NEUROIMMUNE MECHANISMS OF OPIOID-MEDIATED IMMUNOMODULATION

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ABSTRACT
TIMOTHY BENJAMIN SAURER: Neuroimmune Mechanisms of Opioid-Mediated Immunomodulation
(Under the direction of Donald T. Lysle, Ph.D.)

Administration of opioid drugs such as morphine and heroin elicits pronounced effects on the immune system, including decreases in natural killer (NK) cell activity and lymphocyte proliferative responses. Despite a wealth of data indicating that opioids induce such immune alterations by acting through µ-opioid receptors in the brain, there is little known about how opioids interact with neurotransmitter systems to modulate specific immune parameters. The present investigations address how opioid interactions with dopamine transmission in the nucleus accumbens translate into peripheral immune alterations. The experiments presented in Chapter 2 revealed that pharmacological blockade of dopamine D₁ receptors in the nucleus accumbens shell abolished the suppressive effects of both morphine and heroin on the ability of splenic NK cells to lyse tumor cell targets. In contrast, selective stimulation of dopamine D₁ receptors in the nucleus accumbens shell produced reductions in splenic NK cell activity comparable to opioid administration. Additionally, antagonism of D₁ receptors in the nucleus accumbens shell completely prevented heroin’s inhibitory effects on in vivo iNOS expression in spleen, liver, and lung tissues. Chapter 3 showed that these dopamine-dependent immunomodulatory effects are mediated peripherally via the sympathetic peptide transmitter NPY. Administration of a selective NPY Y₁ receptor antagonist blocked opioid-induced decreases of NK activity and
iNOS expression, but did not reverse the suppression of dopamine-independent effects on lymphocyte proliferative responses. Chapter 4 showed that similar dopamine and NPY receptor mechanisms underlie the conditioned immunomodulatory effects of opioids. Rats received conditioning sessions during which an injection of morphine was paired with a distinctive environment that served as the conditioned stimulus (CS). Administration of a either a D₁ antagonist or a Y₁ antagonist prior to CS re-exposure fully blocked the conditioned suppression of NK cell activity. Collectively these findings suggest that opioid-induced increases in dopamine release in the nucleus accumbens shell inhibit splenic NK activity and iNOS production by facilitating the NPY release from sympathetic nerves. Additionally, the findings suggest that conditioned and unconditioned immunomodulatory effects of opioids involve similar receptor mechanisms.
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<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>C</td>
<td>Celsius</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>Con-A</td>
<td>concanavalin A</td>
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<td>CS</td>
<td>Conditioned stimulus</td>
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<td>DAT</td>
<td>dopamine transporter</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DPM</td>
<td>disintegrations per minute</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
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<tr>
<td>i.c.v.</td>
<td>intracerebroventricular</td>
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<td>IL-1</td>
<td>interleukin 1</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NK</td>
<td>natural killer</td>
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<td>NPY</td>
<td>neuropeptide Y</td>
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<td>PAG</td>
<td>periaqueductal gray matter</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>RNA</td>
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<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<td>s.c.</td>
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<td>S.E.</td>
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<td>tumor necrosis factor-alpha</td>
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<td>UCS</td>
<td>unconditioned stimulus</td>
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<td>vol</td>
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<td>VTA</td>
<td>ventral tegmental area</td>
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CHAPTER 1
GENERAL INTRODUCTION

History of Opioids

Opioid drugs have been used in the form of crude opium since prehistoric times, making them among the oldest psychoactive drugs known to man (Booth, 1998). Opium is a naturally occurring substance obtained from the sap of the immature seedpod of the opium poppy *Papaver somniferum*. Because opium use antedates recorded history, it is unknown when and where opium’s psychoactive properties were initially discovered. However, historians generally agree that by ca. 3400 B.C., the Sumerians had become familiar with opium’s euphoric properties as they referred to the opium poppy as “hul gil,” meaning the “joy plant” (Booth, 1998; Scott, 1969). Thus, the Sumerians, renowned as the world’s first civilization and credited with the invention of writing, are also recognized for introducing opium to the ancient world (Brownstein, 1993).

Opium eventually spread from Sumer (the area known as present-day Iraq) to the Egyptians, who first documented the drug’s medicinal properties in ancient medical texts. The *Ebers Papyrus* (ca. 1500 B.C.), mentioned opium in some 700 remedies including a notable “remedy to prevent excessive crying of children,” which required concocting an amalgam of poppy plants and fly excrement (Booth, 1998; Brownstein, 1993). Therapeutic uses of opium were also recognized by the Greek physician Hippocrates (ca. 460-357 B.C.),
the father of modern medicine, and later by Galen (ca. 130-201 A.D.) who extolled opium as a cure for a number of sundry ailments including headaches, deafness, fever, leprosy, jaundice, and melancholy, among others (Scott, 1969). In addition, ancient Greek writers such as Homer and Theophrastus alluded to the use of opium as an epicurean indulgence (Booth, 1998).

Despite the fact that opium was used recreationally in ancient times, there were few references to the problem of opium addiction. The philosopher Diagoros of Melos in the 3rd century B.C. may have been the first to recognize the danger of opium addiction when he declared that it was better to endure pain than to become bound to opium (Booth, 1998). Nonetheless, the social and medical problems associated with opium addiction were not fully realized until nearly 1,500 years later in China (Scott, 1969). The primary reason opium addiction became such a problem in China was that rather than eating or drinking opium, as was common in Europe and the Middle East, the Chinese popularized the practice of smoking opium (Yaroschuk, 2000). Smoking the drug intensified its euphoric effects by increasing potency and accelerating opium’s entry to the brain. Consequently, opium smoking carried a much greater risk of addiction. China’s opium problem became so severe that it led to the first law against opium smoking, declared in 1729 by the Chinese emperor (Scott, 1969).

In spite of the problems in China, opium was entirely legal and readily available in various preparations throughout Western societies for much of the 19th century (Maisto et al., 2004). Opium’s popularity was fueled by authors of the time-period who praised the drug’s effects. Most notably, British poet Thomas DeQuincey’s book *Confessions of an English Opium Eater* undoubtedly inspired many readers to experiment with opium’s so-called “keys
of Paradise” (Maisto et al., 2004). Not surprisingly, as opium’s popularity became more widespread in Europe, so did concern about the grave consequences of opium dependence.

In the early 1800s, the German chemist F.W. Setürner isolated the major active component of opium and called it morphine after Morpheus, the Greek god of dreams (Huxtable and Schwarz, 2001). Morphine was discovered to be about 10 times more potent than opium, and with the invention of the hypodermic syringe by Alexander Wood in 1856, morphine dependence quickly became a major problem (Levinthal, 2005). Wood’s invention permitted a means of injecting morphine to provide rapid and potent pain-relief for severe wounds. As an unfortunate consequence, many soldiers during the American Civil War became dependent on the drug—so many in fact, that morphine dependence became known as “soldier’s disease” (Levinthal, 2005).

In 1898, the German pharmaceutical company Bayer introduced a new compound called heroin, which was created through a chemical modification to morphine (Booth, 1998). Bayer initially marketed heroin as a “safe” cough suppressant, and although it was approximately three times as potent as morphine, it was purported to be free from morphine’s abuse liability (Levinthal, 2005). This was the first of many such claims for new drugs based on dubious evidence and overzealous interpretation¹ (Eddy, 1957). Consequently, doctors began to prescribe heroin as a treatment for morphine addiction. By 1910, it became apparent that heroin was even more addictive than morphine, and by 1913, heroin had replaced morphine as the leading cause of narcotic-related hospital admissions in the United States (Yaroschuk, 2000).

¹ Surprisingly, many physicians of the time-period also heralded cocaine as a cure for morphine addiction. Not surprisingly, this caused a dramatic increase in the number of morphine addicts who were also addicted to cocaine (Karch, 2005).
Rising concerns about the danger of opioid dependence in the early 1900s led to a series of major changes in public policy in the United States. The 1914 Harrison Narcotics Tax Act limited the use of opioids for medical treatment by requiring a physician’s prescription and effectively transformed morphine addiction from a disease into a criminal offense virtually overnight (Yarochuk, 2000). Increasingly strict regulations were enacted shortly thereafter, and heroin was totally banned and permanently removed from medical usage in 1924 (Maisto et al., 2004). By 1970, the escalation of major changes in drug policy culminated with sweeping legislation that consolidated existing drug laws under the Controlled Substances Act and established the Drug Enforcement Agency as the federal drug law enforcement authority (Yarochuk, 2000). This marked the beginning of the so-called ‘war on drugs.’

Despite more stringent laws and severe penalties for illicit drug use, in 2005, it was estimated that a staggering 19.7 million Americans had used illicit drugs within the past month (SAMHSA, 2006). Roughly 108,000 Americans aged 12 or older abused heroin for the first time in 2005, with approximately 164,572 heroin-related emergency department visits reported in the same year (SAMHSA, 2007). In addition to the major problems surrounding the use of illicit opioids, abuse of opioid-based prescription drugs has escalated in recent years, as evidenced by reports that nearly 12 million Americans had used painkillers recreationally in 2005 (SAMHSA, 2006). In fact, of all U.S. emergency room visits in 2005 involving (nonmedical) use of legal pharmaceuticals and supplements, opioid-based painkillers accounted for one-third (196,225), a 24% increase from 2004 (SAMHSA, 2007). There is little question that opioid abuse remains a significant problem in the United States.
Opioids and Health: Historical Background

Infectious disease was not widely associated with opioid addiction until the early 1900s (Friedman et al., 2003). Ironically, for most of the 19th century, opium use was actually believed to prolong life (Risdahl et al., 1998). The association between opium use and disease did not emerge into public consciousness until the 1870s. Literary author Alonzo Calkins noted in that opium addiction was “plagued with disease” in his 1871 publication of *Opium and the Opium Appetite* (Risdahl et al., 1998). Only four years later, Thomas Whipham published a case study of an opium addict that had developed pleuropneumonia and argued that addiction accelerated progression of the disease and increased the likeliness of death (Risdahl et al., 1998). By the turn of the century, it was realized that morphine addiction carried with it a palpable risk for numerous infectious complications including tetanus, pneumonia, and nephritis among others (Crothers, 1902; Norman, 1876; Osburne, 1892).

The list of infectious diseases associated with opioid use grew progressively longer throughout the 20th century—a reality that mirrored the increasingly common problem of heroin addiction. The post-World War II heroin epidemic in the United States spawned a number of clinical studies of heroin addicts, including a landmark study by Hussey and Katz (1950) that described numerous infectious complications such as skin abscesses, septicemia, endocarditis, and malaria. Reports of high infection rates among opiate addicts continued to proliferate with the ‘drug culture explosion’ of the 1960s to include an impressive list of bacterial, viral, and fungal infections including skin sepsis, bacterial and fungal endocarditis, septicemia, viral hepatitis, bacterial bronchopneumonia, malaria, and tetanus (Cherubin, 1971; Hussey and Katz, 1950; Louria et al., 1967; Luttgens, 1949).
As infectious diseases became viewed as common complications of opioid addiction, conventional wisdom held that the high incidence of infections among addicts was primarily due to increased exposure to pathogens resulting from the use of contaminated needles or tainted drugs. In addition, researchers hypothesized that lifestyle practices may also represent a risk factor. For example, opioid addicts commonly engage in insalubrious behavioral practices that result in malnutrition, stress, sleep deprivation, and/or alcohol abuse. Certainly, there are many factors that contribute to the high incidence of infections among addicts. However, the impact of opioids per se on the immune system was not widely considered—or at least did not generate a great deal of research interest—until the emergence of the HIV/AIDS epidemic, when it was revealed that heroin use was a major risk factor for HIV-infection (Ginzburg, 1984; Quagliarello, 1982). This observation drew considerable attention to the possibility that drugs themselves may influence disease resistance, and thus a new directive emerged to identify potential biological mechanisms by which opioids may modify immunological processes.

**Opioid-Induced Immunomodulation**

**Phenomenological Observations**

There is compelling evidence that opioids disrupt immune function in humans. Clinical studies report that heroin users display a variety of immunological abnormalities compared to non-users, including lower numbers of circulating lymphocytes, decreased NK cell activity, and reduced antibody-dependent cellular cytotoxicity (Brown et al., 1974; 2The notion that opioid drugs per se are innocuous persists to this day, as evidenced in the most recent edition of the popular college-level textbook *Biopsychology*. In a chapter on drug addiction, the author states, “…the health hazards of chronic (opioid) exposure are surprisingly minor. The main risks are constipation, pupil constriction, menstrual irregularity, and reduced libido. Many opiate addicts have taken pure heroin or morphine for years with no serious ill effects.” (Pinel, 2006).
These studies were among the first to propose that the high incidence of infectious disease among opioid addicts is related to impaired immune function. Interestingly, the association between increased infection risk and opioid use is not restricted to drug abusing populations. Recent clinical reports indicate that higher doses of opioid analgesics are associated with greater incidence of infectious complications among burn victims and cardiac patients (El Solh et al., 2006; Schwacha et al., 2006). Furthermore, controlled studies indicate that acute morphine exposure produces immunosuppressive effects in healthy, non-drug using individuals (Tubaro et al., 1985; Yeager et al., 1995). Thus, the use of opioids—whether for therapeutic or recreational purposes—appears to increase infection susceptibility independently of addiction-related risk factors such as intravenous drug use.

Although clinical observations and a limited number of controlled human studies support the hypothesis that opioid-induced immune alterations alter disease resistance, the immunomodulatory properties of opioids have been predominantly ascertained from animal models. Interestingly, the immunomodulatory properties of opioids appear to be conserved across mammalian species, as opioid-induced immune alterations have been demonstrated in human, monkey, pig, rabbit, rat, and mouse (e.g., Carr et al., 1994a; Carr and France, 1993; Olin et al., 2007; Shavit et al., 1987; Tubaro et al., 1983; Yeager et al., 1995). A major advantage of animal models is that they permit the experimenter to control for confounding variables inherent to clinical studies that complicate the determination of cause-effect relationships (e.g., differences in age, nutrition, medical history, drug history, etc.). In addition, because human studies are largely limited to the analysis of peripheral blood, the use of animals permits a more comprehensive evaluation of opioid-induced changes in
immune status through examination of other tissues such as the spleen, lymph nodes, lung, and liver. Morphine is the most commonly used opioid in animal studies since it is considered the prototype μ-opioid receptor agonist and because the biological effects of heroin are chiefly attributed to its major active metabolite (morphine). Studies have shown that morphine administration suppresses a number of immune parameters, including NK cell activity (Bayer et al., 1990a; Shavit et al., 1986), lymphocyte proliferation (Bayer et al., 1990b; Lysle et al., 1993), antibody production (Lefkowitz and Chiang, 1975; Lockwood et al., 1994), and interferon production (Hung et al., 1973). Moreover, morphine has been shown to alter host resistance to various infectious and neoplastic diseases (Lewis et al., 1983; Tubaro et al., 1983). Importantly, morphine’s immunomodulatory effects are dose-dependent and can be prevented by the opioid receptor antagonist naltrexone, indicating that morphine’s effects on immunity are due to the drug’s pharmacological activity (Lysle et al., 1993).

Peripheral Mechanisms

Although the immunomodulatory effects of opioids have been extensively documented, the mechanisms by which opioids exert these effects are not entirely understood. Because opioid receptors are expressed by numerous cell types throughout the body, a major research focus has been to determine the site of action of opioids. One direction of this work tests whether opioids alter immune status by acting directly on cells of the immune system via cell-surface opioid receptors. Wybran and colleagues (1979) provided some of the first evidence for the presence of opioid receptors on human peripheral blood T-lymphocytes by showing that naloxone reverses the suppression of active T-cell rosette formation by morphine. Subsequent
investigations implicated the involvement of μ-opioid receptors in particular (Chuang et al., 1995; Mehrishi and Mills, 1983; Sedqi et al., 1995), although others have reported the existence of additional opioid receptor subtypes as well as naloxone-insensitive morphine binding sites (Joseph and Bidlack, 1994; Lawrence et al., 1995; Madden et al., 1987; Roy et al., 1992).

The opioid receptors found on immunocytes appear to be functional, since in vitro exposure to morphine produces concentration-dependent and opioid antagonist-reversible alterations in several assays of immune status. For example, morphine suppresses Con A-stimulated interferon-γ production (Peterson et al., 1987) and enhances the growth of HIV-1 in human peripheral blood mononuclear cell cultures (Peterson et al., 1990). Furthermore, addition of morphine to murine peritoneal macrophage cultures suppresses phagocytic activities (Casellas et al., 1991; Rojavin et al., 1993; Szabo et al., 1993). In contrast, other studies report no direct effect of morphine or effects only at very high concentrations (Fuchs and Pruett, 1993; Pruett et al., 1992; Thomas et al., 1995; Yeager et al., 1992). For example, Bayer et al. (1992) showed in rats that only high concentrations of morphine suppress lymphocyte proliferation when added directly to mitogen-stimulated splenocyte and blood cultures, but these effects are not antagonized by naltrexone. Thus, while there is evidence to support that morphine directly influences the activity of immune cells, these effects are complex and may be specific to certain immune measures. Taken together, these findings indicate that immune cells express functional opioid receptors and provide one pathway through which opioids such as morphine modulate the immune system.
Central Mechanisms

Another site of action for opioids is through the regulatory actions of the central nervous system (CNS) on the immune system. Substantial evidence supports the existence of a complex, bi-directional link between the CNS and the immune system (e.g., Felten et al., 1985), and there is experimental evidence that morphine's immunomodulatory effects involve central opioid receptors. An initial study by Shavit et al. (1986) found that systemic administration of morphine, but not N-methylmorphine (a form of morphine which does not readily penetrate the blood-brain barrier), produces a naltrexone-reversible suppression of splenic natural killer cell activity in the rat. That same study showed that intracerebroventricular (i.c.v.) administration of morphine dose-dependently suppresses splenic natural killer cell activity, and prior administration of naltrexone blocks the effect of i.c.v. morphine. Along similar lines, microinjections of morphine directly into the periaqueductal gray region of the rat brain produce a highly significant, naltrexone-sensitive suppression in splenic natural killer cell activity (Weber and Pert, 1989). Bayer and colleagues showed that microinjection of morphine into the anterior hypothalamus inhibits blood lymphocyte proliferation to mitogen (Hernandez et al., 1993). In addition, studies from our laboratory have shown that a single i.c.v. microinjection of morphine dose-dependently alters lymphocyte proliferation to T- and B-cell mitogens and natural-killer cell cytotoxicity in the spleen (Lysle et al., 1996). Our studies also showed that i.c.v. administration of N-methylnaltrexone dose-dependently antagonizes the immunomodulatory effects of systemic morphine administration, whereas systemic administration of N-methylnaltrexone (at doses that do not act centrally) are ineffective in blocking morphine's effects (Fecho et al., 1996a). Thus, there is an
overwhelming amount of evidence implicating a role of the central nervous system in opioid-induced immune alterations.

