# A heroin/morphine vaccine: mechanism of action and extending its use to other abused opioids

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## Abstract

Heroin is more widely used than any other illicit opioid and mortality rates among heroin users are an average of 13 times higher than the general population. Intravenous heroin use is associated with crime, social disruption, and transmission of blood-born pathogens such as human immunodeficiency virus and hepatitis C. Effective pharmacotherapies are available to treat heroin abuse but have been largely unsuccessful because they require frequent dosing, have a high abuse potential, or have low compliance.

Vaccines against heroin and its metabolites (e.g. morphine) are being considered as a complementary treatment for heroin abuse because they are long-acting, selective, have no abuse potential, and may benefit those unwilling to take the current pharmacotherapies. Vaccination with morphine-conjugate vaccines can elicit a strong immune response that reduces the behavioral effects of heroin in animals, presumably by morphine-specific antibodies binding opioids in blood and reducing their distribution to brain. This thesis explores the use of M-KLH, a morphine hapten conjugated to keyhole limpet hemocyanin (KLH) using a tetraglycine linker and mixed with either Freund's or alum adjuvant for increasing the immune response.

Morphine vaccines present many challenges that make translation to clinical use difficult. Heroin is sequentially metabolized to its active intermediates 6-monoacetylmorphine (6-MAM), morphine, and then to morphine-6-glucuronide (*in vivo* and *ex vivo*). Heroin enters brain and is rapidly converted to 6-MAM, which is presumed to mediate most of heroin's early effects. With regard to the mechanism of action of morphine vaccines, it is unclear whether the antibodies they generate must bind heroin, its downstream metabolites, or both to prevent opioid distribution from plasma to brain and reduce heroin's behavioral effects. However, because analytical assays to measure

heroin and metabolite concentrations in tissues have used a wide range of conditions and varying degrees of stability have been reported, studying the effect of vaccination on heroin distribution is not straightforward. In addition, heroin and metabolite distribution after i.v. heroin administration, the most common route of abuse by humans, has not been well characterized in non-vaccinated rodents. Finally, blockade of heroin by vaccination may not prevent the abuse of structurally distinct opioids.

The overall goal of this thesis was to better understand the mechanism of action of morphine vaccines and to extend their use to other abused opioids. The specific aims were to stabilize heroin in blood and brain tissues for subsequent pharmacokinetic studies, study distribution of heroin and its metabolites in non-vaccinated and vaccinated rats, explore the effects of vaccination on heroin-induced behaviors, and determine if vaccine efficacy is retained when combined with a vaccine targeting oxycodone, another commonly abused opioid. These aims were explored using clinically relevant drug doses.

Heroin and metabolite degradation was significantly reduced by 1) the addition of ice-cold sodium fluoride (a general esterase inhibitor) and formate buffer (pH 3.0) in heroin-spiked tissues, 2) rapid removal of red blood cells via centrifugation, and 3) drying opioids after extraction from tissues prior to measuring their levels. Using these conditions heroin and its metabolites were stabilized in tissues for subsequent distribution studies.

In non-vaccinated rats 6-MAM was the predominant metabolite in brain as early as one minute after administration of 0.26 mg/kg i.v. heroin, which is consistent with previous studies that suggest that 6-MAM mediates heroin's early behavioral effects. Vaccination with a morphine-conjugate vaccine (M-KLH) led to a reduction of 6-MAM and morphine, but not heroin, distribution to brain after heroin administration, suggesting

that morphine vaccines reduce accumulation of 6-MAM in brain. The mechanism by which this occurs is likely through antibody binding of 6-MAM in plasma to prevent its distribution to brain and is consistent with very high plasma 6-MAM concentrations in vaccinated rats after i.v. heroin or 6-MAM administration.

Vaccination with M-KLH led to a reduction of heroin-induced anti-nociception and locomotor activity and remained effective for up to 16 days after repeated dosing suggesting that heroin vaccines may have long-lasting efficacy. These results are consistent with findings from the distribution studies and support the hypothesis that morphine vaccines function by retaining 6-MAM in plasma and prevent its accumulation in brain.

To determine whether opioid vaccines could be combined without reducing individual vaccine efficacy and prevent heroin addicts from abusing structurally distinct opioids, rats were vaccinated with M-KLH, an oxycodone-conjugate vaccine (Oxy-KLH), or the bivalent vaccine (both M-KLH and Oxy-KLH). Total morphine- and oxycodone-specific antibody titers were significantly increased in rats that received the bivalent vaccine compared to rats that received individual vaccines. Concurrent i.v. administration of 6-MAM and oxycodone in M-KLH vaccinated rats led to increased 6-MAM retention in plasma and reduced 6-MAM distribution in brain. A similar effect on oxycodone distribution was seen in Oxy-KLH vaccinated rats. There was a trend towards greater efficacy in altering both 6-MAM and oxycodone distribution in the bivalent group compared to individual vaccine groups. These data suggest that combining opioid vaccines will retain, and possibly enhance, individual vaccine efficacy and might be a viable option to prevent addicts from abusing structurally distinct opioids.

These findings contribute to the understanding of how morphine vaccines elicit their effects on heroin-induced behaviors and suggest that morphine vaccines, alone or

in combination with other pharmacotherapies, may benefit those seeking treatment for heroin addiction.

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# Abbreviations

| | |
|---|---|
| %MPE: | percent maximum possible effect |
| 6-MAM: | 6-monoacetylmorphine |
| AChE: | acetylcholinesterase |
| APC: | antigen presenting cell |
| ANOVA: | analysis of variation |
| BChE: | butyrylcholinesterase |
| BSA: | bovine serum albumin |
| DC: | dendritic cell |
| DOR: | δ-opioid receptor |
| ELISA: | enzyme-linked immunosorbant assay |
| FCA: | Freund's complete adjuvant |
| FIA: | Freund's incomplete adjuvant |
| GPCR: | G protein coupled receptor |
| $IC_{50}$: | half maximal inhibitory concentration |
| IgG: | immunoglobulin G |
| IgM: | immunoglobulin M |
| i.m.: | intramuscular |
| i.p.: | intraperitoneal |
| i.v.: | intravenous |
| kD: | kiloDaltons |
| KLH: | keyhole limpet hemocyanin |
| KOR: | κ-opioid receptor |
| M-3-G: | morphine-3-glucuronide |
| M-6-G: | morphine-6-glucuronide |
| M-KLH: | morphine conjugated to KLH |
| MHC: | major histocompatibility complex |
| MOR: | μ-opioid receptor |
| NaF | sodium fluoride |
| OXY-KLH: | oxycodone conjugated to KLH |
| s.c.: | subcutaneous |

# CHAPTER 1

## Introduction

This chapter summarizes background information and introduces the goals of this thesis.

