, was not found to

involve classical opioid receptor activation, suggesting that morphine-induced neuroimmune signaling is mediated exclusively via the TLR4/MD-2 complex (Wang et al., 2012). Recently, the TLR4/MD-2 complex was also shown to modulate morphine-induced accumbal dopamine release, as antagonism of the TLR4/MD-2 complex decreased dopamine levels in the NAcS (Hutchinson et al., 2012). Dopamine is critical for the expression of heroin-conditioned immunosuppression. Thus, it's possible that dopamine levels are modulated via TLR4 in our model. The cell-types(s) mediating morphine's effect on dopamine are unknown; however, it is likely the result of glial cell activation based on high levels of TLR4 receptor expression observed on glia (Bsibsi et al., 2002, Holm et al., 2012, Schwarz et al., 2011). Morphine has also been shown to increase the activation of microglia and astrocytes. In fact, this effect has been observed up to 10 days following the cessation of chronic morphine treatment (Zhang et al., 2012), suggesting that morphine-induced neuroimmune signaling is not a transient phenomenon. Moreover, microglial activation also increases upon re-exposure to a morphine-paired context in a model of CPP (Schwarz et al., 2011), further implicating a potential role for IL-1 signaling in the expression of conditioned responding. Taken together, these data suggest it is possible that IL-1 signaling in the BLA is mediated via TLR4 receptor activation on microglial cells.

Conclusions

The negative health consequences associated with opioid use have been well characterized. Due to the prevalence of opioid use, such as heroin and medicinal opioids, it is important to understand the mechanisms that mediate the conditioned effects of opioids on immune function. This dissertation provides insight into a novel mechanism mediating heroin-conditioned immunosuppression and raises many questions that could direct future studies aimed

at elucidating other important factors involved in this effect. These studies indicate that following heroin-conditioning IL-1β is expressed in the BLA and mNAcS at levels that may be indicative of exaggerated neuroimmune signaling. It is important to determine if the schedule of heroin administration used in the present studies and/or exposure to a distinct environment can induce pathological levels of central IL-1β. In addition, the present findings are the first to demonstrate that IL-1 signaling in the BLA is a critical mechanism involved in heroin-conditioned immunosuppression. Finally, it does not appear that BLA IL-1β signaling is capable of inducing peripheral immune suppression in response to an immune challenge. These studies further our understanding of the central mechanisms that affect the ability of the immune system to respond to infection. These findings may also provide insight into IL-1-mediated conditioned responding in other models of associative learning, potentially leading to novel treatments aimed at both drug and non-drug disorders.

REFERENCES

ADER, R. & COHEN, N. 1982. Behaviorally conditioned immunosuppression and murine systemic lupus erythematosus. *Science,* 215**,** 1534-6.

ADER, R. & COHEN, N. 1992. Conditioned immunopharmacologic effects on cell-mediated immunity. *Int J Immunopharmacol,* 14**,** 323-7.

ADER, R., COHEN, N. & BOVBJERG, D. 1982. Conditioned suppression of humoral immunity in the rat. *J Comp Physiol Psychol,* 96**,** 517-21.

ADER, R., KELLY, K., MOYNIHAN, J. A., GROTA, L. J. & COHEN, N. 1993. Conditioned enhancement of antibody production using antigen as the unconditioned stimulus. *Brain Behav Immun,* 7**,** 334-43.

AGULHON, C., FIACCO, T. A. & MCCARTHY, K. D. 2010. Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca2+ signaling. *Science,* 327**,** 1250-4.

AVITAL, A., GOSHEN, I., KAMSLER, A., SEGAL, M., IVERFELDT, K., RICHTER-LEVIN, G. & YIRMIYA, R. 2003. Impaired interleukin-1 signaling is associated with deficits in hippocampal memory processes and neural plasticity. *Hippocampus,* 13**,** 826-34.

BAJO, M., VARODAYAN, F. P., MADAMBA, S. G., ROBERT, A. J., CASAL, L. M., OLEATA, C. S., SIGGINS, G. R. & ROBERTO, M. 2015. IL-1 interacts with ethanol effects on GABAergic transmission in the mouse central amygdala. *Front Pharmacol,* 6**,** 49.

BARRIENTOS, R. M., HIGGINS, E. A., SPRUNGER, D. B., WATKINS, L. R., RUDY, J. W. & MAIER, S. F. 2002. Memory for context is impaired by a post context exposure injection of interleukin-1 beta into dorsal hippocampus. *Behav Brain Res,* 134**,** 291-8.

BASER, K. H. C. & ARSAN, N. 2014. Opium Poppy (Papaver somniferum). *In:* YANIV, Z. & DUDAI, N. (eds.) *Medicinal and Aromatic Plants of the Middle-East.* 1 ed.: Springer Netherlands.

BASU, A., KRADY, J. K., O'MALLEY, M., STYREN, S. D., DEKOSKY, S. T. & LEVISON, S. W. 2002. The type 1 interleukin-1 receptor is essential for the efficient activation of microglia and the induction of multiple proinflammatory mediators in response to brain injury. *J Neurosci,* 22**,** 6071-82.

BAYER, B. M., DAUSSIN, S., HERNANDEZ, M. & IRVIN, L. 1990a. Morphine inhibition of lymphocyte activity is mediated by an opioid dependent mechanism. *Neuropharmacology,* 29**,** 369-74.

BAYER, B. M., HERNANDEZ, M. & IRVIN, L. 1990b. Suppression of lymphocyte activity after acute morphine administration appears to be glucocorticoid independent. *Prog Clin Biol Res,* 325**,** 273-82.

BEATTIE, E. C., STELLWAGEN, D., MORISHITA, W., BRESNAHAN, J. C., HA, B. K., VON ZASTROW, M., BEATTIE, M. S. & MALENKA, R. C. 2002. Control of synaptic strength by glial TNFalpha. *Science,* 295**,** 2282-5.

BELLINGER, F. P., MADAMBA, S. & SIGGINS, G. R. 1993. Interleukin 1 beta inhibits synaptic strength and long-term potentiation in the rat CA1 hippocampus. *Brain Res,* 628**,** 227-34.

BERKENBOSCH, F., VAN OERS, J., DEL REY, A., TILDERS, F. & BESEDOVSKY, H. 1987. Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. *Science,* 238**,** 524-6.