**Role of Dopamine**

Despite a wealth of data indicating that morphine modulates immune status by acting at opioid receptors in the brain, little is known about how the opioid system interacts with other neurotransmitter systems to modulate specific immune parameters. Our laboratory has recently provided evidence that the regulation of central dopaminergic pathways is an important mechanism of morphine-induced immune alterations. Morphine exerts a stimulatory effect on midbrain dopamine neurons that project to forebrain structures such as the nucleus accumbens and dorsal striatum, resulting in increased cell firing and elevated extracellular dopamine levels. Because several of the behavioral effects of morphine are dependent on dopamine transmission, we initially hypothesized that dopamine may also play an important role in morphine-induced immunomodulation.

We began testing the hypothesis that dopamine mediates morphine-induced immunosuppression by examining the effect of the dopamine D2-like receptor agonist 7-OH-DPAT on acute morphine treatment in rats (Saurer et al., 2004). Five dopamine receptor subtypes have been identified (D1-D5), and these are divided into D1-like family (D1, D5) and the D2-like family (D2, D3, D4) based on biochemical, pharmacological, and physiological properties. It is generally considered that there are opposing effects of D1 and D2 receptor activation on cAMP-dependent signaling, with D1-like receptors coupling to stimulatory Gs-proteins, and D2-like receptors acting through inhibitory Gi/o proteins (Missale et al., 1998; Stoof and Kebabian, 1981). Because of the complexity of dopamine receptor pharmacology,
we selected 7-OH-DPAT for use in our initial study because it had been shown to antagonize dopamine signaling at the behavioral, cellular, and molecular level, possible due to activity at dopamine autoreceptors. For instance, 7-OH-DPAT inhibits stimulated endogenous dopamine release (Patel et al., 1995) and attenuates a diverse set of morphine-induced behaviors associated with increased dopamine signaling (Cook et al., 1999; Rodriguez et al., 1995; Suzuki et al., 1995). Moreover, 7-OH-DPAT functionally antagonizes dopamine signaling at the molecular level as evidenced by data showing that 7-OH-DPAT abolished morphine-induced expression of c-Fos protein (a marker of neuronal activation) in several dopamine terminal regions (unpublished data). Results from our study showed that administration of 7-OH-DPAT into the lateral ventricle dose-dependently attenuated morphine-induced reductions of splenic NK cell activity at several-fold lower doses than were effective following systemic administration (Saurer et al., 2004). These findings provided the first direct evidence that central dopaminergic mechanisms are involved in morphine-induced immunomodulation.

The mesocorticolimbic system is perhaps the most widely studied dopaminergic system with respect to its interactions with opioids because it is an integral component of the so-called ‘reward circuitry’ of the brain. Dopamine neurons in this system arise from the ventral tegmental area of the midbrain and project to the prefrontal cortex and to limbic areas such as the nucleus accumbens and the amygdala. In particular, the stimulatory influence of morphine on dopamine signaling in the nucleus accumbens has been extensively documented both neurochemically and electrophysiologically. Importantly, dopamine receptors in the nucleus accumbens modulate several of morphine’s behavioral effects including reinforcement (Shippenberg et al., 1993) and analgesia (Altier and Stewart, 1998). The
latter finding is of particular significance because neural pathways subserving morphine’s effect on immune functions have been suggested largely based on studies indicating that the immunoregulatory and analgesic effects of morphine involve similar neural structures. For example, microinjection of morphine into the PAG produces both analgesia (Yaksh et al., 1976) and suppression of NK activity (Weber and Pert, 1989), and the onset of morphine’s immunologic and analgesic effects follows a similar time course (Nelson et al., 1997). More importantly however, is that the nucleus accumbens has been also been shown to regulate immune functions, as lesions (Deleplanque et al., 1994) or pharmacological manipulation (Nistico et al., 1994) of mesoaccumbens dopamine neurons results in altered immune responses. Overall, there is a substantial amount of indirect evidence to suggest that opioids alter components of host defense by modulating the dopaminergic input to the nucleus accumbens.

**Role of Neuropeptide Y**

The efferent mechanisms whereby centrally acting opioids modulate peripheral immunity involves both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). The production of glucocorticoids from the adrenal glands is thought to play an important role in the effects of chronic morphine exposure on immune status (Freier and Fuchs, 1994), but this does not appear to mediate the effects of acute morphine administration (Bayer et al., 1990b). The SNS on the other hand, mediates many of the immunomodulatory effects of acute morphine exposure. For example, the suppressive effects of acute morphine administration on mitogenic responses of T cells and B cells are prevented by pretreatment with β-adrenoceptor antagonists (Fecho et al., 1993a). However,
morphine-induced reductions in splenic NK cell activity are not blocked by either peripheral adrenoceptor antagonists or adrenalectomy, suggesting than neither the HPA axis nor sympathetic catecholamines are involved (Carr et al., 1994b; Fecho et al., 1993a; Fecho et al., 1996b).

Little is known regarding the peripheral mechanism whereby morphine inhibits splenic NK activity. Immunocytochemical studies have suggested that NPY may be an important modulator of splenocyte functions given the presence of NPY in postganglionic sympathetic fibers innervating the spleen (Romano et al., 1991). NPY is released upon sympathetic activation and induces a host of immunomodulatory effects. Notably, there have been several investigations of the relationship between NPY and NK activity. For example, circulating NPY levels, but not catecholamines, were inversely correlated with NK activity in Alzheimer’s caregivers, suggesting that NPY regulates NK activity under physiological conditions in vivo (Irwin et al., 1991). Further support for this hypothesis was provided by the demonstration that NPY produces a direct and dose-dependent inhibition of NK activity in vitro (Nair et al., 1993). Although the mechanism of the effect of NPY on NK cells is not fully understood, the discovery of functional NPY Y1 receptors on rat splenic lymphocytes offers the possibility that NPY may interact directly with its cognate receptors on NK cells to suppress cytolytic activity (Bedoui et al., 2002; Petitto et al., 1994). One of the key objectives of the experiments described in Chapter 3 is to examine the role of the sympathetic peptide transmitter NPY in opioid-induced reductions in NK cell activity.
**Conditioned Immunomodulation**

Studies of Pavlovian conditioning of immunity have provided some of the most convincing evidence for reciprocal communication between the nervous system and the immune system. The basic conditioning paradigm involves the temporal pairing of a neutral conditioned stimulus (CS), such as a novel taste, odor, or context, with a stimulus that actively evokes an immunomodulatory response, termed the unconditioned stimulus (UCS). Following the CS-UCS pairing, re-exposure to the CS alone alters immune functioning in a manner which mimics the normal or unconditioned effect of the UCS. Ader and Cohen (1975) provided one of the earliest demonstrations of conditioned immunomodulation by showing that a gustatory stimulus which has been paired with the immunosuppressive drug cyclophosphamide can acquire immunosuppressive properties in mice. Subsequent investigations have demonstrated that many immune measures are modifiable by conditioning including both humoral and cell-mediated immune responses (for review, see Ader and Cohen, 1993). In addition to animal studies, conditioned immune alterations have been demonstrated in humans as well (Exton et al., 2000b; Goebel et al., 2002). Furthermore, evidence from a number of studies which have investigated the effects of conditioned immune alterations on disease processes suggests that conditioning phenomena are clinically relevant. For example, conditioned effects on the immune system have been shown to significantly influence morbidity and mortality in animal models of lupus (Ader and Cohen, 1982), rheumatoid arthritis (Klosterhalfen and Klosterhalfen, 1983; Lysle et al., 1992b), allergic dermatitis (Exton et al., 2000a), organ transplantation (Exton et al., 1998), and neoplastic disease (Gorczynski et al., 1985). Interestingly, it is possible to condition increases or decreases in immune measures such as NK cell activity, with the direction of the
conditioned effect mirroring that of the unconditioned effect (Ghanta et al., 1985; O'Reilly and Exon, 1986).

While there is undoubtedly a myriad of receptors and signaling molecules involved the mediation of complex processes such as Pavlovian conditioning of immunity, there has been considerable interest in the role of the endogenous opioid system. A number of studies using different conditioning models have shown that many of the behavioral and physiological effects of opioid drugs can be conditioned, suggesting that the opioid system is particularly integral to several conditioning phenomena. For instance, the involvement of opioid receptors has been established in studies of conditioned taste aversion (Leblanc and Cappell, 1975), conditioned analgesia (Miller et al., 1990), and conditioned hyperthermia (Lal et al., 1976). Notably, opioid receptors have also been shown to mediate a number of conditioned immunomodulatory effects. Much of the impetus for investigating the role of endogenous opioids in conditioned immunomodulation was derived from early studies showing that the opioid antagonist naltrexone blocked reductions in NK activity produced by exposure to inescapable electric shock in rats (Cunnick et al., 1988; Shavit et al., 1984). Further studies demonstrated that these stressor-induced, opioid-mediated immune alterations could be conditioned to the environmental context (the CS) associated with the stressful or aversive stimulus. For example, the presentation of a CS which has previously been paired with aversive electric shock produces naltrexone-reversible reductions in NK cell activity and lymphocyte mitogenic responses (Lysle et al., 1992a). These conditioned immune alterations were subsequently shown to be mediated specifically by μ-opioid receptors in the central nervous system, providing direct evidence for the involvement of the central opioid system in conditioned immunomodulation (Perez and Lysle, 1997).
Similar to the effects of stress, morphine’s pharmacological effects on immune status can also be conditioned to environmental stimuli. For example, when rats are re-exposed to a distinctive environment in which they have previously received morphine, immunological alterations occur that are similar to those produced by the drug alone, including decreased mitogen responsiveness of blood and splenic lymphocytes, reduced interleukin-2 production, and decreased NK cell activity in the spleen (Coussons et al., 1992; Coussons-Read et al., 1994a; Coussons-Read et al., 1994b). These conditioned effects are specifically associated with re-exposure to the CS, as extensive control procedures have shown that the immune alterations are not related to extraneous procedural variables (Coussons et al., 1992). Because opioid drugs typically produce immunosuppression, conditioned stimuli paired with opioid administration acquire immunosuppressive properties. Thus, an important implication is that any detrimental health consequences of opioid use may also be conditioned to environmental stimuli and not solely due to pharmacological properties of the drug.

**Goals of the Dissertation**

This dissertation addresses a void in current knowledge regarding the neuroimmune mechanisms of opioid-mediated immunomodulation. The present studies examine effects of both heroin and morphine on several immune parameters to provide a general assessment of the mechanisms involved in opioid-induced immune alterations. Given the paramount importance of NK cells for surveillance and resistance to infections and cancers, NK cell activity is an important parameter of immune function to consider when investigating the effects of opioids. Furthermore, by using a model of Gram-negative bacterial infection to examine the effects of heroin on iNOS production, these studies will provide valuable
information regarding the mechanisms whereby opioids alter resistance to infectious diseases. **Chapter 2** describes the role of dopamine receptors in the nucleus accumbens as a central mediator of opioid-induced immune alterations. **Chapter 3** examines the contributions of the sympathetic transmitter NPY as a potential peripheral mediator of the dopamine-dependent immunomodulatory effects of opioids. Finally, **Chapter 4** evaluates whether conditioned environmental stimuli modulate immune status by acting through similar receptor pathways as exogenous opioids. Collectively, these projects will provide valuable insights to the field of neuroimmunology by elucidating novel receptor mechanisms of opioid-mediated immunomodulation. Furthermore, given that the capacity to increase dopamine levels in the nucleus accumbens is shared by other drugs of abuse, these studies may have important mechanistic implications for how other classes of abused drugs alter immune status.
CHAPTER 2
ROLE OF NUCLEUS ACCUMBENS DOPAMINE IN OPIOID-INDUCED IMMUNE ALTERATIONS

Introduction

Opioids suppress a number of immune parameters indirectly through interactions with the central nervous system. Such neural modulation of the immune system is a precisely regulated phenomenon, as stimulation of opioid receptors in different areas of the brain selectively modifies distinct immunological parameters. To date, opioid receptors in the periaqueductal gray matter and the anterior hypothalamus have been identified as sites that mediate the immunomodulatory effects of morphine. Beyond these initial investigations however, there has been little progress toward the elucidation of neural mechanisms that underlie the immunological effects of opioids. Because peripheral immune responses are regulated at all levels of the neuraxis, it is likely that opioids influence immunity through interactions with numerous neural structures. Surprisingly however, there is virtually nothing known about how the stimulation of opioid receptors interacts with other brain regions to modulate immune functions.

Recently, our laboratory has shown that morphine’s effects on the immune system involve central dopamine systems (Saurer et al., 2004). Midbrain dopamine neurons, which innervate basal ganglia and limbic structures of the forebrain, play an important role in the regulation of peripheral immune responses. Lesion studies in rats indicate that the
destruction of nigrostriatal or mesolimbic dopamine neurons impairs both cellular and humoral immune measures as evidenced by changes in NK cell activity and altered immune responses to foreign antigen (Deleplanque et al., 1994; Devoino et al., 1997). Furthermore, it has been reported that the stimulation of D1 receptors in the nucleus accumbens produces immunosuppressive effects as evidenced by decreased splenic lymphocyte proliferative responses (Nistico et al., 1994). Given that morphine administration patently stimulates dopamine release in the nucleus accumbens, we hypothesized that dopamine receptors in the nucleus accumbens may comprise a critical neural substrate of opioid-mediated immunomodulation.

The studies described in the current chapter begin to characterize the dopaminergic mechanisms involved in opioid-induced immunomodulation by first examining the effects of opioids on splenic NK cell activity. NK cells are a distinct population of lymphocytes that constitute the immune system’s first line of defense against a variety of infections and cancers. It is because of their unique ability to spontaneously (without prior sensitization) and selectively (without damaging normal cells) kill tumor cells that they are called “natural killer” cells (Kiessling et al., 1975). The ability of NK cells to lyse target cells is an important effector mechanism in host defense as it plays an indispensable role in resistance to viral (Biron et al., 1999; Whiteside and Herberman, 1994) and metastatic diseases (Herberman and Ortaldo, 1981; Whiteside and Herberman, 1995). Most importantly with respect to the current study, data from our laboratory and that of many others clearly demonstrate that ex vivo NK cell cytolytic responses are extraordinarily sensitive to the effects of in vivo opioid administration (Lysle et al., 1993; Nelson et al., 1997; Shavit et al., 1986; Weber and Pert, 1989). Therefore, a key objective of this chapter is to elucidate a
central dopaminergic mechanism through which opioids may act to suppress this crucial immune function parameter—NK cell activity.

To test the hypothesis that dopamine receptors in the nucleus accumbens mediate the effects of morphine on NK cell activity, the current studies first examine the contributions of dopamine receptor subtypes using the D₁ receptor antagonist SCH-23390 and the D₂ antagonist raclopride. Immunostaining for c-Fos protein was utilized to identify areas of neuronal activation within the nucleus accumbens (i.e., core vs. shell) following morphine injection, and subsequent investigations examined whether morphine’s NK suppressive effect is contingent upon the activation of D₁ receptors in the core or shell subdivisions of the nucleus accumbens. Furthermore, using the selective D₁ receptor agonist SKF-38393, the present study evaluates the effect of direct D₁ receptor stimulation on immune status.

To determine whether the mechanisms of morphine’s effects generalize to the more commonly abused opioid heroin, the present study also examines the role of the nucleus accumbens in heroin-induced alterations of NK activity and splenic lymphocyte proliferation. Importantly, heroin’s effect on the in vivo expression of inducible nitric oxide synthase (iNOS) was also assessed to determine whether dopaminergic mechanisms underlie opioid effects on in vivo immune measures. The iNOS enzyme plays a critical role in disease resistance because it is responsible for the production of nitric oxide in response to infectious challenges. The generation of nitric oxide by cells of the immune system has potent antimicrobial and anti-tumor effects and may also be involved in regulating the immune response per se (Green et al., 1990; Green and Nacy, 1993; Hibbs et al., 1987; James and Glaven, 1989; Karupiah et al., 1993; Rossi et al., 1999; Vincendeau et al., 1992). Since iNOS is not expressed under normal physiological conditions, but rather must be induced, enzyme levels
are commonly used as a marker of nitric oxide production\textsuperscript{3}. In the present study, we stimulate iNOS production with lipopolysaccharide (LPS), the major immunogenic component expressed on the surface of Gram-negative bacteria. Thus, LPS injection provides a useful model to examine the in vivo effects of heroin on the immune response to Gram-negative infection. Collectively, the studies presented in this chapter provide an extensive evaluation of the role of dopamine D\textsubscript{1} receptors in the nucleus accumbens shell in the effects of opioids on several critical parameters of immune function.

**Materials and Methods**

**Animals**

Adult male Lewis rats weighing approximately 200-250 grams were purchased from Charles River Laboratories (Raleigh, NC). Upon arrival, animals were individually housed and maintained on a reverse 12-hr light/dark cycle. Animals were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Food and water were available *ad libitum* throughout the experiment.

**Drugs**

Morphine sulfate (NIDA, Bethesda, MD), diacetylmorphine sulfate (heroin; NIDA), R(\(+\))-SCH-23390 hydrochloride (Sigma-Aldrich, St. Louis, MO), S(-)-raclopride (+)-tartrate salt (Sigma), and R(\(+\))-SKF-38393 hydrochloride (Sigma) were dissolved in sterile 0.9% saline. For all experiments, morphine was administered s.c. at a dose of 15 mg/kg in a 1.0 ml/kg volume. This dose was selected based on previous studies from our laboratory showing that the effects of morphine are dose-dependent and that the effects were blocked by

\textsuperscript{3} Nitric oxide is a volatile gaseous molecule that is difficult to quantify directly.
naltrexone, indicating the involvement of opioid receptors (Lysle et al., 1993). Heroin was administered s.c. at doses of 1, 3, 5, or 10 mg/kg in a 1.0 ml/kg volume. Dose-effect analyses indicated that 3 mg/kg heroin induced a maximal effect so this dose was used for all subsequent studies. Anti-DAT-saporin (Advanced Targeting Systems, San Diego, CA) was diluted in sterile PBS to a final concentration of 0.5 μg/μl.

**Systemic Dopamine Antagonism**

The effect of D₁ or D₂ dopamine receptor antagonism was determined using SCH-23390 or raclopride respectively. In the first experiment rats were assigned to one of six groups (n = 4 per group) in which they received the D₁ receptor antagonist SCH-23390 (0, 0.1, or 1.0 mg/kg, s.c.) 30 minutes prior to saline or morphine. This experiment was replicated using the same design, and the data was combined for analysis (n = 7 – 8 per group). In the second experiment rats were assigned to one of eight groups (n = 3) in which they received an injection of raclopride (0, 0.1, 1.0, or 3.0 mg/kg, s.c.) 30 minutes prior to injection of morphine or saline.

**C-Fos Immunohistochemistry**

Given the functional distinctions between the core and shell components of the nucleus accumbens, we assessed the pattern of neuronal activation in the core and shell to determine whether morphine differentially activated these areas in the male Lewis rat at a dose of 15 mg/kg. Animals were assigned to two groups (n = 3 per group) in which they received a s.c. injection of morphine or saline in a 1 ml/kg volume. Two hours later, animals were deeply anesthetized with ketamine/xylazine and transcardially perfused with cold saline.
followed by a 4% paraformaldehyde solution. Brains were removed and post-fixed for two days. Immunohistochemistry was performed on free-floating 50 µm cryostat-sliced coronal sections using the avidin-biotin-peroxidase (ABC) method (Vectastain Elite Kit, Vector Laboratories, CA) with DAB detection. The primary antibody used was goat anti-c-Fos IgG (Santa-Cruz Biotechnology, CA, sc-52, diluted 1:20,000). c-Fos labeled cells were counted using the bright field method on an Olympus BX51 microscope interfaced with ImagePro Plus software (Media Cybernetics, Silver Spring, MD). A standard rectangle (size determined by region being analyzed) was placed over the area of interest and the number of labeled cells was counted automatically by the software. Counts were performed bilaterally and averaged over 4 consecutive sections for each animal.