**1.    Heroin addiction: a worldwide problem**

Heroin addiction is classified as a substance-related disorder. Addiction is defined as "a primary, chronic, neurobiological disease, with genetic, psychosocial, and environmental factors influencing its development and manifestations" by the American Society of Addiction Medicine (ASAM, 2001). Addiction behaviors include a complex variety of symptoms including loss of control over its use, compulsive use, continued use despite negative consequences, and drug cravings.

An estimated 13 to 22 million people worldwide abuse opioids (UNODC, 2010). Heroin is more widely used than any other illicit opioid with over 400,000 users in the U.S. alone (SAMHSA, 2010). Easier access to prescription opioids has led to an increase in opioid abuse and serves as a gateway to intravenous heroin abuse (Lankenau et al., 2012). Because the therapeutic window of heroin is extremely small, and because heroin purity varies, heroin-dependent users have a mortality rate that averages 13 times higher than the general population (UNODC, 2010). Intravenous heroin use is associated with crime, social disruption, reduced productivity, and transmission of blood-borne pathogens such as HIV and Hepatitis C (Tang et al., 2006). The estimated total economic cost of heroin in the United States alone was $21.9 billion in 1996 (Mark et al., 2001). These factors support the rationale that treating heroin addiction is necessary.

Pharmacological therapies are available to treat individuals addicted to heroin. These therapies include opioid agonists (methadone and buprenorphine) or opioid antagonists (naltrexone) and function by binding to opioid receptors in the brain to mimic or block the effects of heroin. While these medications are safe, effective, and have led to a decrease in the transmission of HIV transmission (Fiellin et al., 2006), less than 20% of

heroin addicts currently receive these therapies in the US (SAMHSA, 2010) and even fewer in other countries  (Tang and Hao, 2007; Mendelson et al., 2008; Krupitsky et al., 2010; Lobmaier et al., 2010). Compliance with antagonist use is low (O'Malley et al., 2000) and agonists have a high abuse potential, risk of diversion, and relatively short duration of efficacy, limiting their appeal and necessitating tight regulation. Behavioral therapies alone are largely unsuccessful at reducing relapse (Rawson et al., 1979). New approaches are therefore clearly needed for the treatment of heroin addiction.

Vaccination against heroin and its metabolites (denoted as morphine vaccines throughout this thesis) is being explored as an alternative treatment option for heroin addiction. Morphine vaccines have been shown to reduce the behavioral effects of heroin, though little is known about how these effects are achieved. Presumably, these effects are through opioid-selective antibodies binding heroin and its metabolites in plasma and reducing their distribution to brain (i.e. a pharmacokinetic intervention), but this has not yet been demonstrated. The overall goal of this thesis was to better understand the mechanism of action of morphine vaccines and to extend their use to other abused opioids. The following discussion provides the necessary background for the thesis aims.

## 2.    Pharmacological factors that contribute to heroin addiction

In order to develop novel strategies (e.g. immunotherapy) to treat heroin addiction, it is important to understand the pharmacological, behavioral, and social factors contributing to heroin addiction. This section will focus on heroin's disposition and impact on the neurobiological pathways that lead to heroin addiction.

## 2.1 Heroin

Diacetylmorphine, more commonly referred to as heroin, is a synthetic opioid created from O-acetylation of morphine at the C3 and C6 positions. Heroin is usually administered via intravenous injection, smoke inhalation, or intranasal snorting (Klous et al., 2005). Heroin is sequentially metabolized both in the periphery and in the central nervous system as (Fig 1): heroin → 6-monoacetylmorphine (6-MAM) → morphine → morphine-6-glucuronide (M-6-G, not significantly formed in rodents) and morphine-3-glucuronide (M-3-G) (Antonilli et al., 2005). Heroin and its metabolites bind to and activate opioid receptors in the brain, influencing neurotransmitter release that results in pain reduction, euphoric effects, and tolerance. Heroin is presumed to be a pro-drug because it is rapidly metabolized, has considerably lower affinity for opioid receptors than 6-MAM or morphine (Inturrisi et al., 1983), and is generally found at low concentrations in brain shortly after heroin administration (Andersen et al., 2009). 6-MAM is more likely to be the primary mediator of heroin's early behavioral effects, because it is found at high levels in plasma and brain after heroin administration in mice (Way et al., 1960; Andersen et al., 2009) and administration of equimolar heroin or 6-MAM doses results in similar behavioral effects (Andersen et al., 2009).

## 2.2 Opioid receptors

Opioids elicit their effects by activating opioid receptors. Opioid receptors are distributed throughout the periphery and CNS and are part of a group of seven transmembrane-spanning $G_i/G_o$ protein-coupled receptors (GPCRs) that are activated by both endogenous opioid peptides and exogenous opioids. G proteins are heterotrimeric structures composed of α, β and γ subunits. In the inactive state, these subunits are tightly bound together. Binding of an opioid ligand to the GPCR on the cell surface

causes intracellular GDP release from the G protein α subunit and binding of GTP, facilitating the complex dissociation. The α and the βγ subunits can then activate distinct intracellular effectors (reviewed in Law et al., 2000; Bian et al., 2012). Currently, four distinct opioid receptors have been identified: delta (DOR), kappa (KOR), mu (MOR), and nociceptin (NOP). However, most of morphine's rewarding properties are due to the activation of MORs (Matthes et al., 1996; Takita et al., 1997).

MORs are found in the brainstem and medial thalamus and are largely responsible for analgesia, respiratory depression, euphoria, sedation, decreased gastrointestinal motility, and physical dependence associated with heroin use (Arvidsson et al., 1995; Takita et al., 1997; Trescott, 2008). A key neuroanatomical structure related to opioid reward is the mesocorticolimbic dopaminergic system (the reward pathway), which is mainly composed of the dopaminergic projections from the ventral tegmental area (VTA) and the substantia nigra (SN) in the midbrain to the nucleus accumbens (NAcc) in the ventral striatum (Chao and Nestler, 2004).