BERTA, T., LIU, Y. C., XU, Z. Z. & JI, R. R. 2013. Tissue plasminogen activator contributes to morphine tolerance and induces mechanical allodynia via astrocytic IL-1beta and ERK signaling in the spinal cord of mice. *Neuroscience,* 247**,** 376-85.

BEZZI, P., DOMERCQ, M., BRAMBILLA, L., GALLI, R., SCHOLS, D., DE CLERCQ, E., VESCOVI, A., BAGETTA, G., KOLLIAS, G., MELDOLESI, J. & VOLTERRA, A. 2001. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. *Nat Neurosci,* 4**,** 702-10.

BHASKARAN, M., KAPASI, A. A., REDDY, K. & SINGHAL, P. C. 2007. Morphine priming rescues high-dose morphine-induced biological perturbations. *J Infect Dis,* 195**,** 1860-9.

BISSET, N. G., BRUHN, J. G., CURTO, S., HOLMSTEDT, B., NYMAN, U. & ZENK, M. H. 1994. Was opium known in 18th dynasty ancient Egypt? An examination of materials from the tomb of the chief royal architect Kha. *J Ethnopharmacol,* 41**,** 99-114.

BLANKENSHIP, M. R., HUCKFELDT, R., STEINMETZ, J. J. & STEINMETZ, J. E. 2005. The effects of amygdala lesions on hippocampal activity and classical eyeblink conditioning in rats. *Brain Res,* 1035**,** 120-30.

BOGDAN, C., ROLLINGHOFF, M. & DIEFENBACH, A. 2000. The role of nitric oxide in innate immunity. *Immunol Rev,* 173**,** 17-26.

BROWNSTEIN, M. J. 1993. A brief history of opiates, opioid peptides, and opioid receptors. *Proc Natl Acad Sci U S A,* 90**,** 5391-3.

BSIBSI, M., RAVID, R., GVERIC, D. & VAN NOORT, J. M. 2002. Broad expression of Toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol,* 61**,** 1013-21.

BUCHANAN, M. M., HUTCHINSON, M., WATKINS, L. R. & YIN, H. 2010. Toll-like receptor 4 in CNS pathologies. *J Neurochem,* 114**,** 13-27.

BUSSIERE, J. L., ADLER, M. W., ROGERS, T. J. & EISENSTEIN, T. K. 1993. Effects of in vivo morphine treatment on antibody responses in C57BL/6 bgJ/bgJ (beige) mice. *Life Sci,* 52**,** PL43-8.

CHAO, C. C., MOLITOR, T. W., CLOSE, K., HU, S. & PETERSON, P. K. 1993. Morphine inhibits the release of tumor necrosis factor in human peripheral blood mononuclear cell cultures. *Int J Immunopharmacol,* 15**,** 447-53.

CICERO, T. J. & KUEHN, B. M. 2014. Driven by prescription drug abuse, heroin use increases among suburban and rural whites. *JAMA,* 312**,** 118-9.

CIFONE, M. G., CIRONI, L., MECCIA, M. A., RONCAIOLI, P., FESTUCCIA, C., DE NUNTIIS, G., D'ALO, S. & SANTONI, A. 1995. Role of nitric oxide in cell-mediated tumor cytotoxicity. *Adv Neuroimmunol,* 5**,** 443-61.

COOGAN, A. & O'CONNOR, J. J. 1997. Inhibition of NMDA receptor-mediated synaptic transmission in the rat dentate gyrus in vitro by IL-1 beta. *Neuroreport,* 8**,** 2107-10.

COOGAN, A. N., O'NEILL, L. A. & O'CONNOR, J. J. 1999. The P38 mitogen-activated protein kinase inhibitor SB203580 antagonizes the inhibitory effects of interleukin-1beta on long-term potentiation in the rat dentate gyrus in vitro. *Neuroscience,* 93**,** 57-69.

COUSSONS-READ, M. E., DYKSTRA, L. A. & LYSLE, D. T. 1994a. Pavlovian conditioning of morphine-induced alterations of immune status: evidence for opioid receptor involvement. *J Neuroimmunol,* 55**,** 135-42.

COUSSONS-READ, M. E., MASLONEK, K. A., FECHO, K., PEREZ, L. & LYSLE, D. T. 1994b. Evidence for the involvement of macrophage-derived nitric oxide in the modulation of immune status by a conditioned aversive stimulus. *J Neuroimmunol,* 50**,** 51-8.

COUSSONS, M. E., DYKSTRA, L. A. & LYSLE, D. T. 1992. Pavlovian conditioning of morphine-induced alterations of immune status. *J Neuroimmunol,* 39**,** 219-30.

CUMMINS, E., BOUGHNER, E. & LERI, F. 2014. Cue-induced renewal of heroin place preference: involvement of the basolateral amygdala. *Neuroreport,* 25**,** 297-302.

CUNNINGHAM, A. J., MURRAY, C. A., O'NEILL, L. A., LYNCH, M. A. & O'CONNOR, J. J. 1996. Interleukin-1 beta (IL-1 beta) and tumour necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. *Neurosci Lett,* 203**,** 17-20.

DART, R. C., SEVERTSON, S. G. & BUCHER-BARTELSON, B. 2015. Trends in opioid analgesic abuse and mortality in the United States. *N Engl J Med,* 372**,** 1573-4.

DAVIES, C. A., LODDICK, S. A., TOULMOND, S., STROEMER, R. P., HUNT, J. & ROTHWELL, N. J. 1999. The progression and topographic distribution of interleukin-1beta expression after permanent middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab,* 19**,** 87-98.

DELEPLANQUE, B., VITIELLO, S., LE MOAL, M. & NEVEU, P. J. 1994. Modulation of immune reactivity by unilateral striatal and mesolimbic dopaminergic lesions. *Neurosci Lett,* 166**,** 216-20.

DINARELLO, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol,* 27**,** 519-50.

DOMERCQ, M., BRAMBILLA, L., PILATI, E., MARCHALAND, J., VOLTERRA, A. & BEZZI, P. 2006. P2Y1 receptor-evoked glutamate exocytosis from astrocytes: control by tumor necrosis factor-alpha and prostaglandins. *J Biol Chem,* 281**,** 30684-96.