**Surgical and Microinjection Procedures**

For all microinjection experiments, animals were implanted with bilateral guide cannulae (Plastics One, Roanoke, VA) one week prior to drug administration. Stereotaxic surgeries were performed under anesthesia induced with a 0.2 ml intramuscular injection of a 1:1 (vol/vol) mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). On the test day, animals receiving microinjections into the accumbens shell were assigned to one of six groups (n = 3 – 4) in which they received SCH-23390 (0, 0.015, or 0.15 µg/side) 30 minutes prior to morphine or saline administration. This experiment was replicated twice using the same design, and the data was combined for analysis (n = 7 – 12 per group). SCH-23390 was administered in a 0.5 µl volume per side via a 33-gauge injector that protruded 2 mm beyond the tip of the guide cannula (AP +1.7, ML ±1.0, DV –7.4). Coordinates are expressed as millimeters from bregma (Paxinos and Watson, 1986).
A separate experiment using the same design was performed to examine the effect of SCH-23390 administration into the nucleus accumbens core. Animals were assigned to one of six groups (\(n = 4\)) in which they received SCH-23390 (0, 0.015, or 0.15 \(\mu\)g/side) 30 minutes prior to morphine or saline administration. SCH-23390 was administered in a 0.5 \(\mu\)l volume per side via a 28-gauge injector that protruded 2 mm beyond the tip of the guide cannula (AP +1.7, ML ±1.9, DV −6.8).

The effects of D₁ antagonism on heroin-induced immune alterations were examined in a subsequent series of experiments. To determine the effects on NK activity and lymphocyte proliferation, SCH-23390 (0, 0.015, or 0.15 \(\mu\)g/side) was administered 30 minutes prior to heroin or saline (\(n = 5 – 6\) per group). Microinjections were delivered in a 0.5 \(\mu\)l volume per side via a 33-gauge injector that protruded 2 mm beyond the tip of the guide cannula (AP +1.7, ML ±0.8, DV −7.4). The same experimental procedures were used to evaluate the effects of D₁ antagonism on iNOS production. In this experiment, animals were assigned to one of six groups (\(n = 3 – 4\)) in which they received SCH-23390 (0, 0.015, or 0.15 \(\mu\)g/side) 30 minutes prior to heroin or saline in combination with an injection of LPS (1000 \(\mu\)g/kg, s.c.). This experiment was replicated twice using the same design, and the data was combined for analysis (\(n = 6 – 7\) per group).

For all experiments, drug was infused over a 30 second period using a microsyringe pump (Harvard Apparatus, Holliston, MA) and the injector was left in place for one minute to allow diffusion of the drug away from the injection site. Following drug treatment and sacrifice, Alcian Blue dye was injected via the cannula and brains were removed and post-fixed in 4% paraformaldehyde for two days. Brains were transferred to a 30% sucrose solution for cryoprotection, frozen, and stored at -80°C for subsequent analysis. Accurate
cannulae placements were verified by examination of 50 µm thionin-stained tissue sections under a light microscope. Only animals with placements within the targeted region were included in the analysis.

The effect of direct pharmacological activation of nucleus accumbens D1 receptors on NK activity was investigated by implanting rats with bilateral cannulae into the nucleus accumbens shell as described above. Animals were assigned to one of four groups (n = 4 per group) in which they received bilateral microinjections of the D1 agonist SKF-38393 (0, .05, .5, or 5.0 µg/side). SKF-38393 was administered in a 0.5 µl volume per side according to the procedure described previously. Animals were sacrificed one hour later to assess splenic NK activity and splenocyte proliferative responses. Cannulae placements were verified as described above.

**Immunotoxin Administration Procedure**

The immunotoxin anti-DAT-saporin is a highly selective dopaminergic cytotoxin that utilizes a monoclonal antibody to target cells expressing the dopamine transporter (DAT) (Wiley et al., 2003). Under stereotaxic surgery, anesthetized animals received bilateral injections of anti-DAT-saporin (0.5 µg/side) into the nucleus accumbens shell (AP+1.7, ML±1.0, DV-7.4). Immunotoxin injections were performed using a microsyringe pump (Harvard Apparatus) that delivered 1.0 µl/side of the toxin over a 60s interval. Bilateral injections were performed simultaneously via a bilateral 33 gauge injector (Plastics One), and the injectors remained in place for an additional minute to allow diffusion of the toxin into the parenchyma. Sham lesions were performed by injecting an equivalent volume of phosphate buffered saline vehicle. Two weeks following toxin administration, animals
received a subcutaneous injection of morphine (15 mg/kg) or saline. One hour later, animals were sacrificed and splenocytes were used to determine NK activity.

**Tissue Collection**

One hour following morphine, heroin, or saline injections, rats were sacrificed by cervical dislocation. This time point was selected based on previous data from our laboratory indicating a maximal suppression of the measured immune parameters 1 h following morphine administration. The spleen was removed and placed in 7 ml of RPMI-1640 tissue culture media supplemented with 10 mM HEPES, 2 mM glutamine, and 50 µg/ml gentamicin (hereafter referred to as supplemented RPMI; GIBCO, Grand Island, NY). Each spleen was prepared as a single-cell suspension by gently pressing the tissue between two sterile, frosted microscope slides in supplemented RPMI enriched with 10% fetal bovine serum (hereafter referred to as complete RPMI; GIBCO). Splenic leukocytes were counted using a Hemavet 850 cell analyzer (CDC Technologies Inc., Oxford, CT), and cell suspensions were adjusted to 5 × 10⁶ leukocytes/ml by diluting with complete RPMI. Prior work by our laboratory has utilized flow cytometry to demonstrate that acute morphine treatment does not alter the total number of leukocytes or the relative number of each major leukocyte subpopulation (including NK cells) in the spleen (Fecho and Lysle, 1999).

**Natural Killer Cell Assay**

Splenic NK cell activity was assessed using a standard chromium release assay. Adjusted splenocyte suspensions were co-incubated with the murine T-cell lymphoma, YAC-1. The YAC-1 target cells were labeled by incubation for 70 min with 200 µCi of sodium
chromate-51 $[^{51}\text{Cr}]$. YAC-1 cells were then washed three times with complete RPMI to remove exogenous $[^{51}\text{Cr}]$. Splenic leukocytes were used as effectors and were plated in triplicate at 10, 5, 2.5, and $1.25 \times 10^5$ cells/well of a 96 well plate. Labeled targets were diluted and plated at $1 \times 10^4$ cells/well to give effector/target (E:T) ratios of 100:1, 50:1, 25:1 and 12.5:1. Following a 5 h incubation at 37°C in a humidified CO$_2$ incubator, the amount of $[^{51}\text{Cr}]$ released into the supernatant was determined using an LKB gamma counter (model 1272 CliniGamma). Percent specific lysis at all E:T ratios were used to calculate lytic units (Pross and Maroun, 1984). Results are reported as the number of lytic units per $10^7$ effector cells, where a lytic unit is defined as the number of splenic leukocytes necessary to lyse 20% of the target cells.

**Splenocyte Proliferation Assay**

Mitogen stimulation assays were completed using adjusted splenocyte suspensions. Splenic T- and B-lymphocyte proliferation was induced with the mitogens Con-A and LPS (Sigma-Aldrich), respectively. One hundred microliters of the adjusted cell suspensions were pipetted in triplicate into microtiter plate wells containing final concentrations of 0, 0.5, and 5.0 µg/ml Con-A and 0.5 and 5.0 µg/ml LPS. Splenocyte cultures were then incubated for 48 hours at 37°C in a humidified CO$_2$ incubator. Each culture well was pulsed with 1 µCi of $[^3\text{H}]$ thymidine during the last 5 hours of the incubation period. Cultures were then harvested onto glass fiber filter paper using a Tomtec automatic 96-well cell harvester. The amount of $[^3\text{H}]$-thymidine incorporated into the DNA of proliferating cells was measured using a liquid scintillation counter (Wallac, Model 1205) and is expressed as the mean of the triplicate disintegrations per minute (DPM) for the samples from each rat.
Real-Time RT-PCR

To determine iNOS expression, real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted from a section of each spleen, liver, and lung using TRI-Reagent (Molecular Research Center, Cincinnati, OH), a modification of the original method described by Chomczynski and Sacchi (1987). RNA was quantified spectrophotometrically (GeneQuant II, Pharmacia-Biotech, Piscataway, NJ, USA). For the RT-PCR, reverse transcription is performed using Oligo(dT)18 primer and Moloney Murine leukemia virus-reverse transcriptase following the protocol of the advantage RT-for-PCR kit from Clontech (Palo Alto, CA, USA). PCR amplifications were performed using the Fast Start™ DNA Master SYBR Green I real-time PCR kit (Roche) and the LightCycler instrument (Roche). A master mix containing all reaction components was prepared for all reactions, with each reaction using a 20-ml mix placed in glass capillary tubes specifically designed for use in the LightCycler system. The PCR primer set for iNOS, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGGTGTCAGAGTCTTGTGCCTTTGG-3′, was synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, UNC-Chapel Hill). Copy numbers were generated from an external standard curve. Amplifications were carried out for 40 cycles, and curves showing fluorescence at each cycle were determined by the computer software (Roche). Samples were pre-incubated for 10 min at 95°C to activate the Fast-Start Taq DNA polymerase. The cycle temperatures were 95, 60, and 72°C for the denaturing, annealing, and extending, respectively. The cycle times were 15, 5, and 25 s for the denaturing, annealing, and extending, respectively. Fluorescence level was determined at the end of the extending phase for each cycle of PCR. The analysis of the
fluorescence level in standards and samples over the course of 40 cycles was used to derive
the number of copies of the target molecule in each sample. Additionally, assessments of
housekeeping gene expression, cyclophilin, were made to assure comparable quality of RNA
among samples. The sequence of the cyclophilin primers was 5′-CCAAGACTGAGTGCTGCT-
3′ and 5′-AGATTACAGGGTGTTTGC-3′. The data are expressed as the ratio of iNOS
mRNA copies (per 10 ng cDNA) to cyclophilin copy number based on the standard curve
using the LightCycler software (Roche). Furthermore, to confirm the nature of amplification
product, a melt curve analysis was conducted after the final PCR cycle. This analysis
involved denaturing the products by slowly heating them to 95°C, during which fluorescence
is continuously measured.

Statistics

Data analysis for all dopamine antagonist administration studies was performed using
a two-way analysis of variance (ANOVA), with experimental replication entered into the
model as a covariate to control for inter-assay variability in baseline NK activity when
appropriate. For the two-way analysis, the first factor was antagonist dosage and the second
factor was the type of subsequent injection (morphine/heroin or saline). Data from the
immunotoxin study was also analyzed using a two-way ANOVA to assess the impact of
immunotoxin exposure on morphine’s NK suppressive effect. Analysis of c-Fos expression
was performed using independent samples \( t \)-tests. For the SKF-38393 administration and
heroin-dose effect studies, one-way ANOVA was utilized. For all analyses, polynomial
contrasts and planned contrasts were performed in accordance with \( a \ priori \) hypotheses.
Planned contrasts in each analysis consisted of pairwise comparisons of means between
opioid treatment groups and saline treatment groups at each level of the factor “antagonist dosage.” All analyses were performed with the level of significance set at \( p < 0.05 \).

**Results**

**Effects of Morphine**

*Systemic dopamine receptor antagonism*

The first study examined the effect of subcutaneous administration of the D\(_1\) receptor antagonist SCH-23390 or the D\(_2\) receptor antagonist raclopride on morphine-induced decreases in splenic NK activity. Figure 2.1 (panel A) shows the results of experiments examining the effect of SCH-23390. Planned comparisons between the saline/saline and saline/morphine treatment groups showed that morphine produced a significant decrease in NK cell activity \([F(1, 36) = 23.72; p < .0001]\). Analysis of variance revealed a significant interaction between morphine treatment and SCH-23390 dose \([F(2, 36) = 11.02; p < .001]\). Polynomial contrasts showed a significant linear component to the interaction indicating that the suppressive effect of morphine was attenuated by SCH-23390 administration in a dose-dependent manner \([F(1, 36) = 21.26; p < .0001]\). Furthermore, planned comparisons revealed that the high dose of SCH-23390 completely blocked the suppressive effect of morphine, as the groups that received 1.0 mg/kg of SCH-23390 prior to morphine or saline administration were not significantly different \([F(1, 36) = 2.72; p > .05]\).

The effect of subcutaneous raclopride administration on morphine-induced suppression of NK activity is shown in Figure 2.1 (panel B). Analysis of variance yielded a significant main effect of morphine treatment \([F(1, 16) = 73.57; p < .0001]\), indicating that morphine suppressed NK activity. There was not a significant effect of raclopride dose \([F(3,\ldots)]\).
Moreover, planned comparisons indicated that morphine reduced NK activity at each dose of raclopride tested ($p < .001$).

**Figure 2.1** Effect of systemic dopamine receptor antagonism on morphine-induced suppression of splenic NK activity. *A*, Subcutaneous administration of the D$_1$ antagonist SCH-23390 dose-dependently attenuated the effect of morphine. *B*, Treatment with the D$_2$ antagonist raclopride did not alter the effect of morphine on NK activity. Data are expressed as lytic units (mean ± S.E.). *$p < .05$; **$p < .001$ compared with appropriate saline-treated control group that received the same dose of dopamine antagonist.

*Induction of c-Fos expression*

Photomicrographs of c-Fos protein expression in the nucleus accumbens in the core and shell following morphine or saline treatment are displayed in Figure 2.2 (A-D). Compared to saline treatment, morphine did not increase the number of c-Fos positive cells in the core subregion of the nucleus accumbens [$t(4) = 0.58; p > .05$] (Figure 2.2, panel E). In the shell of the nucleus accumbens however, morphine induced a significant increase in
the number of c-Fos labeled cells compared to saline treatment \[ t(4) = 4.61, p < .01 \] (panel F). These results show that the same dose of morphine used to elicit immunosuppression produces an activation of the nucleus accumbens shell, but not the core, in the male Lewis rat.

**Figure 2.2** Expression of c-Fos protein in the nucleus accumbens following injection of saline (A, B) or 15 mg/kg morphine (C, D). Representative photomicrographs of the nucleus accumbens core (A, C) and shell (B, D) are shown. Data in E and F are expressed as c-Fos labeled cells (mean ± S.E.) counted bilaterally and averaged over four consecutive 50 µm sections for each animal. Morphine administration produced a significant increase in c-Fos positive cells in the nucleus accumbens shell but had no effect in the core. Magnification, 10×. *p < 0.01 compared to saline-treated group.
Nucleus Accumbens $D_1$ receptor antagonism

Because the $D_2$ antagonist raclopride—a compound which readily crosses the blood-brain barrier (Hall et al., 1988)—did not attenuate the effect of morphine on NK activity, only the $D_1$ antagonist SCH-23390 was administered in the microinjection studies. Figure 2.3 (panel A) shows the effects of SCH-23390 microinjections into the nucleus accumbens shell on morphine-induced alterations in NK activity. Planned comparisons demonstrated that morphine significantly reduced NK cell activity compared to saline treatment among animals that received microinjections of saline [$F(1, 47) = 18.49; p < .001$]. Analysis of variance revealed a significant interaction between morphine treatment and SCH-23390 dose [$F(2, 47) = 5.65; p < .01$] and polynomial contrasts showed a significant linear component to the interaction indicating that the suppressive effect of morphine was attenuated by SCH-23390 administration in a dose-dependent manner [$F(1, 47) = 11.26; p < .01$]. Furthermore, planned comparisons revealed that the high dose of SCH-23390 completely blocked the suppressive effect of morphine, as the groups that received 0.15 µg/side SCH-23390 prior to morphine or saline administration were not significantly different [$F(1, 47) = 0.19; p > .05$]. These findings show that $D_1$ antagonist administration into the nucleus accumbens shell prevents the suppression of NK activity by morphine.

The effect of SCH-23390 administration into the nucleus accumbens core is shown in Figure 2.3 (panel B). Analysis of variance revealed a significant main effect of morphine [$F(1, 18) = 52.23; p < .0001$]. There was no effect of SCH-23390 dose [$F(2, 18) = 2.31; p > .05$], nor was there a significant interaction between SCH-23390 dose and morphine treatment [$F(2, 18) = 2.32; p > .05$]. Planned comparisons further indicated that morphine decreased NK activity at each dose of SCH-23390 tested ($ps < .01$). These findings suggest
that D₁ receptors located in the nucleus accumbens core do not mediate the effect of morphine on splenic NK activity and provide additional support for the specificity of microinjections in the shell.

**Figure 2.3** Effect of D₁ receptor antagonism in the nucleus accumbens shell (A) or core (B) on morphine-induced suppression of NK cell activity. Rats received bilateral microinjections of saline or SCH-23390 into the nucleus accumbens prior to a subcutaneous injection of morphine. A, Administration of SCH-23390 into the shell dose-dependently attenuated the effect of morphine on NK activity. B, SCH-23390 microinjections into the core did not alter the effect of morphine. Data are expressed as lytic units (mean ± S.E.). *p < .05; **p < .001 compared to the saline-treated control group that received an equivalent dose of SCH-23390.

**Intra-accumbens immunotoxin administration**

An additional study was conducted to determine whether dopaminergic immunotoxin administration into the nucleus accumbens shell would disrupt the effect of morphine on NK activity. Figure 2.4 displays the results of the anti-DAT-saporin immunotoxin experiment. Planned comparisons demonstrated that morphine significantly reduced NK cell activity compared to saline treatment in the sham-lesion group \([F(1, 12) = 17.31; p < .01]\). Analysis
of variance revealed a significant interaction between morphine and immunotoxin treatment \( [F(1, 12) = 10.15; p < .01] \). In addition, planned comparisons show that NK activity in the morphine treated group was not different from the saline group among animals that received immunotoxin infusions \( [F(1, 12) = 0.12; p > .05] \), indicating that the effect of morphine was abolished by anti-DAT-saporin exposure.

**Figure 2.4** Effect of intra-accumbens immunotoxin administration on morphine-induced suppression of NK cell activity. Rats received bilateral microinjections of vehicle (sham-lesions) or the dopaminergic toxin anti-DAT-saporin (immunotoxic lesions) into the nucleus accumbens shell. The effect of morphine on NK activity was assessed two weeks later. Immunotoxin administration prevented the suppressive effect of morphine on NK activity. Data are expressed as lytic units (mean ± S.E.). *\( p < .05 \) compared with sham-lesioned, saline-treated group.