When MORs are activated by morphine G$\alpha_i$ stimulation inhibits adenylyl cyclase, which reduces cAMP signaling, while the Gβγ subunit has a number of downstream effectors that lead to hyperpolarization and inactivation of GABA neurons and interneurons (Di Chiara and North, 1992; Chao and Nestler, 2004; McCleane and Smith, 2007; Bian et al., 2012). Inactivation of GABA neurons in the VTA and SN reduces GABA release causing dopaminergic neurons to increase dopamine release in the NAcc, which leads to the rewarding effects of heroin and morphine. Long-term exposure of opioids leads to tolerance and dependence, the physiological state of an individual in which abrupt cessation in drug acquisition causes aversive withdrawal symptoms. One contributor to

these effects is opioid receptor down-regulation and dysregulation of cAMP signaling (Waldhoer et al., 2004; Bian et al., 2012).

## 2.3    Heroin pharmacokinetics in humans

The reinforcing efficacy of a drug is influenced by its pharmacokinetic properties. Drugs that are lipid soluble rapidly distribute to brain and allow a rapid onset of drug-induced effects (Farre and Cami, 1991). Other properties such as dose, route of administration, terminal half-life, and the presence of active metabolites can influence the reinforcing efficacy of a drug. For example, oral administration of drugs of abuse is typically associated with the lowest abuse potential because of slower entry of drug and reduced bioavailability (Farre and Cami, 1991).  Altering the pharmacokinetic properties of heroin may be a useful target for alternative treatments for heroin addiction.

### 2.3.1   Heroin absorption, dosing, and route of administration

Heroin is typically abused by intravenous injection (the most widely used route), vapor inhalation ("chasing the dragon"), smoking cigarettes containing heroin, or snorting because these methods result in rapid peak opioid concentrations in plasma (Klous et al., 2005). After i.v. administration of heroin in humans, peak blood levels of heroin and 6-MAM occur at 1.1 – 2.8 min and 0.7 – 2.7 min, respectively (Rentsch et al., 2001; Klous et al., 2005), while morphine blood levels peak around 5 -10 min (Comer et al., 1999).

The dose of heroin that is administered also plays a role in its reinforcing efficacy. At high drug doses aversive effects begin to outweigh the rewarding effects, reducing responding for drug (Martin et al., 1998). At low doses the reinforcing effects are much

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lower, which also may reduce drug use (Martin et al., 1998). Humans abusing heroin typically consume between 2.1 to 2.8 mg/kg per injection and up to 7.1 mg/kg per day (Rentsch et al., 2001). In heroin-dependent individuals given i.v. heroin doses ranging from 0.09 – 1.43 mg/kg, only doses above 0.35 mg/kg were associated with rewarding effects (Comer et al., 1999).

## 2.3.2   Heroin and metabolite distribution

Heroin distribution out of blood and into tissues occurs very rapidly after administration and plays a major role in the 'rush' that accompanies heroin's early effects. Heroin has a large volume of distribution (around 60 – 100 L) because the acetyl groups on its C3 and C6 positions make it highly lipophilic (Rook et al., 2006). No data exists regarding brain opioid levels after heroin administration in humans. However, after s.c. administration of heroin, 6-MAM, or morphine in mice, both heroin and 6-MAM effectively pass the blood-brain barrier, while morphine uptake is delayed, suggesting that heroin and/or 6-MAM are largely responsible for heroin's early effects (Andersen et al., 2009). A pharmacokinetic intervention for heroin addiction might function by reducing the rate of heroin and 6-MAM entry into brain and reduce their accumulation in brain.

## 2.3.3   Heroin degradation

Because this thesis focuses on understanding the mechanism of action of vaccines targeting heroin by studying its effects on heroin and metabolite distribution, ensuring accurate measurement of opioid levels in tissues is essential. It is important to understand what factors influence heroin degradation and the steps required to prevent its degradation in analytical samples.

## 2.3.3.1 Spontaneous degradation of heroin

Heroin is stable in aqueous solutions at pH 4.0 and 5.6 with a half-life of up to 14 days, but degrades in alkaline solutions rapidly (Nakamura et al., 1975; Barrett et al., 1992). Temperature may also play a role in spontaneous degradation. In human plasma heroin degradation to 6-MAM has a half-life of 3 min at 37°C and 354 min at 4°C (Barrett et al., 1992). Storage of biological samples containing heroin should be stored at low pH and low temperatures for stability and accurate opioid measurements.

## 2.3.3.2 Esterase degradation of heroin

Esterases play a large role in the degradation of heroin to its active metabolites. Early investigation of heroin degradation showed that 1) human serum rapidly degrades heroin to 6-MAM (Wright, 1940; Nakamura et al., 1975), 2) heroin degrades twice as fast in human whole blood compared to serum (Nakamura et al., 1975), 3) heroin spiked in human serum does not degrade further than 6-MAM (Ellis, 1948), and 4) heroin degradation also occurs in brain, liver, and kidney tissues (Way et al., 1960). Differences between rates of conversion of heroin to 6-MAM and morphine in blood, plasma, and brain may be attributed to specific esterases in these different tissues (Wright, 1940; Way et al., 1960).

Serum butyrylcholinesterase (BChE) and erythrocyte acetylcholinesterase (erythrocyte AChE) are major esterases in blood that can metabolize heroin (Salmon et al., 1999; Bahar et al., 2012). BChE can deacetylate heroin to 6-MAM approximately nine times faster than erythrocyte AChE can (Salmon et al., 1999). However, erythrocyte AChE can further deacetylate 6-MAM to morphine, while BChE cannot (Lockridge et al., 1980; Salmon et al., 1999).

Brain synaptic AChE does not appear to rapidly deacetylate heroin, despite evidence that shows brain homogenates degrade heroin, albeit slower than in other tissues (Way et al., 1960; Salmon et al., 1999). Heroin in brain tissues, but not plasma, is stable at low temperatures (4°C) and after enzymatic inhibition using sodium fluoride (Karinen et al., 2009). These data suggest that heroin degradation in brain occurs via different enzymes than those found in blood. However, no data exists regarding a mechanism of heroin degradation in brain. Blocking esterase activity in tissues (in vitro) containing heroin and its metabolites should be considered when attempting to obtain accurate opioid levels.

2.3.4   Morphine glucuronides

Morphine is metabolized in the liver by UDP-glucuronosyltransferase (UGT) enzymes. The two major metabolites are M-3-G and M-6-G (De Gregori et al., 2012). M-3-G has an affinity to opioid receptors approximately 300 times lower than morphine and does not contribute to the rewarding effects of heroin (Pasternak et al., 1987; Wright et al., 2001). M-6-G is 100-fold more potent than morphine at producing anti-nociception when administered i.c.v. (Pasternak et al., 1987). However, because morphine-6-glucuronide is not significantly formed in rodents (Antonilli et al., 2005) and morphine-3-glucuronide is found at low levels in brain after heroin administration these molecules were not further explored in this thesis.