ERIKSSON, C., NOBEL, S., WINBLAD, B. & SCHULTZBERG, M. 2000. Expression of interleukin 1 alpha and beta, and interleukin 1 receptor antagonist mRNA in the rat central nervous system after peripheral administration of lipopolysaccharides. *Cytokine,* 12**,** 423-31.

FECHO, K., DYKSTRA, L. A. & LYSLE, D. T. 1993a. Evidence for beta adrenergic receptor involvement in the immunomodulatory effects of morphine. *J Pharmacol Exp Ther,* 265**,** 1079-87.

FECHO, K. & LYSLE, D. T. 2000. Heroin-induced alterations in leukocyte numbers and apoptosis in the rat spleen. *Cellular Immunology,* 202**,** 113-23.

FECHO, K., MASLONEK, K. A., DYKSTRA, L. A. & LYSLE, D. T. 1993b. Alterations of Immune Status Induced by the Sympathetic Nervous-System - Immunomodulatory Effects of Dmpp Alone and in Combination with Morphine. *Brain Behavior and Immunity,* 7**,** 253-270.

FECHO, K., MASLONEK, K. A., DYKSTRA, L. A. & LYSLE, D. T. 1996a. Assessment of the involvement of central nervous system and peripheral opioid receptors in the immunomodulatory effects of acute morphine treatment in rats. *Journal of Pharmacology and Experimental Therapeutics,* 276**,** 626-636.

FECHO, K., MASLONEK, K. A., DYKSTRA, L. A. & LYSLE, D. T. 1996b. Evidence for sympathetic and adrenal involvement in the immunomodulatory effects of acute morphine treatment in rats. *Journal of Pharmacology and Experimental Therapeutics,* 277**,** 633-645.

FECHO, K., NELSON, C. J. & LYSLE, D. T. 2000. Phenotypic and functional assessments of immune status in the rat spleen following acute heroin treatment. *Immunopharmacology,* 46**,** 193-207.

FELEDER, C., REFOJO, D., NACHT, S. & MOGUILEVSKY, J. A. 1998. Interleukin-1 stimulates hypothalamic inhibitory amino acid neurotransmitter release. *Neuroimmunomodulation,* 5**,** 1-4.

FERNANDEZ, H. & LIBBY, T. A. 2011. *Heroin,* Center City, Minnesota, Hazelden.

FIACCO, T. A., AGULHON, C., TAVES, S. R., PETRAVICZ, J., CASPER, K. B., DONG, X., CHEN, J. & MCCARTHY, K. D. 2007. Selective stimulation of astrocyte calcium in situ does not affect neuronal excitatory synaptic activity. *Neuron,* 54**,** 611-26.

FLORESCO, S. B., YANG, C. R., PHILLIPS, A. G. & BLAHA, C. D. 1998. Basolateral amygdala stimulation evokes glutamate receptor-dependent dopamine efflux in the nucleus accumbens of the anaesthetized rat. *Eur J Neurosci,* 10**,** 1241-51.

FRIEDMAN, H. & EISENSTEIN, T. K. 2004. Neurological basis of drug dependence and its effects on the immune system. *J Neuroimmunol,* 147**,** 106-8.

FRIEDMAN, H., NEWTON, C. & KLEIN, T. W. 2003. Microbial infections, immunomodulation, and drugs of abuse. *Clin Microbiol Rev,* 16**,** 209-19.

FUCHS, R. A. & SEE, R. E. 2002. Basolateral amygdala inactivation abolishes conditioned stimulus- and heroin-induced reinstatement of extinguished heroin-seeking behavior in rats. *Psychopharmacology (Berl),* 160**,** 425-33.

FUKAGAWA, H., KOYAMA, T., KAKUYAMA, M. & FUKUDA, K. 2013. Microglial activation involved in morphine tolerance is not mediated by toll-like receptor 4. *J Anesth,* 27**,** 93-7.

GEMMA, C., FISTER, M., HUDSON, C. & BICKFORD, P. C. 2005. Improvement of memory for context by inhibition of caspase-1 in aged rats. *Eur J Neurosci,* 22**,** 1751-6.

GIBERTINI, M. 1998. Cytokines and cognitive behavior. *Neuroimmunomodulation,* 5**,** 160-5.

GOMEZ-FLORES, R. & WEBER, R. J. 2000. Differential effects of buprenorphine and morphine on immune and neuroendocrine functions following acute administration in the rat mesencephalon periaqueductal gray. *Immunopharmacology,* 48**,** 145-56.

GOSHEN, I., KREISEL, T., OUNALLAH-SAAD, H., RENBAUM, P., ZALZSTEIN, Y., BEN-HUR, T., LEVY-LAHAD, E. & YIRMIYA, R. 2007. A dual role for interleukin-1 in hippocampal-dependent memory processes. *Psychoneuroendocrinology,* 32**,** 1106-15.

GOVITRAPONG, P., SUTTITUM, T., KOTCHABHAKDI, N. & UNEKLABH, T. 1998. Alterations of immune functions in heroin addicts and heroin withdrawal subjects. *Journal of Pharmacology and Experimental Therapeutics,* 286**,** 883-889.

GRIMM, J. W. & SEE, R. E. 2000. Dissociation of primary and secondary reward-relevant limbic nuclei in an animal model of relapse. *Neuropsychopharmacology,* 22**,** 473-9.

GRIMM, M. C., BEN-BARUCH, A., TAUB, D. D., HOWARD, O. M., WANG, J. M. & OPPENHEIM, J. J. 1998. Opiate inhibition of chemokine-induced chemotaxis. *Ann N Y Acad Sci,* 840**,** 9-20.

HAGEL, J. M., MACLEOD, B. P. & FACCHINI, P. J. 2007. OpiumPoppy. *In:* PUA, E. C. & DAVEY, M. R. (eds.) *Biotechnology in Agriculture and Forestry 61.* Germany: Springer-Verlag Berlin Heidelberg.

HAMRA, J. G. & YAKSH, T. L. 1996. Equianalgesic doses of subcutaneous but not intrathecal morphine alter phenotypic expression of cell surface markers and mitogen-induced proliferation in rat lymphocytes. *Anesthesiology,* 85**,** 355-65.