**Effects of Dopamine D\(_1\) Agonist Administration**

A subsequent experiment examined whether the activation of D\(_1\) receptors in the absence of morphine is sufficient to produce immune alterations. The results of the NK assay displayed in Figure 2.5 show that administration of SKF-38393 into the nucleus accumbens shell induced significant reductions in splenic NK activity \( [F(3, 12) = 4.17; p < \)
Polynomial contrasts showed a highly significant linear component to the SKF-38393 treatment \([F(1, 12) = 10.09; p < .01]\), indicating that the suppressive effect of SKF-38393 on NK cytolytic activity increased in a dose-dependent manner. Conversely, SKF-38393 did not alter T or B cell proliferative responses to Con-A \([F(3, 12) = 0.20; p > .05]\) or LPS \([F(3, 12) = 0.22; p > .05]\), respectively.

![Figure 2.5](image)

**Figure 2.5** Effect of nucleus accumbens dopamine D₁ receptor stimulation on splenic immune measures. Rats received bilateral microinjections of saline or SKF-38393 into the nucleus accumbens shell one hour prior to sacrifice. SKF-38393 treatment produced a suppression of NK activity at the two highest doses tested. Con-A and LPS stimulated proliferative responses were not altered at any dose tested. Data are expressed as lytic units (mean ± S.E.). *\(p < .05\) compared with saline-treated group.

**Effects of Heroin**

*Heroin dose-response analysis*

To expand the generality of the previous findings with morphine, the present study also investigated the role of the nucleus accumbens in mediating heroin-induced immune alterations. The optimal dose of heroin for producing immune alterations was first established by conducting a dose-response analysis. The results of this experiment are displayed in Figure 2.6. Analysis of variance yielded a significant main effect of dose on NK
cell activity, indicating that heroin reduced NK activity \( F(4, 15) = 16.22; p < .0001 \). Furthermore, planned comparisons showed that heroin significantly decreased cytolytic activity at every dose \((ps < .001)\). Figure 2.6 also displays the effects of heroin of splenocyte proliferative responses to Con-A and LPS. There was a significant main effect of heroin on proliferation induced by Con-A \( F(4, 14) = 36.51; p > .0001 \). Planned comparisons further showed that heroin significantly decreased mitogenic responses to Con-A at each dose examined \((ps < .05)\). Analysis of variance also revealed a significant main effect of heroin on LPS-induced proliferation \( F(4, 14) = 33.89; p < .0001 \). The effect of heroin on LPS-induced proliferation was evident at each dose tested as determined by planned comparisons with the saline control group \((ps < .05)\).

**Figure 2.6** Effects of heroin dose on splenic immune measures. Subcutaneous heroin administration significantly suppressed NK activity at each dose. Heroin also significantly decreased splenocyte mitogenic responses to Con-A and LPS at each dose examined, with more pronounced effects at doses of 3 mg/kg or higher. Data from the cytotoxicity assay data are expressed as lytic units (mean ± S.E.). Data from proliferation assays are expressed as disintegrations per minute (mean ± S.E.). \(*p < .05; **p < .001\) compared with appropriate saline-treated control group.

**Effect of D_1 receptor antagonism on NK activity**

The effect of SCH-23390 administration into the nucleus accumbens shell on heroin-induced decreases in NK activity is shown in Figure 2.7. Analysis of variance revealed
significant main effects of heroin treatment \([F(1, 27) = 18.15; p < .001]\) and SCH-23390 dose \([F(2, 27) = 6.02; p < .01]\). Planned comparisons demonstrated that heroin significantly reduced NK cell activity compared to saline treatment in animals that received saline microinjections \([F(1, 27) = 16.48; p < .001]\). Planned comparisons also revealed that both doses of SCH-23390 attenuated the effect of heroin, since there was no significant difference between heroin and saline treated groups in animals that received SCH-23390 at a dose of 0.015 \(\mu g/\text{side}\) \([F(1, 27) = 3.76; p > .05]\), or 0.15 \(\mu g/\text{side}\) \([F(1, 27) = 1.90; p > .05]\). Importantly, among groups that received subcutaneous saline injections, SCH-23390 did not significantly alter NK activity compared to saline microinjections at either dose tested \((ps > .05)\). These findings show that D\(_1\) antagonist administration into the nucleus accumbens shell prevents the suppression of NK activity induced by heroin.

**Figure 2.7** Effect of D\(_1\) receptor antagonism in the nucleus accumbens shell on heroin-induced suppression of NK cell activity. Rats received bilateral microinjections of saline or SCH-23390 into the nucleus accumbens prior to a subcutaneous injection of heroin. Both doses of SCH-23390 attenuated the effect of heroin on NK activity. Data are expressed as lytic units (mean ± S.E.). **\(p < .001\)** compared to the saline-treated control group that received an equivalent dose of SCH-23390.
Effect of D$_1$ receptor antagonism on splenocyte proliferation

Figure 2.8 (left panel) shows the effect of SCH-23390 administration into the nucleus accumbens shell on heroin-induced reductions in proliferative responses to Con-A. The analysis yielded a significant main effect of heroin treatment [$F(1, 27) = 27.00; p < .0001$], but there was not a significant effect of SCH-23390 [$F(2, 27) = 0.33; p > .05$]. Moreover, there was not a significant interaction between heroin and SCH-23390 treatment [$F(2, 27) = 0.01; p > .05$]. Planned comparisons further indicated that heroin decreased Con-A induced proliferation at each dose of SCH-23390 tested ($ps < 0.01$).

The effect of SCH-23390 administration on heroin-induced decreases mitogenic responses to LPS is shown in the right panel of Figure 2.8. Analysis of variance revealed a significant main effect of heroin [$F(1, 27) = 37.62; p < .0001$]. There was no effect of SCH-23390 treatment [$F(2, 27) = 0.44; p > .05$], nor was there a significant interaction between SCH-23390 dose and heroin treatment [$F(2, 27) = 0.02; p > .05$]. Planned comparisons indicated that heroin’s suppressive effect on LPS-induced proliferation was not blocked by SCH-23390 at any dose, as the heroin group was significantly decreased relative to the appropriate saline control group that received the same dose of SCH-23390 ($ps < .01$). These findings suggest that D$_1$ receptors located in the nucleus accumbens shell do not mediate the effect of heroin on splenocyte proliferative responses.
Figure 2.8 Effect of D₁ receptor antagonism in the nucleus accumbens shell on heroin-induced decreases in splenocyte mitogenic responses. Rats received bilateral microinjections of SCH-23390 into the nucleus accumbens prior to a subcutaneous injection of saline or heroin. SCH-23390 did not alter the effect of heroin on splenocyte proliferative responses to Con-A or LPS stimulation. Data are expressed as lytic units (mean ± S.E.). *p < .01 compared to the saline group that received the same dose of SCH-23390.

Effect of D₁ receptor antagonism on iNOS expression

A final study was performed to examine whether D₁ receptors in the nucleus accumbens shell also play a role in heroin’s effects on the production of iNOS in vivo. LPS-induced expression of iNOS mRNA in spleen, liver, and lung tissues is shown in figure 2.9. Planned comparisons demonstrated that, among animals that received saline microinjections, heroin significantly reduced iNOS levels compared to saline in the spleen \([F(1, 33) = 6.02; p < .05]\); liver \([F(1, 33) = 14.07; p < .001]\); and lung \([F(1, 33) = 12.11; p < .01]\). Analysis of variance revealed a significant interaction between SCH-23390 dosage and heroin treatment in the spleen \([F(2, 33) = 4.50; p < .05]\); liver \([F(2, 33) = 9.74; p < .001]\), and lung \([F(2,33) = 7.91; p < .01]\), indicating that SCH-23390 altered the suppressive effect of heroin in all tissues. Importantly, planned comparisons revealed that heroin and saline treated groups
which received 0.15 µg/side SCH-23390 were not significantly different with respect to iNOS expression in the spleen $[F(1, 33) = 0.23; p > .05]$; liver $[F(1, 33) = 3.96; p > .05]$; or lung $[F(1, 33) = 1.37; p > .05]$. These results indicate that the high dose of SCH-23390 abolished heroin’s effect on iNOS expression in all tissues.

**Figure 2.9** Effect of D₁ receptor antagonism in the nucleus accumbens shell on heroin-induced reductions of iNOS mRNA expression. Rats received microinjections of SCH-23390 into the nucleus accumbens prior to subcutaneous injections of saline or heroin in conjunction with LPS administration. SCH-23390 microinjections prevented the suppressive effect of heroin on iNOS expression in the spleen, liver, and lung. Data are expressed as the ratio of iNOS mRNA expression to expression of the housekeeping gene cyclophilin (mean ± S.E.). *$p < .05$; **$p < .001$ compared to the saline group that received an equivalent dose of SCH-23390.

**Discussion**

The findings presented in the current chapter demonstrate that nucleus accumbens dopamine plays an essential role in the effects of morphine and heroin on several immune parameters. Specifically, the results show that the activation of dopamine D₁ receptors in the nucleus accumbens shell is necessary to produce opioid-induced reductions of splenic NK cell activity and in vivo iNOS expression in spleen, liver, and lung tissues. Collectively,
these findings characterize a novel central mechanism whereby opioids modulate critical immune parameters.

The present data show that systemic administration of the dopamine D₁ receptor antagonist SCH-23390 blocked the effect of morphine on splenic NK activity, while the D₂ antagonist raclopride had no effect. These findings suggest that morphine-induced increases in D₁ receptor activation may have detrimental effects on the immune system, since the ability of NK cells to lyse target cells is an important effector mechanism in host defense that correlates highly (73% concordance) with altered host resistance in laboratory animals (Luster et al., 1993). Because NK cells are important for diverse aspects of host defense including anti-tumor and antiviral responses, the production of antipathogenic cytokines and chemokines, and the modulation of other immune effector functions (Biron et al., 1999; French and Yokoyama, 2003; Seaman, 2000; Trinchieri, 1989), opioid-induced decreases in NK cytolytic activity may have significant consequences on disease resistance. To determine whether central dopamine D₁ receptors mediated the effect of morphine, we examined the role of D₁ receptors within the nucleus accumbens specifically. The stimulatory influence of morphine on dopamine transmission within the nucleus accumbens has been extensively documented, and several studies have provided evidence that peripheral immune responses are regulated by mesoaccumbens dopamine neurons (Deleplanque et al., 1994; Devoino et al., 1997; Nistico et al., 1994).

The nucleus accumbens is a heterogeneous structure with two primary divisions, the shell and core, that have been distinguished based on anatomical, connectional, and functional properties. To determine whether morphine differentially activates the core or shell when administered at a dose that produces immunosuppression, we examined the
expression of c-Fos protein as a marker of neuronal activation. The results indicate that morphine selectively induces c-Fos expression in the nucleus accumbens shell. These findings are consistent with other reports that have found preferential c-Fos induction in the shell using various doses of morphine in other rat strains (Barrot et al., 1999; Grabus et al., 2004; Singh et al., 2004). Taken together with evidence that morphine preferentially increases extracellular dopamine in the shell of the nucleus accumbens (Pontieri et al., 1995), the analysis of c-Fos expression suggests that the dopamine receptors mediating morphine’s effect in the present study are located in the shell subregion. Therefore, we initially targeted the nucleus accumbens shell for SCH-23390 microinjections and found that blockade of D1 receptors in the shell prevents morphine-induced reductions of splenic NK activity. However, because the shell is a narrow region situated directly medial to the core, it is possible that injections of antagonist into the shell will diffuse into the core. To control for the possibility of drug diffusion, we also administered SCH-23390 into the core, but the core microinjections had no effect on the ability of morphine to suppress splenic NK activity. Collectively, these findings indicate that the activation of D1 receptors within the nucleus accumbens shell is necessary to elicit morphine-induced reductions of splenic NK activity.

Additional evidence for the role of the nucleus accumbens in opioid-induced immune alterations is provided by data showing that intra-accumbens administration of anti-DAT-saporin prevents morphine’s suppressive effect on NK activity. The anti-DAT-saporin conjugate is a highly selective dopaminergic immunotoxin that utilizes a monoclonal antibody to target cells expressing the dopamine transporter (DAT). Once the conjugate becomes internalized, the saporin toxin moiety is released, leading to ribosome inactivation, inhibition of protein synthesis, and eventually cell death. Full lesion development and
complete cellular dissolution are observed after two weeks (Wiley and Kline IV, 2000). Our data show that morphine administration did not suppress splenic NK activity in rats that received intra-accumbens shell injections of anti-DAT-saporin (0.5 µg/µl) two weeks earlier. It is also important to note that anti-DAT-saporin administration did not alter the baseline immune response in saline treated animals, suggesting that immunotoxin exposure does not produce non-specific effects on NK cell cytotoxicity. The dosage of immunotoxin used in the current study was selected based on reports that doses up to 8 µg produced no observable cellular damage when administered intracerebroventricularly, whereas doses as low as 0.28 µg produced selective dopamine cell loss when administered directly into the striatal parenchyma (Wiley et al., 2003). Taken together with results from the SCH-23390 microinjections, these data provide convincing evidence for the role of the nucleus accumbens in morphine-induced immunosuppression.

Another important finding in the present study is that stimulation of accumbens D1 receptors with SKF-38393 significantly reduces NK cytolytic responses. Thus, the data suggest that not only is D1 activation necessary for morphine-induced reductions in splenic NK cell activity, but that direct D1 activation in the nucleus accumbens shell is sufficient to suppress NK activity. This finding is of particular importance given that a common property of virtually every abused drug is the ability to increase dopaminergic transmission in the nucleus accumbens. Hence, one might hypothesize that in addition to morphine, other drugs of abuse should depress NK cytolytic responses. Indeed, suppressed splenic NK activity has been reported following in vivo administration of heroin (Fecho et al., 2000), amphetamine (Nunez-Iglesias et al., 1996), and cocaine (Pacifici et al., 2003). Though the extent to which a nucleus accumbens D1 receptor-mediated mechanism contributed to the observed NK
suppression in these studies is unknown, the notion that such diverse pharmacological agents may act through a common central mechanism to suppress host immunity is an intriguing hypothesis.

While the immunomodulatory properties of morphine are well known, the effects of heroin on immune status have not been extensively investigated. The paucity of controlled studies of heroin’s effects may be partly due to the fact that heroin (diacetylmorphine) is readily metabolized to morphine in the body. Thus, it is frequently assumed that the biological effects of heroin and morphine are the same. However, though heroin and morphine do produce many similar effects, they also differ in a number of important pharmacological properties. For instance, heroin penetrates the nervous system more rapidly than morphine and produces different subjective psychological effects. Furthermore, several studies have indicated that heroin is capable of producing analgesic effects through different receptor pathways than morphine (Bolger et al., 1988; Harrigan and Downs, 1978; Lange et al., 1980; Scott and Orr, 1969; Switzman et al., 1981; Tasker and Nakatsu, 1984; Uman and Inturrisi, 1981; van Ree et al., 1978). The heroin molecule per se does not possess intrinsic activity at opioid receptors that could account for such discrepancies, but in contrast to morphine, heroin is metabolized to 6-monoacetylmorphine, which has a pharmacological profile distinct from morphine. Thus, because metabolites of heroin may engage different receptor populations than morphine, it is possible that heroin acts through different neural mechanisms to produce immunomodulation. Therefore, in order to generalize our findings with morphine to the more commonly abused opioid heroin, we also investigated whether dopamine receptors mediate the effects of heroin.
The present findings are the first to show that heroin-induced immune alterations involve the activation of nucleus accumbens D₁ receptors. Specifically intra-accumbens D₁ antagonist administration abolished the suppressive effect of heroin on NK activity. Interestingly SCH-23390 administration did not attenuate heroin’s suppressive effects on T and B cell proliferative responses to mitogen stimulation. These results are consistent with the data showing that stimulation of accumbens D₁ receptors with SKF-38393 does not alter splenocyte mitogenic responses. One intriguing aspect of these findings is that nucleus accumbens dopamine receptors appear to be selectively involved in a subset of opioid-induced immune alterations. That is to say, the effects of opioids on certain immune responses seem to be “dopamine-dependent” (e.g., NK cell activity) whereas other responses are not (e.g., lymphocyte proliferative responses).

The current findings also provide evidence that heroin-induced reductions of iNOS expression in spleen, lung, and liver tissues are dopamine-dependent effects. The generation of large amounts of nitric oxide by cells of the immune system, particularly the macrophage, is imperative for host resistance to potentially infectious microbes including bacteria, viruses, and parasites (Green et al., 1990; Green and Nacy, 1993; James and Glaven, 1989; Rossi et al., 1999; Vincendeau et al., 1992). Our data further substantiate the critical involvement of nucleus accumbens dopamine by demonstrating that accumbens D₁ receptors mediate the effects of heroin on the in vivo immune response to LPS, the major immunogenic component expressed on the surface of Gram-negative bacteria. The injection of LPS is a generally accepted model of sepsis, a systemic infection resulting from the proliferation of bacteria in the blood (usually due to a failure of immunological defenses) that often leads to multiple organ failure and results in death. The fact that SCH-23390 microinjections prevented the
suppression of iNOS in several key organs indicates that the heroin-induced increase of nucleus accumbens dopamine has widespread effects on immune system in vivo. Furthermore, an important implication of these findings is that the dopamine response to heroin may be directly involved in the increased susceptibility to bacterial complications among heroin addicts as the result of impaired nitric oxide production.

An important issue for future studies will be to investigate the efferent mechanisms through which increased D₁ receptor activation in the nucleus accumbens translates into altered peripheral immune responses. The two major pathways through which the brain modulates peripheral immune responses are the HPA axis the sympathetic nervous system. The efferent mechanisms that mediate the effect of acute opioid treatment on NK activity and iNOS production specifically have not been elucidated to date, but the involvement of the HPA axis seems improbable since opioid-induced suppression of NK activity has been observed in adrenalectomized animals and in animals treated with glucocorticoid antagonists (Bayer et al., 1990b; Fecho et al., 1996b; Liang-Suo et al., 2002). On the other hand, sympathetic efferent pathways have been implicated based on indirect evidence showing that opioid administration increases central sympathetic outflow (Appel et al., 1986), and activation of the sympathetic nervous system by various stimuli suppresses splenic NK activity (Fecho et al., 1993b; Irwin et al., 1990; Jiang et al., 2004; Wu and Pruett, 1996b). Furthermore, the PAG—a region closely associated with the regulation of sympathetic outflow—has been identified as a site of morphine’s µ-opioid receptor interactions responsible for the suppression of NK cell activity (Lysle et al., 1996; Weber and Pert, 1989).

In conclusion, the present study provides the first demonstration that the nucleus accumbens is involved in opioid-induced immunosuppression. Overall, these data provide substantial
evidence for the involvement of endogenous dopamine pathways in opioid mediated immunosuppression and further suggest that opioid-induced increases in dopamine signaling may have adverse consequences on immune status. Given that the capacity to increase dopamine transmission is a common property of drugs of abuse, these findings may have important implications regarding the mechanisms through which abused drugs compromise host defenses.
CHAPTER 3
NEUROPEPTIDE Y-DEPENDENT MECHANISMS OF OPIOID-INDUCED IMMUNOMODULATION

Introduction

The previous chapter presented evidence that the stimulation of dopamine D1 receptors in the nucleus accumbens shell is necessary to produce opioid-induced decreases in splenic NK cell activity and iNOS expression. The studies described in the current chapter will examine how these central effects of opioids translate into peripheral immune alterations by evaluating the role of the sympathetic peptide transmitter neuropeptide Y (NPY). Although peripheral mediators responsible for the effects of opioids on certain immune measures have been identified, the receptor mechanisms that underlie effects on NK cytolytic activity and iNOS production have not been established. The primary pathways whereby centrally acting opioids modulate peripheral immune responses involve the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. In general, the sympathetic nervous system mediates immune alterations induced by acute opioid exposure, whereas the HPA axis appears to be involved in the immunological effects that result from chronic opioid exposure.