2.3.5   Heroin Elimination

Heroin is eliminated rapidly after administration in humans, with a terminal half-life of around 2 – 3 min in humans (Rook et al., 2006). 6-MAM has a half-life ranging from 5.4 – 52 min (Rook et al., 2006). Drugs with short elimination half-lives have been associated with higher rates of drug self-administration (Griffiths et al., 1981; Farre and

Cami, 1991; Quinn et al., 1997). Morphine has a half-life estimated to be around 100 –

280 min, while its glucuronides have a much longer half-life ranging from 2.0 – 6.4 hr

(Rook et al., 2006). The long half-lives of morphine and M-6-G are thought to contribute

to heroin's long-lasting effects, with subjective effects sustaining out to 270 min after i.v.

administration of 50 mg heroin in humans (Comer et al., 1999).

## 2.4    Heroin pharmacokinetics in rodents

Heroin pharmacokinetic parameters have not been well characterized in rodents. Almost

all pharmacokinetic studies in rodents have been done in mice using the s.c. route (Way

et al., 1960; Pacifici et al., 2000; Andersen et al., 2009), in mice using extremely large

doses i.v. heroin doses (Way et al., 1960), or in rats using morphine (Ngai et al., 1976;

Bhargava et al., 1993). This will be discussed in more detail in the section titled,

"challenges facing preclinical development of opioid vaccines" (see section 5.2).

## 3.    Current treatments for heroin addiction

Current therapies to treat heroin addiction are available and function mainly by binding to

MOR to block heroin's effects. These medications fall into two categories: opioid

agonists (methadone and buprenorphine) and antagonists (naltrexone), based on their

mechanisms of action.

## 3.1    Opioid agonists

Methadone Maintenance Treatment (MMT) has been the principal therapy option for

heroin addicts and is considered an effective treatment of heroin addiction. Methadone is

a full MOR agonist and has a high affinity for MOR. It is highly lipophilic, leading to high

brain levels that stabilize heroin-dependent individuals (Dole and Kreek, 1973). In one

study, 77% of patients that received methadone stopped i.v. heroin use over a 6 month period (Joseph et al., 2000). Buprenorphine is a partial MOR agonist with a long duration of action. It is being more commonly prescribed than methadone because dosing is more convenient, there are fewer restrictions on outpatient dispensing, and allows individuals who don't meet criteria for MMT to receive treatment.

## 3.2    Opioid antagonists

Naltrexone is a potent MOR antagonist that can block the euphoric effects of heroin. As opposed to agonist treatments, naltrexone has no abuse potential. However, oral naltrexone has shown limited efficacy. Patient abstinence from opioid use for 7 to 10 days prior to initiating naltrexone is required and risk of precipitated withdrawal decrease its appeal and may lead to relapse. A depot naltrexone has been approved in the U.S. and has shown higher retention rates (51% continued therapy) in one study (Tetrault and Fiellin, 2012). Naltrexone may have side effects that reduces its desirability including nausea, headaches, insomnia, and epigastric pain (Tetrault and Fiellin, 2012).

While these pharmacotherapies have been shown to be safe, effective, and have been shown to reduce the risk of HIV transmission (Fiellin et al., 2006) less than 20% of addicts in the US currently receive these therapies (SAMHSA, 2010). MMT requires daily dosing under supervised visits to clinics, which is inconvenient, while some areas may not have an infrastructure capable of handling the large number of addicts needing medication (Lewis, 1999). Agonists have a high abuse potential, risk of diversion, and relatively short duration of efficacy, limiting their appeal and necessitating tight regulation. Agonists are also perceived as trading one addiction for another (Campbell and Lovell, 2012). Antagonists bind to opioid receptors and block opioid analgesics,

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which becomes an issue when managing pain in an emergency situation or prior to major surgery (Vickers and Jolly, 2006). Also, compliance with antagonist use is also low (O'Malley et al., 2000). These issues suggest that longer acting treatments that do not elicit their own pharmacological effects could benefit addicts unwilling to use currently available treatments.

3.3     Behavioral therapy

Psychosocial therapy can be used alongside current pharmacotherapies to treat heroin addiction. These treatments include coping skills, changing the patient's environment, and group or individual therapy (Raisch et al., 2002). While some studies have shown efficacy when behavioral therapy is combined with pharmacotherapy, meta-analysis suggests that behavioral therapy may not actually improve pharmacotherapy outcomes (Tetrault and Fiellin, 2012).

4.     Vaccine as a complementary therapy to current treatments

The pharmacotherapies described above bind to opioid receptors in brain and are subject to the same side effects that other opioids have. Vaccination against heroin, on the other hand, targets the drug rather than the brain. Antibodies (large proteins with a molecular weight of ~150 kDa that do not cross the blood-brain barrier) elicited by the immune system after active immunization circulate in the bloodstream and sequester the target drug there, presumably reducing its distribution to brain and ultimately reducing the reinforcing effects of the drug. Opioid vaccines are being studied as an alternative or complementary treatment of heroin addiction because they are long-acting, highly selective, and have few side effects. Below is a discussion of the history, mechanism of

action, and potential benefits of opioid vaccines, as well as challenges facing their translation to human use.

## 4.1    Current addiction vaccines

Drug-specific vaccines have emerged as a potential treatment for addictions to many other drugs of abuse including nicotine and cocaine (Fox, 1997; Hieda et al., 1997). Nicotine and cocaine vaccines have advanced to human clinical trials and have demonstrated the proof-of-concept that addiction vaccines can block the reinforcing effects of psychoactive drugs and reduce drug-seeking behaviors in individuals that achieve high serum antibody concentrations (Haney et al., 2010; Hatsukami et al., 2011).

## 4.2    History of morphine-conjugate vaccines

Immunization as a feasible option to treat heroin addiction was demonstrated in the early 1970's in a rhesus monkey (Bonese et al., 1974). The monkey was trained to self-administer heroin and cocaine and then vaccinated against morphine. Once vaccinated the monkey did not self-administer low doses of heroin but readily administered cocaine, demonstrating antibody specificity towards the targeted molecule. Higher heroin doses were associated with more lever presses, suggesting that antibodies could be overcome by large drug doses. More recently, a study was published showing that a highly immunogenic and selective morphine-conjugate vaccine could prevent reacquisition of heroin self-administration in vaccinated rats (Anton and Leff, 2006).  Other studies have followed showing that morphine-conjugate vaccines can block a variety of heroin-induced effects, including anti-nociception and locomotor activity (Ma et al., 2006; Li et al., 2011; Stowe et al., 2011).