HANISCH, U. K. 2002. Microglia as a source and target of cytokines. *Glia,* 40**,** 140-55.

HOFFMAN, K. E., MASLONEK, K. A., DYKSTRA, L. A. & LYSLE, D. T. 1995. Effects of central administration of morphine on immune status in Lewis and Wistar rats. *Adv Exp Med Biol,* 373**,** 155-9.

HOLDRIDGE, S. V., ARMSTRONG, S. A., TAYLOR, A. M. & CAHILL, C. M. 2007. Behavioural and morphological evidence for the involvement of glial cell activation in delta opioid receptor function: implications for the development of opioid tolerance. *Mol Pain,* 3**,** 7.

HOLM, T. H., DRAEBY, D. & OWENS, T. 2012. Microglia are required for astroglial Toll-like receptor 4 response and for optimal TLR2 and TLR3 response. *Glia,* 60**,** 630-8.

HSUEH, C. M., KUO, J. S., CHEN, S. F., HUANG, H. J., CHENG, F. C., CHUNG, L. J. & LIN, R. J. 1999. Involvement of catecholamines in recall of the conditioned NK cell response. *J Neuroimmunol,* 94**,** 172-81.

HUSSEY, H. H. & KATZ, S. 1950. Infections resulting from narcotic addiction; report of 102 cases. *Am J Med,* 9**,** 186-93.

HUTCHINSON, M. R., LEWIS, S. S., COATS, B. D., REZVANI, N., ZHANG, Y., WIESELER, J. L., SOMOGYI, A. A., YIN, H., MAIER, S. F., RICE, K. C. & WATKINS, L. R. 2010. Possible involvement of toll-like receptor 4/myeloid differentiation factor-2 activity of opioid inactive isomers causes spinal proinflammation and related behavioral consequences. *Neuroscience,* 167**,** 880-93.

HUTCHINSON, M. R., LEWIS, S. S., COATS, B. D., SKYBA, D. A., CRYSDALE, N. Y., BERKELHAMMER, D. L., BRZESKI, A., NORTHCUTT, A., VIETZ, C. M., JUDD, C. M., MAIER, S. F., WATKINS, L. R. & JOHNSON, K. W. 2009. Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). *Brain Behav Immun,* 23**,** 240-50.

HUTCHINSON, M. R., NORTHCUTT, A. L., HIRANITA, T., WANG, X., LEWIS, S. S., THOMAS, J., VAN STEEG, K., KOPAJTIC, T. A., LORAM, L. C., SFREGOLA, C., GALER, E., MILES, N. E., BLAND, S. T., AMAT, J., ROZESKE, R. R., MASLANIK, T., CHAPMAN, T. R., STRAND, K. A., FLESHNER, M., BACHTELL, R. K., SOMOGYI, A. A., YIN, H., KATZ, J. L., RICE, K. C., MAIER, S. F. & WATKINS, L. R. 2012. Opioid activation of toll-like receptor 4 contributes to drug reinforcement. *J Neurosci,* 32**,** 11187-200.

HUTSON, L. W., SZCZYTKOWSKI, J. L., SAURER, T. B., LEBONVILLE, C., FUCHS, R. A. & LYSLE, D. T. 2014. Region-specific contribution of the ventral tegmental area to heroin-induced conditioned immunomodulation. *Brain Behav Immun,* 38**,** 118-24.

ILYIN, S. E., GAYLE, D., FLYNN, M. C. & PLATA-SALAMAN, C. R. 1998. Interleukin-1beta system (ligand, receptor type I, receptor accessory protein and receptor antagonist), TNF-alpha, TGF-beta1 and neuropeptide Y mRNAs in specific brain regions during bacterial LPS-induced anorexia. *Brain Res Bull,* 45**,** 507-15.

JAMES, S. L. 1995. Role of nitric oxide in parasitic infections. *Microbiol Rev,* 59**,** 533-47.

JING, T., WU, L., BORGMANN, K., SURENDRAN, S., GHORPADE, A., LIU, J. & XIONG, H. 2010. Soluble factors from IL-1beta-stimulated astrocytes activate NR1a/NR2B receptors: implications for HIV-1-induced neurodegeneration. *Biochem Biophys Res Commun,* 402**,** 241-6.

JOHN, G. R., CHEN, L., RIVIECCIO, M. A., MELENDEZ-VASQUEZ, C. V., HARTLEY, A. & BROSNAN, C. F. 2004. Interleukin-1beta induces a reactive astroglial phenotype via deactivation of the Rho GTPase-Rock axis. *J Neurosci,* 24**,** 2837-45.

JOHNSON, J. L., ROLAN, P. E., JOHNSON, M. E., BOBROVSKAYA, L., WILLIAMS, D. B., JOHNSON, K., TUKE, J. & HUTCHINSON, M. R. 2014. Codeine-induced hyperalgesia and allodynia: investigating the role of glial activation. *Transl Psychiatry,* 4**,** e482.

JONES, M. E., LEBONVILLE, C. L., BARRUS, D. & LYSLE, D. T. 2015. The role of brain interleukin-1 in stress-enhanced fear learning. *Neuropsychopharmacology,* 40**,** 1289-96.

KATSUKI, H., NAKAI, S., HIRAI, Y., AKAJI, K., KISO, Y. & SATOH, M. 1990. Interleukin-1 beta inhibits long-term potentiation in the CA3 region of mouse hippocampal slices. *Eur J Pharmacol,* 181**,** 323-6.

KAWASAKI, Y., ZHANG, L., CHENG, J. K. & JI, R. R. 2008. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci,* 28**,** 5189-94.

KELM, M. 1999. Nitric oxide metabolism and breakdown. *Biochim Biophys Acta,* 1411**,** 273-89.

KONSMAN, J. P., TRIDON, V. & DANTZER, R. 2000. Diffusion and action of intracerebroventricularly injected interleukin-1 in the CNS. *Neuroscience,* 101**,** 957-67.

KOO, J. W. & DUMAN, R. S. 2009. Interleukin-1 receptor null mutant mice show decreased anxiety-like behavior and enhanced fear memory. *Neurosci Lett,* 456**,** 39-43.