Sympathetic nerve fibers directly innervate primary and secondary lymphoid organs and have even been shown to form synaptic-like contacts with splenic lymphocytes (Felten et al., 1985; Felten and Olschowka, 1987). Activation of the SNS results in the release of
catecholamines, which can modulate immune status directly via activation of cell surface immunocyte adrenoceptors. Several of the immunomodulatory effects induced by morphine are mediated by β-adrenoceptors specifically. For instance, β-adrenoceptor antagonists block the inhibitory effect of morphine on splenocyte proliferative responses to T and B cell mitogens (Fecho et al., 1993a). Catecholamines have also been reported to modulate NK cell activity via β2-adrenoceptors (Irwin et al., 1990; Takamoto et al., 1991), but morphine-induced inhibition of NK responses appears to involve different mechanisms. Studies which have employed peripherally acting adrenergic antagonists show that neither the β-receptor antagonist nadolol (Fecho et al., 1993a), nor the α-receptor antagonist doxazosin (Carr et al., 1994b) prevent morphine’s inhibitory effect on NK activity. Other investigations of SNS involvement in the effects of morphine have shown that the ganglionic blocker chlorisondamine does not prevent reductions in NK activity, but since chlorisondamine alone inhibits NK activity it is difficult to draw conclusions regarding the mechanisms of morphine’s effect (Fecho et al., 1996b). In sum, there is no conclusive evidence that either glucocorticoids or peripheral catecholamines are responsible for the suppressive effect of acute morphine exposure on NK activity.

Several lines of evidence indicate that NPY may be an important modulator of splenic NK cell activity. Immunocytochemical have revealed the presence of NPY in postganglionic sympathetic fibers that innervate the spleen (Romano et al., 1991). Activation of the sympathetic nervous system causes the release of NPY, which in turn induces a host of immunomodulatory effects. Notably, circulating NPY levels, but not catecholamines, were inversely correlated with NK activity in Alzheimer’s caregivers, suggesting that NPY may modulate NK activity under physiological conditions in vivo. Further support for this
hypothesis was provided by the demonstration that NPY produces a direct and dose-dependent inhibition of NK activity \textit{in vitro} (Nair et al., 1993). Although the mechanism of the effect of NPY on NK cells is not fully understood, the discovery of functional NPY Y\textsubscript{1} receptors on rat splenic lymphocytes offers the possibility that NPY may interact directly with its cognate receptors on NK cells to suppress cytolytic activity (Bedoui et al., 2002; Petitto et al., 1994).

An important finding revealed in chapter 2 is that opioid-induced immune alterations which are dependent on central dopamine D\textsubscript{1} receptor activation (i.e., reduced NK activity and iNOS expression), are distinct from the immune alterations mediated by peripheral β-adrenergic receptors (i.e., decreased lymphocyte proliferative responses). In the present chapter, we examine the hypothesis that NPY mediates the dopamine-dependent effects of morphine and heroin on immune status. The first study evaluates the role of NPY Y\textsubscript{1} receptors in morphine-induced alterations of splenic lymphocyte proliferation and NK cell activity. The second study assesses the effect of intravenous NPY administration to determine whether increased levels of NPY are sufficient to alter immune parameters in vivo. A third study was performed to determine whether direct stimulation of nucleus accumbens D\textsubscript{1} receptors regulates NK activity through a peripheral NPY Y\textsubscript{1} receptor mechanism. Finally, the current chapter examines the role of NPY Y\textsubscript{1} receptors in heroin-induced reductions of iNOS expression using the LPS model of Gram-negative sepsis.
Materials and Methods

Animals

Adult male Lewis rats weighing approximately 200-250 grams were purchased from Charles River Laboratories (Raleigh, NC). Upon arrival, animals were individually housed and maintained on a reverse 12-hr light/dark cycle. Animals were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Food and water were available *ad libitum* throughout the experiment.

Drugs

Morphine sulfate (NIDA, Bethesda, MD), R(+)-SKF-38393 hydrochloride (Sigma-Aldrich, St. Louis, MO), and Neuropeptide Y (AnaSpec, San Jose, CA) were dissolved in sterile 0.9% saline. BIBP3226 (Sigma) was dissolved in sterile water. For all experiments, morphine was administered s.c. at a dose of 15 mg/kg in a 1.0 ml/kg volume.

Surgical and Drug Administration Procedures

In the first experiment rats were assigned to one of six groups (*n* = 3-4 per group) in which they received the NPY Y₁ receptor antagonist BIBP3226 (0, 0.1, or 1.0 mg/kg, s.c.) 30 minutes prior to saline or morphine. Animals were sacrificed one hour following morphine or saline administration and spleens were collected for immunological assessments. This time point was selected based on previous data from our laboratory indicating a maximal suppression of the measured immune parameters 1 h following morphine administration. This experiment was replicated using the same design, and the data was combined for analysis (*n* = 7 – 8 per group).
In the second experiment rats were assigned to one of five groups (n = 4) in which they received NPY (0, 0.2, 2, 20, or 200 µg, i.v.) on the test day. Catheterization surgeries were performed under anesthesia induced with a 0.2 ml intramuscular injection of a 1:1 (vol/vol) mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Catheters made of flexible silastic tubing were inserted into the right jugular vein and secured with ligatures around the catheterized vessel. The tubing was tunneled subcutaneously and exteriorized on the dorsal side to allow injection of drugs. A stay suture placed beneath the skin in the scapular region maintained the position of the catheter, and the excess tubing was tucked into a subcutaneous skin pocket made cranial to the scapulae. The exterior portion of the catheter was held in place with two stainless steel suture clips. Patency was tested, and the catheter was filled with 0.2 ml of heparinized dextrose locking solution and sealed with a stainless steel plug. Animals were given a one-week recovery period prior to drug administration. On the test day animals received an i.v. injection of NPY or saline in a 200 µl injection volume, followed by a 300 µl injection of saline to flush through any drug remaining in the tubing. Animals were sacrificed one hour following NPY administration for assessment of immune parameters. This experiment was replicated using the same design, and the data was combined for analysis (n = 8 per group).

For the third experiment, stereotaxic surgeries were performed under anesthesia as described above. Animals receiving SKF-38393 administration into the nucleus accumbens shell were implanted with bilateral 26-gauge guide cannulae (Plastics One, Roanoke, VA) aimed at the following coordinates: AP +1.7, ML ±1.0, DV −5.4. Coordinates are expressed as millimeters from bregma (Paxinos and Watson, 1986). Animals were given a one-week recovery period prior to drug administration. On the test day, animals were assigned to one
of four groups \((n = 4)\) in which they received an injection of saline or BIBP3226 (1 mg/kg, s.c.) 30 minutes prior to a bilateral injection of SKF-38393 (0 or 0.5 \(\mu\)g/ side). One hour following SKF-38393 administration, animals were sacrificed and spleens were collected for assessment of immune status. This experiment was replicated twice using the same design, and the data was combined for analysis \((n = 8\) per group). SKF-38393 was administered in a 0.5 \(\mu\)l volume per side via a 33-gauge injector that protruded 2 mm beyond the tip of the guide cannula (final coordinates: AP +1.7, ML ±1.0, DV –7.4). Intracranial drug microinjections were performed over a 30 second period using a microsyringe pump (Harvard Apparatus, Holliston, MA) and the injector was left in place for one minute to allow diffusion of the drug away from the injection site. Following drug treatment and sacrifice, Alcian Blue dye was injected via the cannula and brains were removed and post-fixed in 4% paraformaldehyde for two days. Brains were transferred to a 30% sucrose solution for cryoprotection, frozen, and stored at -80°C for subsequent analysis. Accurate cannulae placements were verified by examination of 50 \(\mu\)m thionin-stained tissue sections under a light microscope. Only animals with placements within the targeted region were included in the analysis.

**Tissue Collection**

Following sacrifice, spleens were removed and prepared as a single-cell suspension as described in detail in Chapter 2. Splenic leukocytes were counted using a Hemavet 850 cell analyzer (CDC Technologies Inc., Oxford, CT), and cell suspensions were adjusted to \(5 \times 10^6\) leukocytes/ml.
Natural Killer Cell Assay

Splenic NK cell activity was assessed using a standard chromium release assay. A detailed description of the assay is provided in Chapter 2.

Splenocyte Proliferation Assay

Splenocyte proliferative responses were assessed using mitogen stimulation assays. A detailed description of the assay is provided in Chapter 2.

Real-Time RT-PCR

The expression of iNOS mRNA was quantified using real-time reverse transcriptase polymerase chain reaction (RT-PCR) as described in detail in Chapter 2.

Statistics

Data analysis for the experiments involving morphine and heroin administration were performed using a two-way analysis of variance (ANOVA) to assess the impact of BIBP3226 pretreatment on the effect of opioid injection. For the two-way analysis, the first factor was dosage of BIBP3226 and the second factor was the type of subsequent injection (morphine/heroin or saline). Experimental replication was entered into the model as a covariate to control for inter-assay variability in the measured parameters. Data from the i.v. NPY administration study was analyzed using a one-way ANOVA to assess the effect of NPY dosage, with experimental replication included as a covariate. For the third experiment, a two-way ANOVA was utilized. The first factor was dosage of BIBP3226 and the second factor was the dosage of SKF-38393. Again, experimental replication was entered into the
model as a covariate to control for inter-assay variability. For all analyses, general contrast comparisons were performed in accordance with a priori hypotheses. All analyses were performed with the level of significance set at $p < 0.05$.

**Results**

**NPY Y₁ Receptor Antagonism: Morphine-Induced Effects**

The first study examined whether antagonism of NPY Y₁ receptors with BIBP3226 prevented the effect of morphine on immune status. Figure 3.1 shows the effect of BIBP3226 on morphine-induced suppression of NK activity. Morphine produced a significant decrease in splenic NK cell activity as determined by planned comparisons between the vehicle/saline and vehicle/morphine treatment groups [$F(1, 33) = 18.06; p < .001$]. Analysis of variance revealed a significant interaction between morphine treatment and dose of BIBP3226 [$F(2, 33) = 6.75; p < .01$], indicating that the suppressive effect of morphine was reduced by BIBP3226 administration. Furthermore, planned contrasts revealed that Y₁ receptor antagonism altered the suppressive effect of morphine, as the groups that received BIBP3226 (0.1 or 1.0 mg/kg) prior to morphine were not significantly different from the corresponding saline groups.
Figure 3.1  Effect of NPY Y<sub>1</sub> receptor antagonism on morphine-induced suppression of splenic NK activity. Subcutaneous administration of the Y<sub>1</sub> antagonist BIBP3226 reversed the inhibitory effect of morphine on NK activity. Data are expressed as lytic units (mean ± S.E.). **p < .001 compared with the saline group that received the same dose of BIBP3226.

Figure 3.2 shows the effect of BIBP3226 on morphine-induced suppression of splenocyte mitogenic responses to Con-A and LPS. Morphine suppressed proliferation induced by Con-A (Figure 3.2, left panel) as revealed by a significant main effect of morphine treatment [F(1, 33) = 111.84; p < .0001]. LPS-induced mitogenesis (Figure 3.2, right panel) was also significantly reduced by morphine administration [F(1, 33) = 28.62; p < .0001]. There was not a significant main effect of BIBP3226 treatment on proliferative responses to Con-A [F(2, 33) = 0.03; p > .05] or LPS [F(2, 33) = 0.78; p > .05], nor was there a significant BIBP3226 by morphine dose interaction in either analysis. Furthermore, BIBP3226 administration did not block the effect of morphine at any dose tested, as planned comparisons indicated that each morphine treated group displayed significantly lower proliferation compared to the corresponding saline group that received an equivalent dose of antagonist (ps < .05).
Figure 3.2 Effect of NPY Y1 receptor antagonism on morphine-induced suppression of splenic lymphocyte proliferation. Subcutaneous BIBP3226 administration did not attenuate the inhibitory effect of morphine on splenocyte mitogenic responses to Con-A (5.0 µg/ml) or LPS (5.0 µg/ml) stimulation. Data are expressed as disintegrations per minute (mean ± S.E.). *p < 0.05; **p < .001 compared with the saline group that received the same dose of BIBP3226.

Exogenous NPY Administration

The second experiment was carried out to examine the effect of intravenous NPY administration on immune status. The results of the NK assay displayed in Figure 3.3 (left panel) show that NPY produced a dose-dependent reduction in splenic NK activity. Analysis of variance indicated a significant main effect of NPY dosage \([F(4, 33) = 16.23; p < .0001]\). Polynomial contrasts showed a highly significant linear component to the drug treatment \([F(1, 33) = 58.08; p < .0001]\), indicating that the suppressive effect of NPY on NK cytolytic activity increased in a dose-dependent manner.

Figure 3.3 displays the effect of i.v. NPY administration on splenocyte proliferative responses to Con-A (middle panel) and LPS (right panel). The analysis revealed that NPY
treatment did not have a significant effect on splenocyte mitogenesis induced by Con-A \[F(4,32) = 1.53; p > .05\]. Although there appeared to be a trend of increased Con-A induced proliferation in the groups receiving the two highest doses of NPY, planned comparisons revealed no significant differences between the saline and NPY-treated groups at any dose of NPY. There was a significant main effect of NPY dosage on LPS induced splenocyte proliferation \[F(4,32) = 2.69; p < .05\]. Polynomial contrasts revealed a significant linear component \[F(1,32) = 5.11; p < .05\] suggesting that NPY increased proliferation. However, planned comparisons between the saline group and each NPY-treatment group failed to reveal a significant between-group difference on LPS-induced proliferation at any dose of NPY.

**Figure 3.3** Effect of NPY administration on splenocyte immune measures. Intravenous administration of NPY reduced splenic NK cell activity in a dose-dependent manner (left panel). NPY administration did not suppress lymphocyte proliferative responses to Con-A (middle panel) or LPS (right panel) stimulation. Data from the cytotoxicity assay data are expressed as lytic units (mean ± S.E.). Data from proliferation assays are expressed as disintegrations per minute (mean ± S.E.). **\(p < .0001\) compared to saline treatment.
NPY Y\textsubscript{1} Receptor Antagonism: Dopamine Agonist-Induced Effects

Data presented in Chapter 2 showed that morphine’s effect on splenic NK activity is mediated through dopamine D\textsubscript{1} receptor activation in the nucleus accumbens shell. Moreover, injection of the D\textsubscript{1} agonist SKF-38393 into this region was shown to mimic the suppressive effect of morphine on NK activity. Based on those findings, we investigated whether agonist stimulation of accumbens D\textsubscript{1} receptors would produce an NPY Y\textsubscript{1} receptor-mediated inhibition of NK cell activity. Figure 3.4 displays the effect of BIBP3226 on D\textsubscript{1} agonist-induced reductions of NK activity. Planned comparisons demonstrated that SKF-38393 administration significantly reduced NK activity in animals that received subcutaneous saline injections \([F(1, 27) = 48.30; p < .0001]\) Analysis of variance revealed a significant interaction between SKF-38393 and BIBP3226 treatments \([F(1, 27) = 33.97; p < .0001]\), indicating that the suppressive effect of SKF-38393 was attenuated by BIBP3226 pretreatment. Furthermore, planned contrasts revealed that BIBP3226 completely blocked the suppressive effect of SKF-38393, as there was no significant difference between saline and SKF-38393 treatment groups among animals pretreated with BIBP3226 \([F(1, 27) = 1.66; p > .05]\). These findings show that systemic BIBP3226 administration prevents the suppression of NK activity induced by stimulation of D\textsubscript{1} receptors in the nucleus accumbens shell.
**Figure 3.4** Effect of NPY Y₁ receptor antagonism on NK activity in rats that received microinjections of the dopamine D₁ receptor agonist SKF-38393 into the nucleus accumbens shell. Rats received a subcutaneous injection of BIBP3226 or vehicle prior to microinjection of saline or SKF-38393. Administration of BIBP3226 prevented the suppression of NK activity induced by SKF-38393 microinjection. Data are expressed as lytic units (mean ± S.E.). **p < .0001 compared to the vehicle-saline control group.

**NPY Y₁ Receptor Antagonism: Heroin-Induced Effects**

A final study examined whether NPY Y₁ receptors play a role in the effects of heroin on iNOS production in vivo. LPS-induced expression of iNOS mRNA in spleen, liver, and lung tissues is shown in Figure 3.5. Planned comparisons demonstrated that heroin significantly reduced iNOS levels compared to saline in the spleen \( F(1, 37) = 5.66; p < .05 \) and liver \( F(1, 37) = 6.54; p < .05 \) among animals that received vehicle injections but did have a significant effect on lung iNOS expression \( F(1, 37) = 0.99; p > .05 \). Analysis of variance revealed a significant main effect of BIBP3226 dosage in the spleen \( F(2, 37) = 5.57; p < .01 \) and lung \( F(2, 37) = 4.10; p < .05 \). Although the overall interaction between BIBP3226 dosage and heroin treatment was significant in only the liver \( F(2, 37) = 3.29; p < .05 \), polynomial contrasts showed a significant linear interaction in the spleen \( F(1, 37) = \)}
4.50; \( p < .05 \), liver \( F(1, 37) = 6.57; \ p < .05 \), and lung \( F(1, 37) = 4.34; \ p < .05 \) indicating that BIBP3226 altered the effect of heroin in all three tissues. Importantly, planned comparisons between heroin and saline treated groups that received 0.1 or 1.0 mg/kg BIBP showed no significant in the spleen, liver, or lung \( (p s > .05) \). Overall, these results indicate that BIBP3226 abolished heroin’s suppressive effect on iNOS expression in the spleen and liver.

![Graph showing the effect of BIBP3226 on iNOS expression in the spleen, liver, and lung](image)

**Figure 3.5** Effect of NPY \( Y_1 \) receptor antagonism on heroin-induced alterations of iNOS mRNA expression. Rats received subcutaneous injections of BIBP3226 prior to saline or heroin in conjunction with LPS administration. BIBP3226 prevented the suppressive effect of heroin on iNOS expression in the spleen and liver. Data are expressed as the ratio of iNOS mRNA expression to expression of the housekeeping gene cyclophilin (mean ± S.E.). \* \( p < .05 \) compared to the vehicle-saline control group.

**Discussion**

The present study demonstrates that administration of the selective NPY \( Y_1 \) receptor antagonist BIBP3226 prevents the suppressive effects of acute opioid exposure on splenic NK cell activity and the production of iNOS. Opioid-induced effects on various immune parameters such as NK cytolytic responses do not occur via direct interactions between
opioids and immune cells but rather as an indirect consequence of μ-opioid receptor stimulation in the CNS (Fecho et al., 1996a; Shavit et al., 1986; Weber and Pert, 1989). Glucocorticoids and catecholamines have been identified as the major peripheral mediators that directly modulate immunocyte functions in response to signals from the nervous system. Glucocorticoids have been shown play an important role in the immunomodulatory effects induced by chronic morphine exposure, but they do not appear to mediate the effects of acute morphine administration. For example, the glucocorticoid receptor antagonist mifepristone blocks the decrease of NK activity in mice implanted with morphine pellets (Freier and Fuchs, 1994), but neither mifepristone nor adrenalectomy block the reduction in NK activity following acute morphine exposure (Fecho et al., 1996b; Liang-Suo et al., 2002). Moreover, mifepristone does not prevent the effect of acute morphine administration on the proliferative responses of blood, splenic, or thymic lymphocytes (Bayer et al., 1990b; Liang-Suo et al., 2002).