4.3     Morphine vaccine

Opioids are too small to be recognized as foreign by the immune system, but they can be rendered immunogenic by conjugating them to foreign carrier proteins. The vaccine used in this thesis was M-KLH and will be briefly described here. Morphine was modified at its C6 position to attach a tetraglycine ($Gly_4$) linker. This morphine-linker complex is called the hapten. The hapten was covalently attached by carbodiimide linkage to lysine amine groups on keyhole limpet hemocyanin (KLH), a large and immunogenic carrier protein. The hapten-carrier protein complex forms the immunogen. The immunogen was mixed with an adjuvant, Freund's adjuvant or an aluminum salt, to form the vaccine M-KLH (Fig 2). Other heroin/morphine vaccines may differ in linker length and structure, carrier protein, hapten-linker attachment site, or adjuvants used but are similar in design and function (Bonese et al., 1974; Anton and Leff, 2006; Li et al., 2011; Stowe et al., 2011). Therefore, the data shown in this thesis regarding the effects of M-KLH should be generalizable to other heroin and morphine vaccines.

4.3.1   Immunology of hapten-conjugate vaccines

Addiction vaccines are designed to generate high concentration of high affinity serum antibodies that reduce distribution of drug to brain. Although reaching high antibody concentrations in serum may be achieved by the administration of antibodies intravenously (passive immunization), this thesis used active vaccination to elicit drug-specific antibodies and the following discussion will focus on this method.

Active immunization is accomplished by administering an immunogen with an adjuvant into a human or animal, which induces the immune system to respond and develop antibodies against the immunogen. The benefit of this method of immunization is that it

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is relatively inexpensive and provides long-lasting protection against the immunogen. The main limitations are that 1) the immune system must generate these antibodies over time so there is a lag between vaccination and protection against the target and 2) not every individual achieves clinically effective concentrations (Hatsukami et al., 2011).

4.3.2   Role of APCs, B cells, and T cells

Active immunization requires the involvement of many immune cell types to elicit circulating immunogen-specific antibodies. After exposure to the conjugate vaccine, antigen presenting cells (APC) such as dendritic cells (DC), macrophages, and B-cells are recruited to the injection site and transport the immunogen to lymph nodes (Owen et al., 2013; Plotkin et al., 2013). APCs internalize and digest the immunogens in endosomes and present them as small, degraded peptides immunogens on the class II major histocompatibility complex (MHC) in order to activate T cells (Plotkin et al., 2013). T cells that are activated differentiate into T helper ($T_H$) cells are crucial for humoral and cell-mediated immune responses.

Circulating high affinity antibodies are derived from specialized B cell populations. B cell receptors (BCR) on the surface of B cells recognize and bind to the immunogen with low affinity, internalize it, and display portions of the immunogen as small peptides on class II MHC receptors (Owen et al., 2013; Plotkin et al., 2013). These B cells can interact with mature $T_H$ cells specific for the peptide. This interaction results in the release of cytokines by the T cells that induce B cells to differentiate into plasma cells, which produce high affinity antibodies that circulate in the bloodstream, and memory B cells, which express high-affinity BCRs that respond quickly to antigen presentation to differentiate into plasma cells (Taylor et al., 2012; Owen et al., 2013).

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4.3.3    Role of adjuvants

Adjuvants are mixed with an immunogen to enhance the immune response by protecting the immunogen from rapid degradation, recruiting and activating immune cells, and encouraging antigen presentation (Stills, 2005). The two adjuvants used in this thesis were aluminum salts and Freund's adjuvant and their mechanisms of action are described below.

4.3.3.1 Freund's adjuvant

Freund's adjuvant is a potent adjuvant that can elicit a very strong immune response. There are two preparations of Freund's adjuvant; Freund's Complete adjuvant (FCA) and Freund's Incomplete adjuvant (FIA). Freund's adjuvant is an oil-in-water emulsion consisting of light mineral oil, mannide monooleate (a surfactant), and heat-killed mycobacterial cells (in FCA only). Freund's adjuvant produces many non-specific effects including 1) creating a 'depot' effect whereby antigen release is sustained, 2) improving transportation of an antigen to lymph nodes, and 3) increasing interaction of antigens with antigen presenting cells (Stills, 2005). FCA has also been shown to induce IL-6, IL-23 and TGF-$\beta$, which are cytokines involved in the differentiation and activation of T-cells (McGeachy and Cua, 2008).

4.3.3.2 Aluminum salt adjuvants

Aluminum containing adjuvants (alum) are among the few clinically used adjuvants and have been used for the last 80 years (Lambrecht et al., 2009). Alum provides a 'depot' effect, similar to Freund's adjuvant, by adsorption of the immunogen onto alum (due to electrostatic forces) and stimulates uptake by antigen-presenting cells (Brunner et al., 2010). Alum also promotes T-cell differentiation to $T_H$-cells that primarily activate B-cells

to become plasma or memory B-cells (Stills, 2005). The mechanism by which this occurs may be through alum interacting directly with membrane lipids on the surface of DCs, promoting internalization of soluble hapten and enhancing its presentation via class II MHC (Flach et al., 2011). Alum itself does not appear to be internalized.

Freund's Adjuvant, though far more potent in eliciting high antibody titers, is not clinically used because it causes a number of unpleasant side effects. The goal of this thesis was to better understand the mechanism of action of morphine vaccines, so to this end it was useful to administer an adjuvant that elicited a strong antibody response. However, effects on drug distribution were shown in animals vaccinated using alum, demonstrating its potential use in translating morphine vaccines to human use.

## 5.     Thesis goals

### 5.1     Summary

The overall goal of this thesis was to understand how morphine vaccines reduce heroin-induced behavioral effects in rats and to extend their use to other abused opioids. Morphine vaccines were the first addiction vaccines to be studied and showed blockade of heroin's behavioral effects, demonstrating the proof-of-concept that a vaccine strategy could be used to treat heroin addiction. Despite this, morphine vaccines are less well understood than other addiction vaccines due to a variety of challenges that impact their translation to humans. Below is a discussion of those challenges.

### 5.2     Challenges facing preclinical development of opioid vaccines

Compared to nicotine and cocaine, heroin is a more challenging drug target for vaccination therapy. First, heroin metabolism is very complex and many active

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metabolites are generated (see section 2.3). With regard to the mechanism of action of morphine vaccines, it is unclear whether the antibodies they generate must bind heroin, its downstream metabolites, or both to prevent opioid distribution from plasma to brain and reduce heroin's behavioral effects. It is also unknown what antibody concentrations and affinities are required for a morphine-conjugate vaccine to reduce heroin's behavioral effects. This information will be important when translating morphine vaccines to human use because it will provide criteria for determining vaccine efficacy. This thesis provides data that begins addressing the mechanism of action of a morphine-conjugate vaccine.