KRONCKE, K. D., FEHSEL, K. & KOLB-BACHOFEN, V. 1998. Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol,* 113**,** 147-56.

KRUZICH, P. J. & SEE, R. E. 2001. Differential contributions of the basolateral and central amygdala in the acquisition and expression of conditioned relapse to cocaine-seeking behavior. *J Neurosci,* 21**,** RC155.

KUO, J. S., CHEN, S. F., HUANG, H. J., YANG, C. S., TSAI, P. J. & HSUEH, C. M. 2001. The involvement of glutamate in recall of the conditioned NK cell response. *J Neuroimmunol,* 118**,** 245-55.

LIPARI, R. N. & HUGHES, A. 2015. The NSDUH Report: Trends in Heroin Use in the United States: 2002 to 2013. Rockville, MD: Substance Abuse and Mental Health Services Administration.

LIU, C. H., CHERNG, C. H., LIN, S. L., YEH, C. C., WU, C. T., TAI, Y. H. & WONG, C. S. 2011a. N-methyl-D-aspartate receptor antagonist MK-801 suppresses glial pro-inflammatory cytokine expression in morphine-tolerant rats. *Pharmacol Biochem Behav,* 99**,** 371-80.

LIU, L., COLLER, J. K., WATKINS, L. R., SOMOGYI, A. A. & HUTCHINSON, M. R. 2011b. Naloxone-precipitated morphine withdrawal behavior and brain IL-1beta expression: comparison of different mouse strains. *Brain Behav Immun,* 25**,** 1223-32.

LIU, Y., BLACKBOURN, D. J., CHUANG, L. F., KILLAM, K. F., JR. & CHUANG, R. Y. 1992. Effects of in vivo and in vitro administration of morphine sulfate upon rhesus macaque polymorphonuclear cell phagocytosis and chemotaxis. *J Pharmacol Exp Ther,* 263**,** 533-9.

LOURIA, D. B., HENSLE, T. & ROSE, J. 1967. The major medical complications of heroin addiction. *Ann Intern Med,* 67**,** 1-22.

LU, L., FANG, Y. & WANG, X. 2008. Drug abuse in China: past, present and future. *Cell Mol Neurobiol,* 28**,** 479-90.

LUK, W. P., ZHANG, Y., WHITE, T. D., LUE, F. A., WU, C., JIANG, C. G., ZHANG, L. & MOLDOFSKY, H. 1999. Adenosine: a mediator of interleukin-1beta-induced hippocampal synaptic inhibition. *J Neurosci,* 19**,** 4238-44.

LUTTGENS, W. F. 1949. Endocarditis in main line opium addicts; report on 11 cases. *Arch Intern Med (Chic),* 83**,** 653-64.

LYSLE, D. T., COUSSONS, M. E., WATTS, V. J., BENNETT, E. H. & DYKSTRA, L. A. 1993a. Morphine-induced alterations of immune status: dose dependency, compartment specificity and antagonism by naltrexone. *J Pharmacol Exp Ther,* 265**,** 1071-8.

LYSLE, D. T., COUSSONS, M. E., WATTS, V. J., BENNETT, E. H. & DYKSTRA, L. A. 1993b. Morphine-induced modulation of immune status: evidence for opioid receptor mediation and compartment specificity. *Adv Exp Med Biol,* 335**,** 53-9.

LYSLE, D. T. & HOW, T. 2000. Heroin modulates the expression of inducible nitric oxide synthase. *Immunopharmacology,* 46**,** 181-92.

LYSLE, D. T. & IJAMES, S. G. 2002. Heroin-associated environmental stimuli modulate the expression of inducible nitric oxide synthase in the rat. *Psychopharmacology (Berl),* 164**,** 416-22.

MAIER, S. F., GOEHLER, L. E., FLESHNER, M. & WATKINS, L. R. 1998. The role of the vagus nerve in cytokine-to-brain communication. *Ann N Y Acad Sci,* 840**,** 289-300.

MCMAHAN, C. J., SLACK, J. L., MOSLEY, B., COSMAN, D., LUPTON, S. D., BRUNTON, L. L., GRUBIN, C. E., WIGNALL, J. M., JENKINS, N. A., BRANNAN, C. I. & ET AL. 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J,* 10**,** 2821-32.

MEIL, W. M. & SEE, R. E. 1997. Lesions of the basolateral amygdala abolish the ability of drug associated cues to reinstate responding during withdrawal from self-administered cocaine. *Behav Brain Res,* 87**,** 139-48.

MENZEBACH, A., HIRSCH, J., NOST, R., MOGK, M., HEMPELMANN, G. & WELTERS, I. D. 2004. [Morphine inhibits complement receptor expression, phagocytosis and oxidative burst by a nitric oxide dependent mechanism]. *Anasthesiol Intensivmed Notfallmed Schmerzther,* 39**,** 204-11.

MILLER, L. G. & FAHEY, J. M. 1994. Interleukin-1 modulates GABAergic and glutamatergic function in brain. *Ann N Y Acad Sci,* 739**,** 292-8.

MILLER, L. G., GALPERN, W. R., DUNLAP, K., DINARELLO, C. A. & TURNER, T. J. 1991. Interleukin-1 augments gamma-aminobutyric acidA receptor function in brain. *Mol Pharmacol,* 39**,** 105-8.

MILLIGAN, E. D. & WATKINS, L. R. 2009. Pathological and protective roles of glia in chronic pain. *Nat Rev Neurosci,* 10**,** 23-36.

MOHANKUMAR, P. S., THYAGARAJAN, S. & QUADRI, S. K. 1991. Interleukin-1 stimulates the release of dopamine and dihydroxyphenylacetic acid from the hypothalamus in vivo. *Life Sci,* 48**,** 925-30.

MULLER, J., CORODIMAS, K. P., FRIDEL, Z. & LEDOUX, J. E. 1997. Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. *Behav Neurosci,* 111**,** 683-91.

MURRAY, C. A. & LYNCH, M. A. 1998. Evidence that increased hippocampal expression of the cytokine interleukin-1 beta is a common trigger for age- and stress-induced impairments in long-term potentiation. *J Neurosci,* 18**,** 2974-81.