Catecholamines, which are released from sympathetic nerves, have been implicated in many of morphine’s acute immunomodulatory effects. For example, acute morphine administration suppresses the proliferative responses of splenic lymphocytes to concanavalin A (Con-A), lipopolysaccharide (LPS), phytohemagglutinin (PHA), and ionomycin/phorbol myristate acetate (PMA); and these effects are all prevented by pretreatment with β-adrenergic receptor antagonists (Fecho et al., 1993a). However, acute morphine-induced reductions in splenic NK cell activity are not blocked by either peripheral adrenergic receptor antagonists or adrenalectomy, suggesting than neither the HPA axis nor sympathetic catecholamines are involved (Carr et al., 1994b; Fecho et al., 1993a; Fecho et al., 1996b). Still, sympathetic mechanisms have been implicated based on indirect evidence showing that
morphine increases central sympathetic outflow (Appel et al., 1986) and that activation of the sympathetic nervous system by a diverse array of stimuli suppress splenic NK activity. Thus, it is likely that multiple sympathetic mechanisms are involved in the regulation of NK cell activity.

In addition to catecholamines, NPY is another important sympathetic transmitter that modulates a host of immunological parameters. The present study indicates that NPY mediates morphine-induced reductions of splenic NK activity via peripheral Y$_1$ receptors, as BIBP3226 reportedly does not penetrate the blood-brain barrier (Doods et al., 1996). Given that NPY suppresses NK activity in vitro (Nair et al., 1993), it is very possible that Y$_1$ receptors located on NK cells mediate the observed effects, although future studies are needed to resolve this issue. The current study also shows that intravenous NPY administration induces a suppression of NK activity, further supporting an in vivo role for NPY in modulating NK responses. The finding that both the 20 µg and 200 µg doses of NPY induced a comparable suppression indicates that the 20 µg dose is sufficient to produce the maximal inhibitory effect of NPY (an approximately 50% decrease) on NK activity. Interestingly, NPY not only inhibits splenic NK activity directly, but central NPY administration has also been shown to suppress NK responses (von Horsten et al., 1998). Although the intravenous route of NPY administration does not completely differentiate between central and peripheral sites of action, peripheral NPY apparently does alter NK cell responses in vivo, as evidenced by the ability of BIBP3226 to block morphine’s inhibitory effect on NK cell activity. The current findings support the hypothesis that the SNS mediates the acute immunomodulatory effects of opioids and further suggests that NPY does not simply potentiate the effect of catecholamines on NK responses since Y$_1$ receptor antagonism
completely prevented the suppression of NK activity. Both catecholamines and NPY administration have been shown to modulate NK cytotoxicity and these effects appear to occur through distinct receptor mechanisms. An important question for future studies to address is the conditions under which catecholamines or NPY modulate NK activity.

The results of the present study show that $Y_1$ receptor antagonism does not attenuate morphine’s effect on splenocyte proliferative responses to Con-A or LPS, nor did intravenous NPY administration suppress splenocyte proliferation. This is consistent with previous demonstrations indicating that β-adrenergic receptors mediate morphine’s effects on lymphocyte proliferative responses (Fecho et al., 1993a). Although our findings suggest that NPY is not involved in the effects of morphine on lymphocyte proliferation, NPY has been shown to alter a number of other T and B cell activities including T cell production of IL-2 (Medina et al., 2000), and specific antibody responses to keyhole limpet hemocyanin (Friedman et al., 1995). Thus, it is possible that more subtle and sensitive measures of T and B cell status will reveal additional modulatory effects of NPY on morphine-induced immune alterations. Intravenous NPY administration did produce a slight overall enhancement of LPS induced proliferation, but it should be noted that planned pairwise comparisons with the saline control group and each NPY dose group failed to reveal a significant effect of NPY at any individual dose. It is possible that intravenously administered NPY produces indirect effects on lymphocyte proliferative responses by altering the composition or density of lymphocytes in the spleen. For example, intravenous NPY administration has been shown to alter blood leukocyte numbers in a dose-dependent manner (Bedouï et al., 2001). Thus, potential effects of NPY on the distribution of splenic B-cells offers one potential explanation for the slight alterations of LPS induced proliferation. It is important to
emphasize however, that with respect to the effects of morphine, neither the total number of splenic leukocytes nor the relative numbers of lymphocyte subtypes are altered (Fecho and Lysle, 1999). Therefore, it is unlikely that the mobilization of NK cells in response to increased endogenous NPY levels accounts for morphine’s effects on NK activity since spleen NK cell numbers do not change following morphine administration anyway (Fecho and Lysle, 1999).

The discrepancy between the peripheral receptor mechanisms involved in morphine’s effect on NK activity and lymphocyte proliferative responses provides an intriguing example of the ability of the nervous system to selectively modulate discrete immunological parameters. Just as precise neural circuits are involved in a particular behavioral response, specific neural circuits also regulate discrete immunological responses. Data presented in the previous chapter showed that the nucleus accumbens shell is critically involved in mediating the effects of morphine on NK activity. Specifically, intra-accumbens injections of the dopamine D₁ receptor antagonist SCH-23390 was shown to block the effect of morphine and heroin on NK activity but not lymphocyte proliferation, whereas administration of the D₁ agonist SKF-38393 into the accumbens suppressed NK activity similarly to morphine but did not alter splenocyte mitogenesis. The neuroimmune efferent pathway through which nucleus accumbens dopamine receptor activation leads to altered splenic NK responses has not yet been characterized, but the close association with the nucleus accumbens to areas involved in autonomic regulation suggest that sympathetic efferent pathways may be involved. The results of the present study indicate that pretreatment with a peripherally acting Y₁ receptor antagonist prevents the suppression of NK activity induced by D₁ agonist administration into
the nucleus accumbens shell. These findings suggest that the activation of D₁ receptors in the accumbens inhibits NK activity by increasing the peripheral release of NPY.

The present study also shows that Y₁ receptor blockade prevents heroin-induced reductions of iNOS expression in the spleen and liver. Taken together with our previous data showing that heroin’s effect on iNOS expression requires the activation of D₁ receptors in the nucleus accumbens, the current findings suggest that the production of iNOS in response to LPS involves the same neural mechanisms responsible for opioid-induced alterations of NK cell activity. It should be noted that in the present investigation, although there was a trend toward suppression in the lung, heroin did not have a significant effect on iNOS in this tissue. The reason for a lack of effect in the lung tissue only is not immediately apparent. Typically, the spleen and liver show the most robust effect of iNOS expression, but prior investigations such as those presented in the previous chapter do show that heroin inhibits lung iNOS levels under similar experimental parameters. It is possible that the batch of LPS used in the present studied varied in potency, or that perhaps there were ancillary stress effects in the colony room on the day of the experiment. In any case, the findings that heroin-induced reductions of iNOS in the spleen and liver are abolished by BIBP3226 suggest that NPY plays an important role in the in vivo immunological effects of heroin. Furthermore, these data provide additional evidence for the hypothesis that NPY is a peripheral mediator of the dopamine-dependent immunomodulatory effects of opioids.

The SNS modulates the immune system by regulating sympathoadrenal activity or through direct innervation of lymphoid organs. Acute morphine administration has been shown to increase plasma catecholamine levels by activating central sympathetic outflow (Appel et al., 1986). Sympathoadrenal activity appears to mediate morphine’s suppressive
effect on lymphocyte mitogenic responses, because both adrenalectomy and β-adrenoceptor antagonists block this effect (Fecho et al., 1993a; Fecho et al., 1996b). Since adrenalectomy does not alter morphine’s effect on NK activity, it is unlikely that adrenally derived NPY mediates the NK cell suppression. As no studies have examined the peripheral mechanisms of opioid-induced effects on iNOS production, the adrenals cannot be ruled out as a potential source of NPY responsible for effects on spleen and liver iNOS levels. However, adrenalectomy in the rat has been shown to reduce the increase in plasma epinephrine elicited by stress without concomitant effects on increased NPY levels, indicating that the adrenals are not a major source of circulating NPY (Mormede et al., 1990). Thus, nucleus accumbens D₁ receptor activation may modulate splenic NK activity and iNOS expression by increasing the activity of NPY-releasing sympathetic nerves, but this effect does not seem to involve sympathoadrenal activity. Further studies are needed to determine whether opioids alter immune measures by increasing the local release of NPY in target tissues such as the spleen and liver specifically.

Collectively, data from the present study along with the results of prior investigations strongly suggest that the stimulation of CNS μ-opioid receptors following acute opioid administration produces immune alterations via the SNS. Evidence shows that opioid-induced reductions of splenic NK cell activity and LPS-induced iNOS expression are mediated peripherally by NPY Y₁ receptors, whereas morphine’s effects on splenic T and B cell proliferative responses to Con-A and LPS are mediated by peripheral β-adrenoceptors. The adrenal medulla appears to be the source of catecholamines that activate β-adrenoceptors to inhibit splenocyte proliferative responses, whereas the peripheral source of NPY that modulates immune parameters via Y₁ receptors is not the adrenal medulla but possibly
sympathetic nerves such as those that innervate the spleen and liver. The results of the current and prior studies further suggest that opioid administration induces the activation of dopamine D₁ receptors in the nucleus accumbens shell, leading to the suppression of NK cell activity and iNOS production as a result of increased NPY release from sympathetic nerves.
CHAPTER 4
NEUROIMMUNE MECHANISMS OF OPIOID-MEDIATED CONDITIONED IMMUNOMODULATION

Introduction

The results presented in chapters 2 and 3 characterized novel central and peripheral mechanisms that underlie the pharmacological effects of opioids on splenic NK cell activity and nitric oxide production. Those findings suggest that opioid-induced stimulation of dopamine D₁ receptors in the nucleus accumbens shell produces peripheral immune alterations by increasing NPY release from sympathetic nerves. The current chapter draws on the knowledge gained from the previous chapters to examine whether similar receptor mechanisms mediate the conditioned effects of opioids on the immune system. This chapter is based on our recent publication (Saurer et al., in press).

Pavlovian (classical) conditioning of immune responses is a truly fascinating illustration of the dialogue between the brain and the immune system in which the brain associates a novel cue with concomitant immunological changes and subsequently reproduces those precise immune changes when re-exposed to the cue at a later point in time. The basic paradigm involves paired presentations of an initially neutral conditioned stimulus (CS), such as a novel taste or context, with a stimulus that actively evokes an immunomodulatory response, termed the unconditioned stimulus (UCS). Upon repeated CS-UCS pairings, re-exposure to the CS alone (i.e., in the absence of the UCS) alters immune
functioning in a manner which mimics the normal or unconditioned effect of the UCS. Investigators have successfully employed a variety of unconditioned stimuli to demonstrate that a broad range of immune measures are susceptible to Pavlovian conditioning, including both innate and adaptive responses (for reviews see Ader and Cohen, 2001; Kusnecov et al., 1989).

While Pavlovian conditioning of immune responses has been established in a number of models, only a limited number of studies have investigated the biological mechanisms of this phenomenon. The precise mechanisms undoubtedly differ depending on the nature of the UCS as well as that of the CS, but the opioid system appears to play an important role in several paradigms. The presentation of a CS previously paired with aversive electric shock produces conditioned stress-induced reductions in NK cell activity and lymphocyte mitogenic responses that are mediated by endogenous opioids (Lysle et al., 1992a). These conditioned immune alterations were later shown to be mediated specifically by µ-opioid receptors in the CNS, providing direct evidence for the involvement of the central opioid system in conditioned immunomodulation (Perez and Lysle, 1997).

The pharmacological effects of exogenous opioids can also be conditioned to environmental stimuli. For example, when rats are re-exposed to a distinctive environment associated with morphine administration, immunological alterations occur that are similar to those produced by the drug alone, including decreased mitogen responsiveness of blood and splenic lymphocytes, reduced interleukin-2 production, and decreased NK cell activity in the spleen (Coussons et al., 1992). Previous studies have established that these conditioned effects are due to the learned psychological state induced by the CS, as development of the conditioned immunomodulatory response requires the explicit pairing of morphine
administration with the CS and is not attributable to ancillary effects of the conditioning procedure (Coussons et al., 1992). Importantly, it has been recently demonstrated that the conditioned effects of heroin on nitric oxide expression are susceptible to extinction and latent inhibition, indicating that opioid-conditioned immune alterations conform to major principles of associative learning (Szczytkowski and Lysle, 2007). Given that conditioned immune alterations modify disease progression in numerous models of clinical disease (e.g., Ader and Cohen, 1982; Exton et al., 1998; Klosterhalfen and Klosterhalfen, 1983; Lysle et al., 1992b), an important implication is that—in addition to the pharmacological effects of opioids—any detrimental health consequences of opioid use may also be conditioned to environmental stimuli associated with the drug.

There is accumulating evidence that the conditioned effects of morphine on immune status are mediated by neural processes similar to those which underlie the drug’s pharmacological effects. For example, both conditioned and unconditioned immunomodulatory effects of morphine are initiated by the activation of central opioid receptors and involve increased activity of the sympathetic nervous system (Coussons-Read et al., 1994a; Coussons-Read et al., 1994b; Fecho et al., 1996b). The sympathetic nervous system provides a “hardwired” neuroimmune communication pathway as sympathetic fibers directly innervate lymphoid organs and form synaptic-like contacts with splenic lymphocytes (Felten et al., 1985; Felten and Olschowka, 1987). Thus, activation of sympathetic nerves directly modulates immune status via the release of catecholamines and neuropeptide Y (NPY), which interact with immunocyte cell surface receptors. Sympathetic efferent nerves appear to be the major neuroimmune pathway responsible for alterations in peripheral immune parameters induced by morphine and morphine-conditioned stimuli. For instance,
administration of the peripherally acting β-adrenoceptor antagonist nadolol abolished both the conditioned and unconditioned effects of morphine on splenocyte proliferative responses to T and B cell mitogens (Coussons-Read et al., 1994b; Fecho et al., 1993a). However, these studies reported that neither the conditioned nor unconditioned effects of morphine on splenic NK cell activity are attenuated by antagonism of β-adrenoceptors.

The findings presented in the previous chapters demonstrate that the pharmacological effect of morphine on NK cell activity is mediated by mechanisms distinct from those which govern decreases in lymphocyte proliferation. Specifically, it was shown that the activation of dopamine D₁ receptors in the nucleus accumbens shell is necessary for morphine-induced suppression of splenic NK activity and that peripheral NPY Y₁ receptors mediate these dopamine-dependent effects (Saurer et al., 2006b). However, whether similar mechanisms play a role in conditioned immunomodulation is unknown. The goal of the present study was to determine whether exposure to a CS previously associated with morphine would elicit conditioned immunomodulatory effects by acting via similar dopamine and NPY receptor-dependent mechanisms. To address this issue, we evaluated the effects of the dopamine D₁ antagonist SCH-23390 and the NPY Y₁ antagonist BIBP3226 on the expression of conditioned immune alterations which were induced by environmental stimuli previously paired with the administration of morphine.

**Materials and Methods**

**Animals**

Adult male Lewis rats weighing approximately 200-250 grams were purchased from Charles River Laboratories (Raleigh, NC). Upon arrival, animals were individually housed
and maintained on a reverse 12-hr light/dark cycle. Animals were habituated to handling and the colony room environment for two weeks prior to any experimental manipulation. Food and water were available *ad libitum* throughout the experiment.

**Drugs**

Morphine sulfate (NIDA, Bethesda, MD) and R(+)-SCH-23390 hydrochloride (Sigma-Aldrich, St. Louis, MO) were dissolved in sterile 0.9% saline. For all experiments, morphine was administered subcutaneously at a dose of 15 mg/kg in a 1.0 ml/kg volume. This dose was selected based on previous studies from our laboratory showing that the effects of morphine are dose-dependent and that the effects were blocked by naltrexone, indicating the involvement of opioid receptors (Lysle et al., 1993). BIBP3226 (Sigma) was dissolved in sterile water.

**Conditioning Procedures**

The conditioning apparatus consisted of standard rodent conditioning chambers individually contained within sound-attenuating cubicles. The conditioning chambers provided distinctive visual (stainless steel and Plexiglas walls), tactile (wire grid floors), auditory (white noise), and olfactory (cedar chips) cues to distinguish this environment from the home cage.

The first experiment examined the effect of systemic administration of the dopamine D₁ antagonist SCH-23390 on the expression of conditioned morphine-induced immune alterations. All animals received two conditioning sessions separated by 48 hours, during which an injection of morphine was paired with a distinctive environment, the conditioning
chamber. Thus, the injection of morphine served as the unconditioned stimulus (US), and the distinctive environment served as the conditioned stimulus (CS). During each session, rats were administered morphine immediately prior to being placed into the conditioning chambers for 1 hour. Animals were returned to their home cages following each conditioning session. This training phase was separated from the test day by a 12-day recovery period during which the animals received only handling. On the test day, rats were assigned to one of three groups \((n = 8)\) in which they were administered saline or SCH-23390 (0.05, or 0.5 mg/kg, s.c.). Thirty minutes following SCH-23390 administration, half of the animals in each group were re-exposed to the CS and the other half remained in the home cage. Thus, there were six treatment groups \((n = 4)\) in this experiment. A previous study using extensive control manipulations showed that the immune alterations observed following exposure to the conditioning chamber are the result of conditioning processes (Coussons et al., 1992). Immediately following the test session (one hour after the onset of CS re-exposure) animals were sacrificed by cervical dislocation and spleens were collected for immunological assessment. Animals that remained in the home cages on the test day were sacrificed concurrently with those that were exposed to the CS.

The second experiment examined the effect of SCH-23390 administration into the nucleus accumbens shell prior to CS re-exposure on the conditioned immunomodulatory effects of morphine. The conditioning procedures were identical to those described in the first experiment. On the test day, rats received a microinjection of saline or SCH-23390 (0.15 µg/side) into the nucleus accumbens shell. Thirty minutes following microinjection, animals were either re-exposed to the conditioning chamber or remained in the home cage.
One hour later, animals were sacrificed to assess immune status. This experiment was replicated, yielding an experimental design consisting of four groups \((n = 10\) per group).

A third experiment was performed to control for the possibility that the effect of SCH-23390 was due to diffusion of the drug into other dopamine terminal regions near the injection site. In this experiment, the effect of bilateral SCH-23390 administration into the nucleus accumbens core was assessed using the same procedures as described above. Animals were assigned to one of four groups \((n = 5)\) in which they received saline or SCH-23390 \((0.15 \mu g/\text{side})\) into the nucleus accumbens core. Thirty minutes following microinjection, animals were either re-exposed to the conditioning chamber or remained in the home cage. One hour later, animals were sacrificed to assess immune status.