Second, quantification of heroin in biological samples is very difficult due to rapid degradation of heroin to its active metabolites, 6-MAM and morphine (Nakamura et al., 1975; Garrett and Gurkan, 1979; Lockridge et al., 1980; Barrett et al., 1992). In order to better understand how vaccine-generated antibody alter heroin and metabolite distribution in vaccinated rodents, heroin and its metabolites need to be stabilized in drug tissues. This thesis provides studies that address this issue.

Third, distribution of heroin and its metabolites after i.v. administration of heroin has not been well characterized in non-vaccinated rodents. Distribution studies have primarily focused on the s.c. route (Umans and Inturrisi, 1982; Pacifici et al., 2000; Andersen et al., 2009), which may subject heroin to increased peripheral degradation and slower distribution to brain compared to the i.v. route used by humans (Klous et al., 2005). Heroin distribution after i.v. administration has been studied, but an extremely high dose was used to compensate for less sensitive analytical methods and these studies were

performed in mice (Way et al., 1960). The use of rats could allow a wider range of pharmacokinetic and behavioral studies to be performed.

Fourth, there are many structurally distinct opioids available to heroin users if the effects of heroin were blocked. Prescription opioids are widely available and becoming more widely abused than heroin itself (Compton and Volkow, 2006a). A vaccine that targets only heroin and its metabolites may not offer enough protection against other non-targeted opioids, allowing opioid addicts to switch. By combining vaccines that target different opioids, broader selectivity may be achieved. This thesis explores the use of combining a morphine and oxycodone vaccine to address this problem.

5.3     Thesis goals

The following specific aims of this thesis provide insight into how morphine-conjugate vaccines reduce the behavioral effects of heroin. The first goal was to develop an assay to stabilize heroin and its metabolites in blood and brain so that these opioids could be accurately measured after intravenous opioid administration. Second, heroin and metabolite distribution was studied after i.v. administration in both non-vaccinated and vaccinated rats to determine the relative levels of heroin and metabolites in plasma and brain. Third, effect of vaccination on opioid-induced behaviors was examined to determine if M-KLH performed similarly to other morphine-conjugate vaccines. The fourth goal was to evaluate the efficacy of M-KLH when combined with an oxycodone-conjugate vaccine.

**FIGURES**

**Figure 1.** Metabolic pathway of heroin in humans. Butyrylcholinesterases (BChE) and erythrocyte acetylcholinesterases (eAChE) are highly involved in the degradation of heroin in vivo. Temperature and pH also play a role in spontaneous degradation of heroin. Morphine is further metabolized to morphine-3-glucuronide and morphine-6-glucuronide in the liver by UDP-glucuronosyltransferase (UGT) enzymes.
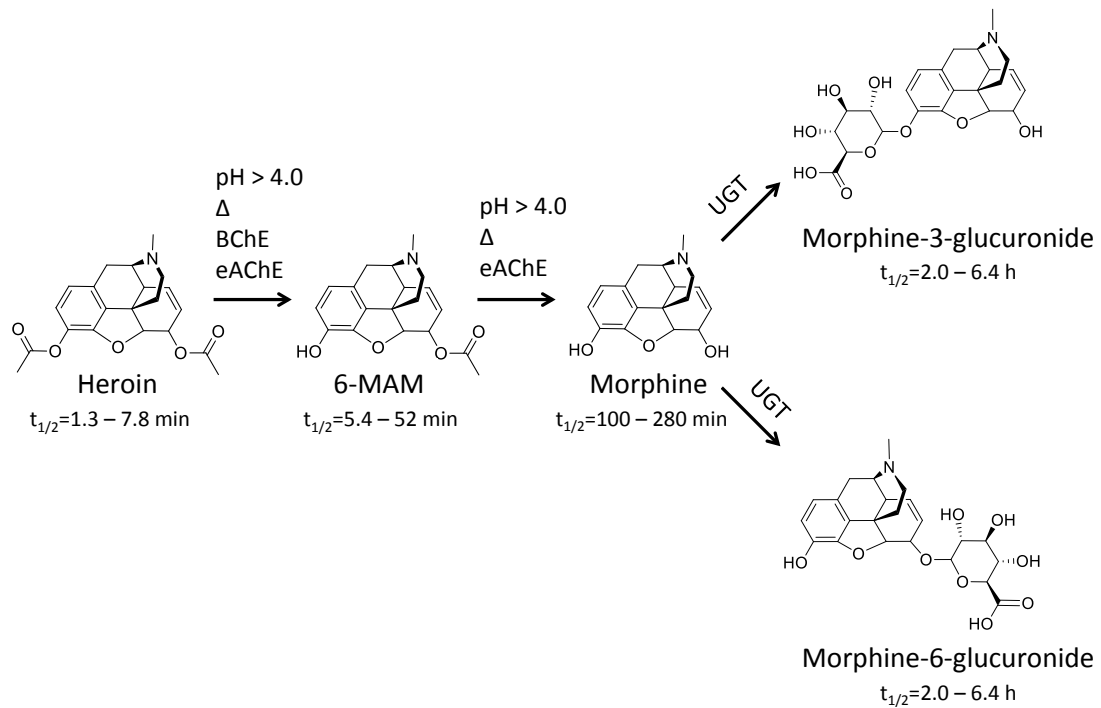
**Figure 2.** Composition of the Morphine-Gly$_4$-KLH (M-KLH) vaccine. KLH; Keyhole limpet hemocyanin, FCA; Freund's complete adjuvant, FIA; Freund's incomplete adjuvant
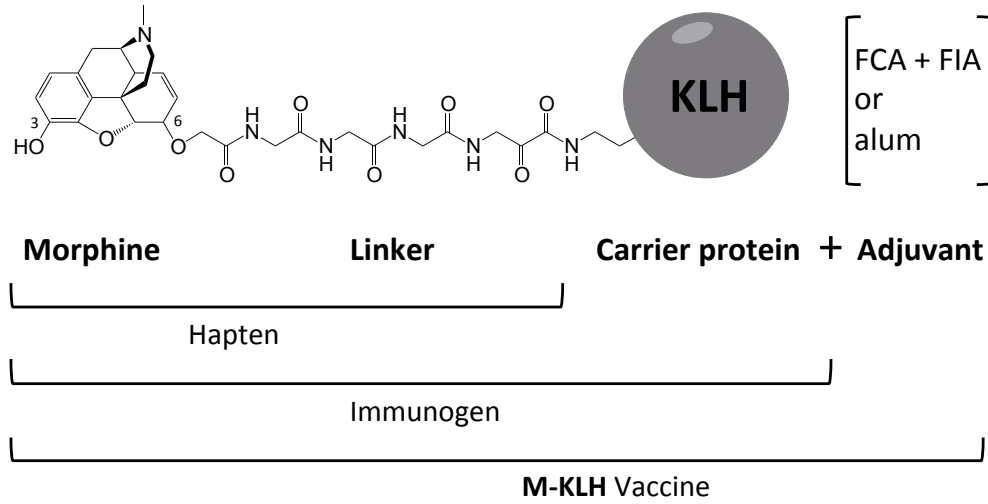
# CHAPTER 2