NAIR, M. P., LAING, T. J. & SCHWARTZ, S. A. 1986. Decreased natural and antibody-dependent cellular cytotoxic activities in intravenous drug abusers. *Clin Immunol Immunopathol,* 38**,** 68-78.

NGUYEN, K. T., DEAK, T., OWENS, S. M., KOHNO, T., FLESHNER, M., WATKINS, L. R. & MAIER, S. F. 1998. Exposure to acute stress induces brain interleukin-1beta protein in the rat. *J Neurosci,* 18**,** 2239-46.

NISTICO, G., CAROLEO, M. C., ARBITRIO, M. & PULVIRENTI, L. 1994. Evidence for an involvement of dopamine D1 receptors in the limbic system in the control of immune mechanisms. *Neuroimmunomodulation,* 1**,** 174-80.

O'CONNOR, J. J. & COOGAN, A. N. 1999. Actions of the pro-inflammatory cytokine IL-1 beta on central synaptic transmission. *Exp Physiol,* 84**,** 601-14.

OITZL, M. S., VAN OERS, H., SCHOBITZ, B. & DE KLOET, E. R. 1993. Interleukin-1 beta, but not interleukin-6, impairs spatial navigation learning. *Brain Res,* 613**,** 160-3.

OKAMOTO, S., IBARAKI, K., HAYASHI, S. & SAITO, M. 1996. Ventromedial hypothalamus suppresses splenic lymphocyte activity through sympathetic innervation. *Brain Res,* 739**,** 308-313.

OLEKHNOVITCH, R., RYFFEL, B., MULLER, A. J. & BOUSSO, P. 2014. Collective nitric oxide production provides tissue-wide immunity during Leishmania infection. *J Clin Invest,* 124**,** 1711-22.

PACHECO-LOPEZ, G., NIEMI, M. B., KOU, W., HARTING, M., FANDREY, J. & SCHEDLOWSKI, M. 2005. Neural substrates for behaviorally conditioned immunosuppression in the rat. *J Neurosci,* 25**,** 2330-7.

PACIFICI, R., DI CARLO, S., BACOSI, A., PICHINI, S. & ZUCCARO, P. 2000. Pharmacokinetics and cytokine production in heroin and morphine-treated mice. *Int J Immunopharmacol,* 22**,** 603-14.

PACIFICI, R., DI CARLO, S., BACOSI, A. & ZUCCARO, P. 1993. Macrophage functions in drugs of abuse-treated mice. *Int J Immunopharmacol,* 15**,** 711-6.

PASCUAL, O., BEN ACHOUR, S., ROSTAING, P., TRILLER, A. & BESSIS, A. 2012. Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proc Natl Acad Sci U S A,* 109**,** E197-205.

PEREZ, L. & LYSLE, D. T. 1995. Corticotropin-releasing hormone is involved in conditioned stimulus-induced reduction of natural killer cell activity but not in conditioned alterations in cytokine production or proliferation responses. *J Neuroimmunol,* 63**,** 1-8.

PETRAVICZ, J., FIACCO, T. A. & MCCARTHY, K. D. 2008. Loss of IP3 receptor-dependent Ca2+ increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity. *J Neurosci,* 28**,** 4967-73.

PRINGLE, A. K., GARDNER, C. R. & WALKER, R. J. 1996. Reduction of cerebellar GABAA responses by interleukin-1 (IL-1) through an indomethacin insensitive mechanism. *Neuropharmacology,* 35**,** 147-52.

PUGH, C. R., FLESHNER, M., WATKINS, L. R., MAIER, S. F. & RUDY, J. W. 2001. The immune system and memory consolidation: a role for the cytokine IL-1 beta. *Neurosci Biobehav Rev,* 25**,** 29-41.

PUGH, C. R., KUMAGAWA, K., FLESHNER, M., WATKINS, L. R., MAIER, S. F. & RUDY, J. W. 1998. Selective effects of peripheral lipopolysaccharide administration on contextual and auditory-cue fear conditioning. *Brain Behav Immun,* 12**,** 212-29.

PUGH, C. R., NGUYEN, K. T., GONYEA, J. L., FLESHNER, M., WAKINS, L. R., MAIER, S. F. & RUDY, J. W. 1999. Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation. *Behav Brain Res,* 106**,** 109-18.

QUAGLIARELLO, V. J., WISPELWEY, B., LONG, W. J., JR. & SCHELD, W. M. 1991. Recombinant human interleukin-1 induces meningitis and blood-brain barrier injury in the rat. Characterization and comparison with tumor necrosis factor. *J Clin Invest,* 87**,** 1360-6.

QUAN, N., STERN, E. L., WHITESIDE, M. B. & HERKENHAM, M. 1999. Induction of pro-inflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat. *J Neuroimmunol,* 93**,** 72-80.

RAGHAVENDRA, V., RUTKOWSKI, M. D. & DELEO, J. A. 2002. The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J Neurosci,* 22**,** 9980-9.

REISS, C. S. & KOMATSU, T. 1998. Does nitric oxide play a critical role in viral infections? *J Virol,* 72**,** 4547-51.

RISHDAL, J. M., PETERSON, P. K. & MOLITOR, T. W. 1996. *Drugs of Abuse, Immunity, and Infections,* New York, CRC Press.

ROJAVIN, M., SZABO, I., BUSSIERE, J. L., ROGERS, T. J., ADLER, M. W. & EISENSTEIN, T. K. 1993. Morphine treatment in vitro or in vivo decreases phagocytic functions of murine macrophages. *Life Sci,* 53**,** 997-1006.

ROTHWELL, N. J., LUHESHI, G. & TOULMOND, S. 1996. Cytokines and their receptors in the central nervous system: physiology, pharmacology, and pathology. *Pharmacol Ther,* 69**,** 85-95.

ROTHWELL, N. J. & LUHESHI, G. N. 2000. Interleukin 1 in the brain: biology, pathology and therapeutic target. *Trends Neurosci,* 23**,** 618-25.

ROUBENOFF, R., HARRIS, T. B., ABAD, L. W., WILSON, P. W., DALLAL, G. E. & DINARELLO, C. A. 1998. Monocyte cytokine production in an elderly population: effect of age and inflammation. *J Gerontol A Biol Sci Med Sci,* 53**,** M20-6.