In the fourth experiment, the effect of subcutaneous injections of the NPY \(Y_1\) antagonist BIBP3226 was examined. Rats received two conditioning sessions and subsequent re-exposure to the CS exactly as described in the procedures of the first experiment, but in this study animals received a subcutaneous injection of vehicle or BIBP3226 \((0.1\) or \(1.0\) mg/kg) on the test day. Thirty minutes following injection, half of the animals in each dose group were re-exposed to the CS and the other half remained in the home cages. Animals were sacrificed one hour later. This experiment was replicated, giving an experimental design comprised of six treatment groups \((n = 4 – 6\) per group).

**Surgical and Microinjection Procedures**

For the intra-accumbens microinjections, stereotaxic surgeries were performed under anesthesia induced with a 0.2 ml intramuscular injection of a 1:1 (vol/vol) mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Animals were implanted with bilateral 26-
gauge guide cannulae (Plastics One, Roanoke, VA) directed toward the nucleus accumbens shell (AP +1.7, ML ±0.8, DV –5.4) or the nucleus accumbens core (AP +1.7, ML ±1.5, DV –4.8). Coordinates are expressed as millimeters from bregma (Paxinos and Watson, 1986). Animals were given a one-week recovery period prior to the start of the conditioning trials.

On the testing day, animals received a bilateral injection of saline or SCH-23390 (0.15 µg/side). SCH-23390 was administered in a 0.5 µl volume per side via a 33-gauge injector that protruded 2 mm beyond the tip of the guide cannula (final coordinates: AP +1.7, ML ±0.8, DV –7.4 for the shell; or AP +1.7, ML ±1.5, DV –6.8 for the core). Intracranial drug microinjections were performed over a 30s period using a microsyringe pump (Harvard Apparatus, Holliston, MA) and the injector was left in place for one minute to allow diffusion of the drug away from the injection site. Immediately following the injection, animals were either returned to the home cage or re-exposed to the CS, depending on the treatment group. Alcian blue dye was injected via the cannula following sacrifice, and brains were removed and post-fixed in 4% paraformaldehyde. Brains were transferred to a 30% sucrose solution for cryoprotection, frozen, and stored at -80°C for subsequent analysis. Accurate cannulae placements were verified by examination of unstained 50 µm tissue sections under a dissecting microscope. Only animals with placements within the targeted region were included in the analysis.

Tissue Collection

Following sacrifice, spleens were removed and prepared as a single-cell suspension as described in Chapter 2. Splenic leukocytes were counted using a Hemavet 850 cell analyzer
(CDC Technologies Inc., Oxford, CT), and cell suspensions were adjusted to $5 \times 10^6$ leukocytes/ml.

**Natural Killer Cell Assay**

Splenic NK cell activity was assessed using a standard chromium release assay. A detailed description of the assay is provided in Chapter 2.

**Splenocyte Proliferation Assay**

Splenocyte proliferative responses were assessed using mitogen stimulation assays. A detailed description of the assay is provided in Chapter 2.

**Statistics**

Data analysis for each experiment was performed using two-way analysis of variance (ANOVA) to assess the impact of drug antagonist treatment on conditioned immune alterations. For the two-way analysis, the first factor was drug dosage and the second factor was the type of treatment on the test day, (i.e., re-exposure to the CS or home cage). Experimental replication was entered into the model as a covariate to control for inter-assay variability in the measured parameters for both the second and fourth experiments. For all data sets, planned contrasts were performed in accordance with *a priori* hypotheses that SCH-23390 and BIBP3226 would antagonize the effect of the CS. Planned contrasts in each analysis consisted of pairwise comparisons of means between CS re-exposed groups and home cage groups at each level of the factor “drug dosage.” All analyses were performed with the alpha-level of significance set at $p < 0.05$
Results

Systemic D₁ Receptor Antagonism

The first study examined the effect of systemic SCH-23390 administration on alterations of immune status induced by exposure to a distinctive environment (the CS) that had been previously paired with morphine administration. The results of the NK cell assay are shown in Figure 4.1. One animal was excluded from this analysis due to technical difficulties. Analysis of variance revealed a significant main effect of CS re-exposure \([F(1, 17) = 26.89; p < 0.001]\), indicating that exposure to the CS suppressed NK activity. More importantly, the analysis revealed a significant interaction between SCH-23390 dosage and CS re-exposure \([F(2, 17) = 12.54; p < 0.001]\), indicating that the reduction in NK activity elicited by CS re-exposure was attenuated by SCH-23390 treatment. Planned contrasts

![Figure 4.1](image-url) Effect of systemic dopamine D₁ receptor antagonism on conditioned suppression of splenic NK cell activity. Subcutaneous administration of SCH-23390 at a dose of 0.5 mg/kg reversed the inhibition of splenic NK activity induced by CS re-exposure. Data are expressed as lytic units (mean ± S.E.). Solid bars indicate rats that remained in their home cages on the test day and open bars represent groups re-exposed to the CS on the test day. *p < 0.01; **p < 0.001 compared with the home cage group that received the same dose of SCH-23390.
indicated that the CS re-exposed group differed from the corresponding home cage control group in animals that received saline ($p < 0.01$) or 0.05 mg/kg of SCH-23390 ($p < 0.0001$). However, at the high dose of SCH-23390 (0.5 mg/kg), planned comparisons showed that there was no difference between the groups exposed to the CS or the home cage on the test day, indicating that this dose of SCH-23390 fully blocked the conditioned suppression of NK activity.

Figure 4.2 shows the results of the mitogen stimulation assays from the first experiment. Re-exposure to the CS produced a significant decrease in splenocyte proliferation induced by Con-A (left panel) as revealed by a significant main effect of CS re-exposure [$F(1, 18) = 54.23; p < 0.0001$]. Splenocyte proliferative responses to LPS (Figure 4.2, right panel) were also significantly reduced in animals re-exposed to the CS as indicated by a significant main effect of CS re-exposure [$F(1, 18) = 45.14; p < 0.0001$]. There was no main effect of SCH-23390 dosage on the proliferative responses to Con-A [$F(1, 18) = 2.71; p > 0.05$] or LPS [$F(1, 18) = 0.73; p > 0.05$], nor was there a significant interaction between SCH-23390 dosage and CS re-exposure in either analysis ($F's \leq 1.01$). Furthermore, SCH-23390 administration did not antagonize the effect of CS re-exposure on responses to Con-A or LPS at any dose tested, as planned comparisons indicated that all groups re-exposed to the conditioning chamber on the test day displayed significantly lower proliferation compared to the corresponding home cage group ($ps < 0.01$).
**Figure 4.2** Effect of systemic D₁ receptor antagonism on conditioned suppression of lymphocyte proliferation. Subcutaneous SCH-23390 administration did not attenuate the inhibitory effect of CS re-exposure on splenocyte mitogenic responses to Con-A (5.0 µg/ml) or LPS (5.0 µg/ml). Data are expressed as disintegrations per minute (mean ± S.E.). Solid bars indicate rats that remained in their home cages on the test day and open bars represent groups re-exposed to the CS on the test day. *p < 0.01; **p < 0.001 compared with the home cage group that received the same dose of SCH-23390.

**Nucleus Accumbens D₁ Receptor Antagonism**

The second experiment was conducted to investigate the effect of bilateral SCH-23390 microinjections into the nucleus accumbens shell on the conditioned suppression of NK cell activity. The results of the NK cell activity assay displayed in Figure 4.3 show that re-exposure to the CS elicited a significant reduction in NK activity compared to animals that remained in the home cage on the test day, as evidenced by a significant main effect of CS re-exposure \( F(1, 32) = 18.34; p < 0.001 \). Analysis of variance further revealed a significant interaction between SCH-23390 administration and CS re-exposure \( F(1, 32) = 4.92; p < 0.05 \), indicating that the conditioned suppression of NK activity was attenuated by intra-accumbens SCH-23390 injections. Planned contrasts indicated that the CS re-exposed group
differed from the corresponding home cage control group in animals that received saline ($p < 0.001$). However, in rats that received SCH-23390 microinjections, planned comparisons showed that there was no difference between the groups exposed to the CS or the home cage on the test day ($p > 0.05$). These findings show that bilateral administration of SCH-23390 at a dose of 0.15 $\mu$g/0.5 $\mu$l is sufficient to fully antagonize the conditioned suppression of splenic NK activity.

**Figure 4.3** Effect of D$_1$ receptor antagonism in the nucleus accumbens shell (A) or core (B) on conditioned suppression of NK cell activity. A, Bilateral injections of SCH-23390 into the nucleus accumbens shell blocked the inhibitory effect of CS re-exposure on NK activity. B, Administration of SCH-23390 into the nucleus accumbens core did not prevent the effect of CS re-exposure. Solid bars indicate rats that remained in their home cages on the test day and open bars represent groups re-exposed to the CS on the test day. Data are expressed as lytic units (mean ± S.E.). *$p < 0.01$; **$p < 0.001$ compared with the home cage group that received the same dose of SCH-23390.
NPY Y₁ Receptor Antagonism

Figure 4.4 displays the effect of subcutaneous BIBP3226 administration on the conditioned suppression of NK activity. There was a significant main effect of CS re-exposure \(F(1, 22) = 19.04; p < 0.001\), indicating that re-exposure to the CS reduced NK cytolytic activity. The suppressive effect of CS re-exposure was attenuated by BIBP3226 treatment, as determined by a significant interaction between CS re-exposure and BIBP3226 dosage \(F(2, 22) = 4.01; p < 0.05\). Planned comparisons indicated that the CS re-exposed group differed from the corresponding home cage group in animals that received saline \(p < 0.001\) or 0.1 mg/kg of BIBP3226 \(p < 0.05\). However, in animals that received the high dose of BIBP3226 (1.0 mg/kg), planned contrasts showed that there was no difference between the home cage and CS re-exposed groups \(p > 0.05\). These findings indicate that the 1.0 mg/kg dose of BIBP3226 fully prevents the reduction in NK activity induced by re-exposure to the CS.

**Figure 4.4** Effect of NPY Y₁ receptor antagonism on conditioned reductions of NK cell activity. Subcutaneous administration of BIBP3226 at a dose of 1.0 mg/kg blocked the inhibition of splenic NK activity induced by CS re-exposure. Solid bars indicate rats that remained in their home cages on the test day and open bars represent groups re-exposed to the CS on the test day. \*\(p < 0.05\); \**\(p < 0.001\) compared with the home cage group that received the same dose of BIBP3226.
**Discussion**

Exposure to environmental stimuli previously paired with morphine induces conditioned immune alterations that mimic morphine’s pharmacological effects. The present study shows that the conditioned suppression of NK activity is blocked by systemic administration of the D₁ antagonist SCH-23390 prior to CS re-exposure, suggesting that morphine conditioned stimuli modulate NK activity through increased D₁ receptor-mediated signaling. In contrast, SCH-23390 did not attenuate the conditioned suppression of splenocyte proliferative responses to Con-A or LPS. This finding is notable because it shows that the learned association between morphine and the CS is not disrupted by D₁ antagonist treatment and furthermore indicates that neuroimmune efferent communication is not impaired in a nonspecific manner. Moreover, the selective role of D₁ receptors in mediating conditioned effects on NK activity mirrors our previous findings with morphine administration in which SCH-23390 was shown to block the effect of morphine on NK activity (Saurer et al., 2006a) but not lymphocyte proliferation (unpublished observations).

The current findings further demonstrate that microinjection of SCH-23390 into the nucleus accumbens shell prior to CS re-exposure completely blocks the suppression of NK activity. Thus, these data indicate that the activation of D₁ receptors in the nucleus accumbens is necessary for the expression of conditioned reductions in splenic NK cell activity. Because increased dopamine transmission in the nucleus accumbens shell has been widely implicated in the acquisition of associative (Pavlovian) learning (Di Chiara et al., 2004; Pezze and Feldon, 2004), an important issue for future investigations to address is whether the nucleus accumbens also plays a role in the development of associative learning processes which establish conditioned effects on immunity. One of the major advantages of
the Pavlovian conditioning model is that it permits the separation of afferent and associative processes from efferent control mechanisms. For example, although β-adrenoceptor antagonists block the expression (recall) of conditioned effects on mitogen-induced lymphocyte proliferation, they do not affect the conditioned response if administered during acquisition, indicating that β-adrenoceptor activity is not required for the development of the conditioned response (Coussons-Read et al., 1994b; Lysle et al., 1991). Since blocking the unconditioned response does not impair the UCS-CS associative processes that establish the conditioned response, the interpretation is that increased catecholamine release (and the subsequent activation of β-adrenoceptors) represents the efferent phase of the unconditioned response. Whether the blockade of D₁ receptors in the nucleus accumbens shell during acquisition disrupts conditioned effects on the immune system is presently unknown. If for example, such manipulations did not influence conditioned responses, these results would indicate that D₁ activation is intimately involved in efferent neuroimmune signaling, but not in associative learning per se. Based on the established role of the nucleus accumbens in learning processes, an important avenue for future investigations will be to evaluate the role of D₁ receptor during the acquisition of UCS-CS associations responsible for conditioned immune alterations.

Although further studies are necessary to elucidate the role of the nucleus accumbens in the conditioning of immunity, the involvement of the accumbens may simply represent a more fundamental efferent neuroimmunoregulatory mechanism, as morphine’s pharmacological effects on NK activity are also abrogated by blocking the activation of D₁ receptors in the nucleus accumbens shell. The precise neuroimmune pathway through which accumbens D₁ receptors activation causes an inhibition of splenic NK cell activity is not fully
understood, but converging evidence suggests that it may involve the modulation of efferent sympathetic nerve outflow. For instance, neural control of splenic NK cell activity is a well documented phenomenon which occurs primarily via the sympathetic nervous system (Irwin et al., 1990; Katafuchi et al., 1993a), and the nucleus accumbens shell has known efferent projections to hypothalamic autonomic regulatory centers (Heimer et al., 1991; Usuda et al., 1998). Moreover, several hypothalamic nuclei have been shown to modulate splenic NK activity including those which receive direct afferent projections from the nucleus accumbens shell (Katafuchi et al., 1993a; Wenner et al., 1996; Wrona and Trojniar, 2003; Wrona and Trojniar, 2005). Thus, one possibility is that dopamine transmission in the nucleus accumbens modulates sympathetic outflow, thereby suppressing NK activity.

There is accumulating evidence that the conditioned effects of morphine on immune status are mediated by neural processes similar to those which underlie the drug’s pharmacological effects. This is not entirely surprising, considering that both conditioned and unconditioned effects are mediated through opioid receptors (Coussons-Read et al., 1994a; Fecho et al., 1996a). The imperative distinction however, is that conditioned immune alterations are produced by endogenous opioids, as no drug is actually administered on the test day. Thus, conditioned effects on immunity reflect the inherent capacity of the brain to modify immune functions. Given the ability of endogenous opioids to modulate immune status, it is no coincidence that stimuli which induce the release of opioids, such as aversive or stressful stimuli, produce similar opioid-dependent immunological alterations which can also be conditioned to environmental stimuli (Lysle et al., 1992a; Perez and Lysle, 1997; Shavit et al., 1984). The essential mechanistic feature shared by unconditioned and
conditioned effects of both aversive stimuli and opioid drugs is the requisite activation of opioid receptors—specifically µ-opioid receptors.

The present findings provide additional support for the hypothesis that morphine’s conditioned and unconditioned effects are mediated by similar mechanisms. The inhibitory effects of morphine on splenic T and B cell proliferative responses are mediated through β-adrenoceptors specifically, whereas NPY Y₁ receptors mediate morphine’s effect on NK cell activity (Fecho et al., 1993a; Saurer et al., 2006b). In the present study, we show that the Y₁ antagonist BIBP3226 blocks the conditioned suppression of NK cell activity, indicating that Y₁ receptors also mediate CS-induced effects on NK activity. Because BIBP3226 reportedly does not penetrate the blood-brain barrier when administered systemically, these findings implicate the involvement of peripheral Y₁ receptors specifically (Doods et al., 1996). Given the results of prior studies which indicate that NPY Y₁ receptors mediate the dopamine-dependent effects of morphine on immune status, the current findings suggest that conditioned increases in nucleus accumbens D₁ receptor activation lead to the suppression of splenic NK activity by facilitating the release of NPY from sympathetic nerves. NPY may in turn, directly interact with NK cells to inhibit cytolysis, as splenic lymphocytes express functional Y₁ receptors (Bedoui et al., 2002; Petitto et al., 1994) and NPY produces direct and dose-dependent suppressive effects on NK activity in vitro (Nair et al., 1993).

The involvement of NPY provides further evidence for the role of the sympathetic nervous system in conditioned immune alterations. Although early theories suggested that conditioned immunomodulation was simply a nonspecific stress reaction involving activation of the hypothalamic-pituitary-adrenal axis and the release of adrenocortical steroids, this explanation does not adequately account for many, if not most, conditioned effects on the
immune system (e.g., Ader et al., 1979; Roudebush and Bryant, 1991). For example, the conditioned immunosuppressive effects of cyclosporine A on T-cell proliferation and cytokine production have been shown to be mediated solely via the sympathetic innervation of the spleen (Exton et al., 1998). Additionally, prior studies from our laboratory have shown that peripheral β-adrenoceptor activity mediates the conditioned effects of both aversive stimuli and morphine on the immune system (Coussons-Read et al., 1994b; Luecken and Lysle, 1992). Thus, the sympathetic nervous system may represent the principal neuroimmune pathway through which a variety of conditioned stimuli induce immune alterations, as sympathetic efferent mechanisms have been established in several paradigms.

In conclusion, the present study demonstrates that the expression of morphine’s conditioned effects on splenic NK cell activity requires the activation of dopamine D₁ receptors in the nucleus accumbens. Furthermore, the current results show that antagonism of NPY Y₁ receptors with BIBP3226 also prevents the conditioned suppression of NK activity. Taken together with the results of previous investigations, the present findings provide additional support for the hypothesis that the conditioned and unconditioned defects of morphine involve the same neural pathways by demonstrating the involvement of similar dopamine and NPY receptor mechanisms. In addition, the present data suggest that conditioned increases in the activation of nucleus accumbens D₁ receptors may induce reductions in splenic NK activity by stimulating the release of NPY. These findings add to our current knowledge regarding the neural mechanisms responsible for conditioned modulation of immunity by providing the first demonstration for the role of the nucleus accumbens in Pavlovian conditioned immunomodulation.
CHAPTER 5
GENERAL DISCUSSION

Experimental Findings

Collectively, the studies comprising the current dissertation provide important new data regarding the neurobiological mechanisms of opioid-induced immune alterations. The investigation of interactions between opioids and neurotransmitter systems has led to significant advancements in our present understanding of how opioids produce analgesia and why opioid drugs are highly addictive. However, a similar research approach has not yet been widely exploited to ascertain how opioid drugs modulate immune function. The present dissertation has drawn upon existing data concerning interactions between opioids and central dopamine systems to evaluate the role of the nucleus accumbens dopamine in opioid-induced immunomodulation.

The findings of Chapter 2 reveal that the nucleus accumbens shell is an essential neural substrate underlying the effects of opioids on immunity. Those studies showed that the stimulation of dopamine D₁ receptors in the nucleus accumbens shell is necessary for opioid-induced suppression of splenic NK cell activity. Moreover, in the absence of opioid administration, the stimulation of D₁ receptors alone produced comparable reductions in NK activity, supporting a fundamental role of the nucleus accumbens in neuroimmune regulation. Additional experiments further demonstrated that heroin’s widespread inhibitory effects on
LPS-induced iNOS expression in peripheral tissues also require the activation of D₁ receptors in the nucleus accumbens.