**Specific Aims and Introduction to Chapters 3, 4, and 5**

This chapter provides an overview of the Specific Aims of this thesis and hypotheses generated that address these Aims. The subsequent chapters 3, 4, and 5 are also introduced.

## 1.	Overall goal

The overall goal of this thesis was to study the mechanism of action of morphine vaccines while extending their use to other abused opioids. The specific goals of this thesis were to 1) stabilize heroin and its metabolites in rat tissues for subsequent distribution studies, 2) study heroin and metabolite distribution after i.v. heroin administration in non-vaccinated and vaccinated rats to better understand how morphine vaccines alter heroin-induced behaviors, 3) study effects of vaccination with M-KLH on heroin-induced behaviors, and 4) study the effects of combining two opioid vaccines on individual vaccine efficacy.

**Specific aim #1 was to develop an assay to stabilize heroin and its metabolites in tissues after collection to accurately measure drug levels in rats.** Because esterase activity and physiological conditions in blood lead to rapid degradation of heroin and its metabolites in tissues, it was important to develop an assay that would reduce degradation so that drug distribution studies in rats would reflect accurate drug levels. The hypothesis tested was that <u>addition of sodium fluoride, low temperature, and rapid extraction of drug from tissues would significantly reduce heroin and metabolite degradation in rat blood and brain.</u>

**Specific aim #2 was to study heroin and metabolite distribution after i.v. heroin administration in non-vaccinated rats.** Heroin distribution studies in non-vaccinated rats after i.v. administration had not previously been performed. To this end, it was important to characterize heroin distribution in rats after i.v. administration to determine relative opioid levels shortly after administration and to guide subsequent distribution

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studies in vaccinated rodents. The hypothesis was that <u>6-MAM was the predominant</u> <u>opioid in plasma and brain shortly after i.v. administration in non-vaccinated rats.</u>

**Specific aim #3 was to study the effects of vaccination with M-KLH on heroin and metabolite distribution after i.v. heroin administration.** Others have shown that morphine-conjugate vaccines can reduce behavioral effects of heroin (Bonese et al., 1974; Li et al., 2011; Stowe et al., 2011), yet the pharmacokinetic mechanisms mediating these effects have not been examined. Understanding how morphine vaccines alter drug distribution will be useful for determining antibody concentrations required to reduce drug distribution to brain, which will provide quantitative parameters for effective morphine vaccines when translated to humans. The hypothesis was that <u>heroin and metabolite distribution in brain would be significantly decreased in rats</u> <u>vaccinated with M-KLH compared to controls.</u>

**Specific aim #4 was to study the effect of vaccination with M-KLH on heroin-induced behaviors.** Because M-KLH is a novel morphine-conjugate vaccine it will be important to show that vaccination with M-KLH will block behavioral effects of heroin. The hypothesis was that <u>heroin-induced locomotor activity and anti-nociception would be</u> <u>significantly reduced in rats vaccinated with M-KLH compared to controls.</u>

**Specific aim #5 was to study the effect of combining opioid vaccines on individual vaccine efficacy.** Because there are many opioids available, and because prescription opioid abuse is becoming more prevalent, it is possible that heroin addicts may switch to other opioids if effects of heroin were blocked after vaccination. Combining multiple opioid vaccines may provide greater protection for opioid addicts willing to quit. The

hypothesis was that <u>M-KLH and oxycodone-KLH (OXY-KLH) vaccine efficacy would be</u> <u>retained in animals vaccinated with both immunogens compared with the individual</u> <u>vaccine groups.</u>

**2.      Summary of how Chapters 3, 4, and 5 address these Aims.**

Chapter 3 (Jones et al., 2013) includes data that address Aim 1. Stability of heroin was studied in vitro using a variety of matrices (rat blood, plasma, and brain, human plasma, and bovine serum), conditions (sodium fluoride, temperatures, and centrifuge speeds), and processed at different times. Using the conditions that provided the highest stability, naïve rats were injected with heroin and heroin and metabolite concentrations in blood and brain were measured to support the in vitro data.

Chapter 4 (Raleigh et al., 2013) includes data that addresses Aims 2, 3, and 4. Heroin and metabolite distribution in blood and brain at early time-points after 0.26 mg/kg i.v. administered heroin was studied in non-vaccinated rats to address Aim 2. Effects of vaccination on heroin and metabolite distribution 4 min after 0.26 mg/kg i.v. administered heroin were studied in vaccinated and non-vaccinated rats to address Aim 3. Effects of vaccination on heroin-induced anti-nociception and on heroin-induced locomotor activity in vaccinated and non-vaccinated rats were studied to address Aim 4.

Chapter 5 (Pravetoni et al., 2012b) addresses Aim 5. This study looked at the effects of combining M-KLH and Oxy-KLH on individual vaccine efficacy. Rats were placed into 4 groups and vaccinated with KLH (control), M-KLH, Oxy-KLH, or M-KLH and Oxy-KLH and antibody titers were compared after vaccination. Rats were given equimolar doses of 6-MAM and oxycodone over a 1-min infusion and blood and brain levels were

measured to determine how combining opioid vaccines would affect individual vaccine efficacy.