ROY, S., BARKE, R. A. & LOH, H. H. 1998. MU-opioid receptor-knockout mice: role of mu-opioid receptor in morphine mediated immune functions. *Brain Res Mol Brain Res,* 61**,** 190-4.

SANTELLO, M., BEZZI, P. & VOLTERRA, A. 2011. TNFalpha controls glutamatergic gliotransmission in the hippocampal dentate gyrus. *Neuron,* 69**,** 988-1001.

SAPOLSKY, R., RIVIER, C., YAMAMOTO, G., PLOTSKY, P. & VALE, W. 1987. Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. *Science,* 238**,** 522-4.

SAURER, T. B., CARRIGAN, K. A., IJAMES, S. G. & LYSLE, D. T. 2004. Morphine-induced alterations of immune status are blocked by the dopamine D2-like receptor agonist 7-OH-DPAT. *J Neuroimmunol,* 148**,** 54-62.

SAURER, T. B., CARRIGAN, K. A., IJAMES, S. G. & LYSLE, D. T. 2006a. Suppression of natural killer cell activity by morphine is mediated by the nucleus accumbens shell. *J Neuroimmunol,* 173**,** 3-11.

SAURER, T. B., IJAMES, S. G., CARRIGAN, K. A. & LYSLE, D. T. 2008a. Neuroimmune mechanisms of opioid-mediated conditioned immunomodulation. *Brain Behav Immun,* 22**,** 89-97.

SAURER, T. B., IJAMES, S. G. & LYSLE, D. T. 2006b. Neuropeptide Y Y1 receptors mediate morphine-induced reductions of natural killer cell activity. *J Neuroimmunol,* 177**,** 18-26.

SAURER, T. B., IJAMES, S. G. & LYSLE, D. T. 2009. Evidence for the nucleus accumbens as a neural substrate of heroin-induced immune alterations. *J Pharmacol Exp Ther,* 329**,** 1040-7.

SAURER, T. B., JAMES, S. G., CARRIGAN, K. A. & LYSLE, D. T. 2008b. Neuroimmune mechanisms of opioid-mediated conditioned immunomodulation. *Brain Behavior and Immunity,* 22**,** 89-97.

SCHMID, A. W., LYNCH, M. A. & HERRON, C. E. 2009. The effects of IL-1 receptor antagonist on beta amyloid mediated depression of LTP in the rat CA1 in vivo. *Hippocampus,* 19**,** 670-6.

SCHNEIDER, G. M. & LYSLE, D. T. 1998. Role of central mu-opioid receptors in the modulation of nitric oxide production by splenocytes. *J Neuroimmunol,* 89**,** 150-9.

SCHNEIDER, H., PITOSSI, F., BALSCHUN, D., WAGNER, A., DEL REY, A. & BESEDOVSKY, H. O. 1998. A neuromodulatory role of interleukin-1beta in the hippocampus. *Proc Natl Acad Sci U S A,* 95**,** 7778-83.

SCHWARZ, J. M., HUTCHINSON, M. R. & BILBO, S. D. 2011. Early-life experience decreases drug-induced reinstatement of morphine CPP in adulthood via microglial-specific epigenetic programming of anti-inflammatory IL-10 expression. *J Neurosci,* 31**,** 17835-47.

SEE, R. E., FUCHS, R. A., LEDFORD, C. C. & MCLAUGHLIN, J. 2003. Drug addiction, relapse, and the amygdala. *Ann N Y Acad Sci,* 985**,** 294-307.

SEE, R. E., KRUZICH, P. J. & GRIMM, J. W. 2001. Dopamine, but not glutamate, receptor blockade in the basolateral amygdala attenuates conditioned reward in a rat model of relapse to cocaine-seeking behavior. *Psychopharmacology (Berl),* 154**,** 301-10.

SHAVIT, Y., DEPAULIS, A., MARTIN, F. C., TERMAN, G. W., PECHNICK, R. N., ZANE, C. J., GALE, R. P. & LIEBESKIND, J. C. 1986. Involvement of brain opiate receptors in the immune-suppressive effect of morphine. *Proc Natl Acad Sci U S A,* 83**,** 7114-7.

SHINTANI, F., KANBA, S., NAKAKI, T., NIBUYA, M., KINOSHITA, N., SUZUKI, E., YAGI, G., KATO, R. & ASAI, M. 1993. Interleukin-1 beta augments release of norepinephrine, dopamine, and serotonin in the rat anterior hypothalamus. *J Neurosci,* 13**,** 3574-81.

SONG, C., HORROBIN, D. F. & LEONARD, B. E. 2006. The comparison of changes in behavior, neurochemistry, endocrine, and immune functions after different routes, doses and durations of administrations of IL-1beta in rats. *Pharmacopsychiatry,* 39**,** 88-99.

SONG, C., PHILLIPS, A. G. & LEONARD, B. 2003. Interleukin 1 beta enhances conditioned fear memory in rats: possible involvement of glucocorticoids. *Eur J Neurosci,* 18**,** 1739-43.

SONG, P. & ZHAO, Z. Q. 2001. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res,* 39**,** 281-6.

SUNDAR, S. K., BECKER, K. J., CIERPIAL, M. A., CARPENTER, M. D., RANKIN, L. A., FLEENER, S. L., RITCHIE, J. C., SIMSON, P. E. & WEISS, J. M. 1989. Intracerebroventricular infusion of interleukin 1 rapidly decreases peripheral cellular immune responses. *Proc Natl Acad Sci U S A,* 86**,** 6398-402.

SUSCHEK, C. V., SCHNORR, O. & KOLB-BACHOFEN, V. 2004. The role of iNOS in chronic inflammatory processes in vivo: is it damage-promoting, protective, or active at all? *Curr Mol Med,* 4**,** 763-75.

SZCZYTKOWSKI, J. L., FUCHS, R. A. & LYSLE, D. T. 2011. Ventral tegmental area-basolateral amygdala-nucleus accumbens shell neurocircuitry controls the expression of heroin-conditioned immunomodulation. *J Neuroimmunol,* 237**,** 47-56.