Chapter 3 explored potential efferent pathways involved in opioid-induced immune alterations by investigating the role of NPY as a peripheral determinant of the central effects of opioids. The results of these studies showed that peripheral NPY Y₁ receptors mediate opioid-induced immunosuppressive effects on splenic NK activity, as well as the production of iNOS in response to LPS challenge. Because NPY Y₁ receptor antagonism selectively blocked opioid effects on immune parameters previously shown to be dependent on nucleus accumbens dopamine, these findings suggest that NPY mediates the dopamine-dependent immunomodulatory effects. Moreover, opioid-induced reductions in the mitogenic responses of T-cells and B-cells—immune responses that are modulated independently of accumbens D₁ receptor stimulation, as shown in Chapter 2—were not influenced by NPY Y₁ receptor antagonist manipulations. Taken together, the studies of Chapter 3 provide convincing evidence that the sympathetic peptide transmitter NPY is an important peripheral mediator of the dopamine-dependent immunomodulatory effects of opioids.

Whereas Chapters 2 and 3 revealed novel mechanisms whereby opioids produce pharmacological effects on the immune system, Chapter 4 demonstrated that similar neural pathways underlie the conditioned effects of opioids. These studies showed that re-exposure to environmental stimuli previously paired with morphine produced conditioned reductions in NK cell activity that were mediated through D₁ receptors in the nucleus accumbens shell. Furthermore, the conditioned effects on NK activity were likewise prevented by blocking NPY Y₁ receptors prior to CS re-exposure. Remarkably, these findings suggest that stimuli
associated with the opioids are capable of inducing immune alterations by engaging the same neural mechanisms responsible for the pharmacological effects of opioid drugs.

**Potential Mechanisms**

**Opioid/Dopamine Interactions**

Collectively, the present studies highlight the role of the nucleus accumbens shell as a neural substrate of opioid-induced immune alterations. These studies show that stimulation of dopamine D₁ receptors in particular, is necessary for the expression of opioid effects on ex vivo and in vivo immune responses. Although the brain mechanisms that regulate immunological functions are only beginning to be understood, these findings provide important clues regarding the neural circuitry involved in immunomodulation.

The periaqueductal gray matter (PAG) has been identified as a site of morphine’s µ-opioid receptor interactions responsible for the suppression of NK cell activity (Lysle et al., 1996; Weber and Pert, 1989). In light of the present findings, the relationship between the PAG and the nucleus accumbens is of significant interest. It is currently believed that morphine primarily increases extracellular dopamine in the nucleus accumbens by suppressing the tonic inhibitory influence of GABA interneurons on dopamine neurons that emanate from the ventral tegmental area (VTA) (Johnson and North, 1992; Klitenick et al., 1992). However, given that morphine has been shown to suppress NK activity via µ-opioid receptors in the PAG, the role of VTA µ-opioid receptors is unclear. Similar to its actions in the VTA, morphine is also believed to increase the activity of PAG efferents by suppressing the tonic influence of inhibitory GABA interneurons (Renno et al., 1992; Stiller et al., 1996). One possibility is that a direct dopaminergic projection from the PAG to the accumbens is
involved in morphine’s suppressive effect on NK activity, as there is evidence that dopamine neurons project from the PAG to the nucleus accumbens shell (Hasue and Shammah-Lagnado, 2002). The significance of this pathway is uncertain however, since the majority of the dopaminergic input to the nucleus accumbens appears to originate from the VTA (Hasue and Shammah-Lagnado, 2002).

An alternative hypothesis is that opioid receptors in multiple sites are capable of modulating immune status. It is possible that the stimulation of μ-receptors in the VTA or the nucleus accumbens inhibit immune parameters in addition to PAG stimulation as part of a complex circuitry involved in neuroimmune regulation. Studies performed in rabbits have shown that microinjection of morphine into the nucleus accumbens induces the release of endogenous opioids in the PAG, whereas morphine injection into the PAG induces opioid peptide release in the nucleus accumbens (Ma et al., 1992; Ma and Han, 1992). Furthermore, naloxone administration into either the PAG or the nucleus accumbens prevents the analgesic effect of morphine microinjection into the other structure (Ma and Han, 1991). Thus, it appears that opioid activities in the nucleus accumbens and PAG are connected through an interrelated positive feedback system. Interestingly, both reinforcing and analgesic effects of morphine can be elicited by injecting morphine into the PAG, the VTA, or the nucleus accumbens (Corrigall and Vaccarino, 1988; Devine and Wise, 1994; Manning et al., 1994; Olmstead and Franklin, 1997). It is likely that opioid-induced immune alterations similarly involve interactions between multiple brain nuclei as part of a complex circuitry involved in neuroimmune modulation. An exciting issue for future studies will be to determine whether the stimulation of μ-opioid receptors in the nucleus accumbens and VTA also produce reductions in NK activity and iNOS production.
**Nucleus Accumbens Efferent Pathways**

Another important issue raised by the current studies concerns the output pathway through which dopamine receptor activity in the nucleus accumbens translates into peripheral immune alterations. Because the current findings suggest that dopamine-dependent immune alterations are mediated peripherally via NPY Y₁ receptors, this pathway appears to involve the sympathetic nervous system. However, precisely how the nucleus accumbens modulates sympathetic outflow in the context of immunomodulation has not been clearly delineated. While little is known about the central mechanisms that modulate iNOS expression in peripheral tissues, neural control of splenic NK cell activity is a well documented phenomenon which is mediated primarily by the sympathetic nervous system via tonic inhibition from the splenic nerve (Irwin et al., 1990; Katafuchi et al., 1993a). The activity of the splenic nerve, which releases norepinephrine and NPY, appears to be under the central control of the hypothalamus (Katafuchi et al., 1993b; Take et al., 1995). The nucleus accumbens shell has direct connections to several hypothalamic autonomic regulatory centers (Heimer et al., 1991; Usuda et al., 1998), suggesting that this may be one pathway involved in the effects of opioids. Moreover, several hypothalamic nuclei have been shown to modulate splenic NK activity including the lateral hypothalamus, which receives extensive afferent input from the nucleus accumbens shell (Okamoto et al., 1996; Wenner et al., 1996; Wrona and Trojniar, 2003; Wrona and Trojniar, 2005). Thus, one possibility is that dopamine transmission in the nucleus accumbens shell alters splenic NK activity through interactions with hypothalamic nuclei that modulate the activity of the sympathetic innervation to peripheral tissues.
**NPY Mechanisms**

The sympathetic nervous system modulates immunocyte functions via direct innervation of target tissues or by inducing the release of signaling molecules into the systemic circulation via the adrenal medulla. Some evidence indicates that the sympatho-adreno-medullary pathway mediates a subset of opioid-induced effects. For instance, opioid effects on splenocyte mitogenic responses are abrogated by β-adrenoceptor blockers and by adrenalectomy, suggesting that these effects are involve the release of catecholamines from the adrenal medulla (Fecho et al., 1993a; Fecho et al., 1996b). However, adrenalectomy does not prevent all opioid induced effects, such as the suppression of NK cell activity, so it is unlikely that the adrenals are the source of NPY that mediates the effects observed in the present studies. Moreover, other studies have found that removal of the adrenals does not attenuate the increase of plasma NPY levels in response to stress (Mormede et al., 1990). A more plausible explanation is that the effects on splenic NK cell activity and iNOS production observed in the present studies are mediated directly through sympathetic innervation of target tissues. As discussed in Chapter 3, sympathetic fibers innervating the spleen provide a “hard-wired” connection between the nervous system and immune cells. Moreover, sympathetic nerves also supply the lung and liver—an important observation considering heroin’s effects on iNOS expression in these tissues. Thus, the local release of NPY in target tissues by sympathetic nerves may modulate NK activity and iNOS production.

NPY can potentially influence immune measures at the cellular level through a number of pathways, as it has pleiotropic effects on immunocyte physiology including the modulation of leukocyte mobilization (Bedoui et al., 2002), cytokine expression (Bedoui et
al., 2003; Straub et al., 2000b), monocyte chemotaxis (Straub et al., 2000a), and antibody production (Friedman et al., 1995). With respect to NK cell activity it appears that NPY acts directly via NK cell-expressed Y₁ receptors to inhibit cytolytic activity, as functional NPY Y₁ receptors have been cloned from rat lymphocytes (Petitto et al., 1994) and in vitro studies indicate that NPY induces a direct and dose-dependent inhibition of NK cell activity (Nair et al., 1993). However, there is less certainty regarding the effects of NPY on iNOS expression. Because NPY has widespread effects on immune parameters, it is possible that Y₁ receptors indirectly modulate iNOS production via leukocyte mobilization or by altering the production of cytokines that induce iNOS. The most parsimonious explanation however, is that the NPY acts directly via Y₁ receptors on iNOS producing cell types such as the macrophage. NPY Y₁ receptors have been shown to modulate macrophage functions in vitro (Dimitrijevic et al., 2005), and macrophages appear to be the major site of iNOS expression in LPS-treated rats (Buttery et al., 1994; Cook et al., 1994). Thus, Y₁ receptors expressed by NK cells and macrophages may directly underlie the inhibitory effects of opioids on cytolytic activity and iNOS production, respectively.

The observation that the proposed dopamine/NPY pathway mediates a subset of opioid-induced effects (i.e., NK activity and iNOS production) raises important questions about the relationship between these immune measures. Although it appears that NK activity and iNOS production are two distinct parameters modulated in a similar manner by the nervous system, another possibility is that these two measures reflect the same underlying immunological perturbation. In addition to macrophages, other cell types such as NK cells are capable of generating iNOS and producing nitric oxide (Bandaletova et al., 1993; Cifone et al., 1999; Salvucci et al., 1998). Thus, reduced iNOS expression in NK cells, for example,
could potentially contribute to heroin’s effects. Even so, it is unlikely that effects on NK cell-derived iNOS could fully account for the effects of heroin observed in the current studies since the production of large quantities of iNOS in response to LPS is largely attributable to macrophage cell types.

Because NK cells are capable of nitric oxide production, another interesting possibility is that Y₁ receptor activation inhibits NK cytotoxic activity by suppressing iNOS production. NK cell-mediating killing is primarily believed to involve the release of cytotoxic granules (e.g., perforin and granzyme) or the activation of cell membrane death receptors (e.g., Fas) (for review see Russell and Ley, 2002), but recent evidence indicates that the induction of iNOS is also involved in NK cell cytotoxic activity (Cifone et al., 1999; Filep et al., 1996; Jyothi and Khar, 1999). While it is well known that nitric oxide can induce apoptosis directly, it is presently unclear if NK cell-derived nitric oxide performs a similar function or simply modulates other cytotoxic pathways. In any case, Y₁ receptors are clearly important in NK cell cytotoxic activity. Whether the intracellular mechanism involves modulating iNOS production will need to be addressed by future studies.

**Biological Implications**

These studies are the first to show that the modulation of nucleus accumbens dopamine is directly involved in the effects of opioids on immune physiology. Researchers in the field of drug addiction have long appreciated that the reinforcing effects of addictive drugs are intimately related to the activation of dopamine neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens. Because the present findings show that stimulation of D₁ receptors in the shell of the nucleus accumbens activates central circuits
that modulate peripheral immune responses, a major implication is that addictive drugs may produce immunological perturbations through common pathways. Although few studies have systematically examined the immunological consequences of different classes of drugs under the same experimental parameters, virtually every addictive drug produces detrimental effects on facets of the immune response. In particular, reductions in splenic NK cell activity have been reported following in vivo administration of alcohol (Boyadjieva et al., 2001; Wu and Pruett, 1996a), cannabinoids (Massi et al., 2000; Patrini et al., 1997), amphetamine (Nunez-Iglesias et al., 1996), and cocaine (Pacifici et al., 2003). Although it is unknown whether nucleus accumbens D₁ receptor mechanisms contributed to the observed NK suppression in these studies, the notion that different classes of abused drugs modulate immunity by acting through a common central mechanism is an intriguing hypothesis worthy of further investigation.

Another major implication of the present findings is that the same neural processes responsible for morphine’s pharmacological effects may mediate the conditioned effects of the drug on immune status. Since conditioned effects on immunity reflect the inherent capacity of the brain to modify immune functions, the data further imply that the proposed dopamine/NPY pathway is a component of normal physiological regulatory mechanisms involved in modulating the immune response. In particular, the current findings may be relevant to certain types of stress-induced immune alterations. For example, it is known that both physical and psychological stressors produce immune alterations that are mediated by brain opioid receptors (Cunnick et al., 1988; Lysle et al., 1992a; Shavit et al., 1984). Interestingly, it appears that certain types of stress also increase the release of nucleus accumbens dopamine (Barrot et al., 1999; Kalivas and Duffy, 1995; Young et al., 1993).
Therefore, in addition to demonstrating neural mechanisms of immunomodulation produced by opioid drugs and drug-conditioned stimuli, the present findings may also have implications regarding the ability of stress and emotional processes to modify the immune response.

The involvement of the nucleus accumbens shell in modulating immune responses raises important questions about the functions of nucleus accumbens dopamine. Historically, the nucleus accumbens has been construed as a “limbic-motor interface” that integrates limbic inputs from the amygdala, hippocampus, and prefrontal cortex, and translates this information into behavioral action via outputs to autonomic and motor nuclei (Mogenson et al., 1980). The proximate question as to what specific information is encoded by nucleus accumbens dopamine has generated a multitude of competing hypotheses. These hypotheses, which are not necessarily mutually exclusive, have largely addressed the role of nucleus accumbens in learning and motivational processes as they relate to natural or drug rewards (e.g., Berridge and Robinson, 1998; Di Chiara, 2002; Koob and Le, 2005; Salamone and Correa, 2002; Wise, 2004). However, because the nucleus accumbens has been implicated in modulating a diversity of physiological functions—including blood pressure (Sakata et al., 2002), stress responses (Ikemoto and Goeders, 1998), wakefulness (Ponzoni et al., 1995), and immunity (Nistico et al., 1994)—a broader perspective is warranted to account for its participation in these more global functions. Recently it has been postulated that dopaminergic projections to the nucleus accumbens are part of a set of coordinated neuronal mechanisms that enables organisms both to find biologically important stimuli from the environment as well as avoid or escape danger (Ikemoto, 2007; Ikemoto and Panksepp, 1999). This hypothesis states that nucleus accumbens dopamine plays a major role in
incentive learning and in the regulation of “action-arousal,” which is defined as a state of arousal that energizes organisms for interactions with the environment. This explanation satisfactorily explains the role of the nucleus accumbens in neuroimmune modulation, because the concept of action-arousal encompasses reciprocal interactions between cognitive/psychological processes and behavioral/physiological responses. Thus, increases in nucleus accumbens dopamine may serve to energize organisms for interactions with the environment in part, by producing an activation of the sympathetic nervous system and HPA axis. Evidence to support this notion comes from studies showing that electrical stimulation of the VTA/medial forebrain bundle elicits increases of both plasma catecholamine and glucocorticoid levels (Burgess et al., 1993). Importantly, increased states of physiological arousal also modulate nucleus accumbens dopamine in a reciprocal manner, as glucocorticoids and mild stressors have been shown to increase dopamine activity in the nucleus accumbens shell (Barrot et al., 1999; Barrot et al., 2000; Piazza et al., 1996). Immunomodulation thus, may be construed as a consequence of the heightened state of autonomic arousal that accompanies increased nucleus accumbens dopamine.

While increased arousal states may explain the link between nucleus accumbens dopamine and altered immune measures, existing theoretical frameworks do not address the ultimate “why” question as to the adaptive value of suppressed immune states. To understand why immunosuppression may be adaptive under certain environmental contexts, it should be emphasized that the immune system and the nervous system do not operate autonomously, but rather communicate in a reciprocal manner. Immune cells produce molecules and receptors for classically defined neurotransmitters, and the nervous system produces and molecules and receptors traditionally categorized as cytokines. This point is
critical, because during states of infection, activated immune cells release proinflammatory cytokines (e.g., IL-1 and TNF-α) that act upon the brain to elicit a constellation of non-specific infection symptoms collectively termed “sickness behaviors” (for review, see Dantzer and Kelley, 2007). These symptoms include both fever and behavioral changes such as fatigue, exaggerated pain responses, anhedonia, listlessness, hypersomnia, reduced appetite, inability to concentrate, and loss of interest in sexual and social activities. Collectively, sickness behaviors have been conceptualized as the expression of a motivational state that enhances survival and promotes recovery from infection (Kelley et al., 2003). However, there are palpable negative consequences of sickness behaviors if the organism is in a temporal situation or environmental context that requires increased arousal, attention, energy expenditure, or vigilance; for example, during periods of increased predation risk, or during migratory or mating seasons. Thus, it may be adaptive in certain contexts to delay the onset of sickness behaviors produced by the immune response if the benefits (e.g., short-term survival, enhanced reproduction) exceed the costs (e.g., heightened risk of infectious disease).

Perhaps not coincidentally, the motivational states modulated by nucleus accumbens dopamine diametrically oppose the motivational states that result from immune activation during sickness. As discussed previously, nucleus accumbens dopamine is believed to play an important role in action-arousal states that enable the organism to interact with biologically significant stimuli. In line with the adaptive value of heightened action-arousal states, increased nucleus accumbens dopamine in response to relevant environmental stimuli may function to limit the production of proinflammatory cytokines that could compromise the performance of adaptive behavioral responses. In other words, suppression of the
immune response and the prolongation of sickness behaviors could serve to promote immediate survival or to enhance reproductive opportunities, thereby increasing the organism’s evolutionary fitness. This proposed conceptual framework offers an evolutionary explanation for a number of empirically observed phenomenon, and it is consistent the fact that the nucleus accumbens integrates information from limbic structures about salient changes in the environment with respect to self-preservation and procreation (MacLean, 1990).

Conclusions

Substantial evidence indicates that opioid use is associated with negative health consequences. Given the widespread medical and recreational uses of opioid drugs, it is important to understand the effects of opioids on immunity and health, as well as the physiological mechanisms of those effects. The present dissertation has proposed a novel neuroimmune pathway that underlies the effects of opioid drugs on imperative immunological functions. These findings show that the interaction of opioids with dopamine signaling in the nucleus accumbens shell is critically involved in modulating immune status. Furthermore, the present studies suggest that increased nucleus accumbens dopamine transmission modulates specific immune parameters by facilitating the release of NPY from sympathetic nerves. Importantly, the dissertation shows that this pathway not only underlies the immunomodulatory effects of exogenous opioids, but also the effects of stimuli associated with opioid administration. As the proposed pathway is involved in the effects of morphine, heroin, and conditioned stimuli on both functional and in vivo immune measures, these findings have significant implications for the mechanisms whereby opioids and
behavioral factors modulate the immune system. Additionally, the proposed mechanisms may also have mechanistic implications for the immunomodulatory effects of other addictive drugs or stressful stimuli that activate mesoaccumbens dopamine neurons. Collectively, these findings provide important insights into how the actions of opioids in the brain translate into peripheral immune alterations.
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