SZCZYTKOWSKI, J. L., LEBONVILLE, C., HUTSON, L., FUCHS, R. A. & LYSLE, D. T. 2013. Heroin-induced conditioned immunomodulation requires expression of IL-1beta in the dorsal hippocampus. *Brain Behav Immun,* 30**,** 95-102.

SZCZYTKOWSKI, J. L. & LYSLE, D. T. 2007. Conditioned effects of heroin on the expression of inducible nitric oxide synthase in the rat are susceptible to extinction and latent inhibition. *Psychopharmacology (Berl),* 191**,** 879-89.

SZCZYTKOWSKI, J. L. & LYSLE, D. T. 2008. Conditioned effects of heroin on proinflammatory mediators require the basolateral amygdala. *Eur J Neurosci,* 28**,** 1867-76.

SZCZYTKOWSKI, J. L. & LYSLE, D. T. 2010. Dopamine D1 receptors within the basolateral amygdala mediate heroin-induced conditioned immunomodulation. *J Neuroimmunol,* 226**,** 38-47.

THEODOROU, S. & HABER, P. S. 2005. The medical complications of heroin use. *Curr Opin Psychiatry,* 18**,** 257-63.

TODA, Y., TSUKADA, J., MISAGO, M., KOMINATO, Y., AURON, P. E. & TANAKA, Y. 2002. Autocrine induction of the human pro-IL-1beta gene promoter by IL-1beta in monocytes. *J Immunol,* 168**,** 1984-91.

TREATMENT, C. F. S. A. 2005. Medication-Assisted Treatment for Opioid Addiction in Opioid Treatment Programs. *In:* SERVICES, U. S. D. O. H. A. H. (ed.). Rockville, MD: Substance Abuse and Mental Health Services Administration.

TUBARO, E., BORELLI, G., CROCE, C., CAVALLO, G. & SANTIANGELI, C. 1983. Effect of morphine on resistance to infection. *J Infect Dis,* 148**,** 656-66.

TUTEJA, N., CHANDRA, M., TUTEJA, R. & MISRA, M. K. 2004. Nitric Oxide as a Unique Bioactive Signaling Messenger in Physiology and Pathophysiology. *J Biomed Biotechnol,* 2004**,** 227-237.

VEREKER, E., CAMPBELL, V., ROCHE, E., MCENTEE, E. & LYNCH, M. A. 2000a. Lipopolysaccharide inhibits long term potentiation in the rat dentate gyrus by activating caspase-1. *J Biol Chem,* 275**,** 26252-8.

VEREKER, E., O'DONNELL, E. & LYNCH, M. A. 2000b. The inhibitory effect of interleukin-1beta on long-term potentiation is coupled with increased activity of stress-activated protein kinases. *J Neurosci,* 20**,** 6811-9.

WANG, S., CHENG, Q., MALIK, S. & YANG, J. 2000. Interleukin-1beta inhibits gamma-aminobutyric acid type A (GABA(A)) receptor current in cultured hippocampal neurons. *J Pharmacol Exp Ther,* 292**,** 497-504.

WANG, X., LORAM, L. C., RAMOS, K., DE JESUS, A. J., THOMAS, J., CHENG, K., REDDY, A., SOMOGYI, A. A., HUTCHINSON, M. R., WATKINS, L. R. & YIN, H. 2012.

Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc Natl Acad Sci U S A,* 109**,** 6325-30.

WEBER, R. J. & PERT, A. 1989. The periaqueductal gray matter mediates opiate-induced immunosuppression. *Science,* 245**,** 188-90.

WILSON, C. J., FINCH, C. E. & COHEN, H. J. 2002. Cytokines and cognition--the case for a head-to-toe inflammatory paradigm. *J Am Geriatr Soc,* 50**,** 2041-56.

YABUUCHI, K., MINAMI, M., KATSUMATA, S. & SATOH, M. 1994a. Localization of Type-I Interleukin-1 Receptor Messenger-Rna in the Rat-Brain. *Molecular Brain Research,* 27**,** 27-36.

YABUUCHI, K., MINAMI, M., KATSUMATA, S. & SATOH, M. 1994b. Localization of type I interleukin-1 receptor mRNA in the rat brain. *Brain Res Mol Brain Res,* 27**,** 27-36.

YARDENI, I. Z., BEILIN, B., MAYBURD, E., ALCALAY, Y. & BESSLER, H. 2008. Relationship between fentanyl dosage and immune function in the postoperative period. *J Opioid Manag,* 4**,** 27-33.

YIRMIYA, R., WINOCUR, G. & GOSHEN, I. 2002. Brain interleukin-1 is involved in spatial memory and passive avoidance conditioning. *Neurobiology of Learning and Memory,* 78**,** 379-89.

YOKOTA, T., UEHARA, K. & NOMOTO, Y. 2004. Addition of noradrenaline to intrathecal morphine augments the postoperative suppression of natural killer cell activity. *J Anesth,* 18**,** 190-5.

YU, B. & SHINNICK-GALLAGHER, P. 1994. Interleukin-1 beta inhibits synaptic transmission and induces membrane hyperpolarization in amygdala neurons. *J Pharmacol Exp Ther,* 271**,** 590-600.

ZEISE, M. L., ESPINOZA, J., MORALES, P. & NALLI, A. 1997. Interleukin-1beta does not increase synaptic inhibition in hippocampal CA3 pyramidal and dentate gyrus granule cells of the rat in vitro. *Brain Res,* 768**,** 341-4.

ZEISE, M. L., MADAMBA, S. & SIGGINS, G. R. 1992. Interleukin-1 beta increases synaptic inhibition in rat hippocampal pyramidal neurons in vitro. *Regul Pept,* 39, 1-7.

ZHANG, X., CUI, Y., JING, J., CUI, Y., XIN, W. & LIU, X. 2011. Involvement of p38/NF-kappaB signaling pathway in the nucleus accumbens in the rewarding effects of morphine in rats. Behav Brain Res, 218, 184-9.

ZHANG, X. Q., CUI, Y., CUI, Y., CHEN, Y., NA, X. D., CHEN, F. Y., WEI, X. H., LI, Y. Y., LIU, X. G. & XIN, W. J. 2012. Activation of p38 signaling in the microglia in the nucleus accumbens contributes to the acquisition and maintenance of morphine-induced conditioned place preference. Brain Behav Immun, 26, 318-25.