# CHAPTER 3

**Stability of heroin, 6-monoacetylmorphine, and morphine in biological samples and validation of an LC-MS assay for delayed analyses of pharmacokinetic samples in rats**

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Degradation of heroin to 6-monoacetylmorphine (6-MAM) and then morphine happens rapidly in vivo and in vitro. The rates of heroin and 6-MAM degradation depend on the type of biological samples, and the duration and conditions of storage. In order to optimize conditions for measuring heroin and its metabolites in samples collected for pharmacokinetic studies in rats, we investigated the time course of degradation of heroin, 6-MAM, and morphine in four biological matrices: rat blood, rat brain homogenate, bovine serum, and human plasma under various conditions. Analyte concentrations were measured by LC–MS. The goal was to identify conditions that allow maximum flexibility in scheduling sample collection and analysis, as well as gain more information on the stability of heroin in blood and tissue samples. A solid-phase extraction method with ice-cold solvents, sodium fluoride (NaF) and a low pH (3.0) maintained sample stability. Quality controls were within 94.0–105% of the target value. Variability was 4.0–8.9% for all analytes within the range of 5–200 ng/mL for heroin, 5–1000 ng/mL for 6-MAM, and 10–200 ng/mL for morphine. Heroin degradation to 6-MAM was faster in rat whole blood than in plasma, and faster in rat plasma than in rat brain homogenate. Maintaining NaF at 4 mg/mL throughout processing enhanced stability; higher NaF concentrations added to whole blood caused hemolysis. Samples processed through solid phase extraction and stored as dried pellets at 80 ◦ C constituted the most stable environment for heroin, and was superior to the storing of samples in solution prior to or after extraction. Nevertheless, post-extraction heroin and 6-MAM levels declined by 6.7–8.3% over one week in rat plasma under these conditions, and by <1–4.7% in bovine serum or human plasma.

## 1.    INTRODUCTION

Quantification of heroin in biological specimens can prove challenging due to the rapid deacetylation of heroin (half-life of approximately 5 minutes in plasma) to its active metabolites 6-monoacetylmorphine (6-MAM) and morphine (Nakamura et al., 1975; Garrett and Gurkan, 1979; Lockridge et al., 1980; Barrett et al., 1992).  In humans, heroin degradation is catalyzed by esterases present in blood and tissue (Lockridge et al., 1980; Salmon et al., 1999).  However, esterase activity and concentrations differ between species resulting in differential degradation of opioids across species and biological matrices within species (Minagawa et al., 1995; Liederer et al., 2005; Koitka et al., 2010).  Degradation of heroin *in vitro* is also dependent on pH and temperature (Barrett et al., 1992).  Therefore, measurement of heroin after collection of biological samples is problematic if conditions are not properly controlled.

Analytical methods that include quantification of heroin recognize that heroin and 6-MAM can be unstable in particular matrices and suggest the need to use fresh standards and controls in order to avoid degradation (Zuccaro et al., 1997; Karinen et al., 2009). Conditions that favor heroin stability include use of general esterase inhibitor(s), most commonly NaF, low pH, and low temperature with the use of ice-cold reagents and/or immediate freezing of samples in liquid nitrogen (Barrett et al., 1992; Karinen et al., 2009). While these measures are useful, a wide range of conditions has been studied, and varying degrees of stability reported. One method incorporated the freezing of samples with liquid nitrogen and a protein precipitation protocol followed by analysis by LC-MS/MS (Karinen et al., 2009). The use of liquid nitrogen is not always convenient, and does not appear to prevent significant degradation of heroin in blood samples stored for up to seven days.  Analyzing samples immediately after collection to minimize

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analyte degradation is difficult when scheduling animal or clinical experiments between laboratories, when transportation of samples is required, or in laboratories with shared instrumentation requiring coordination across multiple laboratories and projects. In our study, heroin and 6-MAM stability were investigated under a variety of conditions and an alternative solid phase extraction method was developed that enabled storage of extracted samples collected in several blood matrices (rat plasma, bovine serum, and human plasma) and rat brain homogenate for up to one week, thus enabling flexibility in scheduling of sample analysis and minimizing the degradation of heroin.

## 2. METHODS

**2.1 Chemicals.** Heroin, 6-monoacetylmorphine, morphine, methadone, codeine, hydrocodone, meperidine, and oxycodone and their deuterated analogues used for analysis were purchased from Cerilliant Analytical Reference Standards (Round Rock, TX). Ammonium formate, formic acid 88%, glacial acetic acid, sodium fluoride, methanol (HPLC grade), and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Human plasma was purchased from Biological Specialty Corporation (Colmar, PA) and bovine serum was purchased from Sigma Chemical Company (St. Louis, MO).

**2.2 Preparation of Stock Standards and Quality Controls.** Stock standards (1 mg/mL) of heroin and 6-MAM were stored in acetonitrile. The stock standard for morphine was stored in methanol (at 1 mg/mL). All working solutions were stored in 10 mM formate (pH 3.0): methanol (50/50, v/v) at -20°C. Working solutions for calibration samples and quality controls were prepared independently and made fresh every two weeks. Heroin standards were prepared separately from the other drugs. Due to the limited availability of rat plasma, bovine serum was used for preparing calibration standards and quality control samples. Standard solutions were prepared by addition of 10 µL of working stock to blank bovine serum to obtain final concentrations of 5, 10, 25, 50, 100, and 200 ng/mL for heroin, 5, 10, 25, 50, 100, 200, 500, 1000 ng/mL for 6-MAM, and 10, 25, 50, 100, 150, and 200 ng/mL for morphine. Quality control samples were prepared by addition of 10 µL of separate quality control working stocks to 200 µL of bovine serum. Final concentrations were 5.6, 40.0, and 160 ng/mL for heroin, 5.6, 40, 160, and 400 ng/mL for 6-MAM, and 16.0, 40, and 160 ng/mL for morphine. The internal

standard working solution was prepared at a concentration of 50 ng/mL in 10 mM formate buffer pH 3.0, aliquoted into screw-top cryogenic vials, and stored at -80°C.

## 2.3    Collection of Blood and Brain Tissue Samples from Rats:

*The relevant conditions for each experiment are presented in Table 1 and indicated in the section below.*

**2.3.1    Blood Collection (Experiments A, B, C, D, F, and G, Table 1).** General procedures and the amount of processing time are represented in Fig. 1.  For *in vitro* stability studies, male Holtzman Sprague-Dawley rats (300-324 g) were decapitated after being anesthetized with isofluorane and, within 1 minute, blood was collected into a vial. Blood was drawn into a syringe containing ice-cold NaF and heparin (final concentration 4 mg/mL NaF) in two 4 mL aliquots (total 8 mL) and transferred to a 15 mL polypropylene tube.  Samples were centrifuged at 3100 g on a Beckman J-6B centrifuge for 3 minutes at 4°C.  Plasma (0.5 mL) was transferred to a 5 mL polypropylene tube and diluted 1:1 with ice cold 10 mM formate buffer (pH 3.0, with 4 mg/mL NaF, unless noted elsewhere).

**2.3.2    Preparation of Samples from Dosed Animals (Experiments E and F, Table 1).** Rats were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine both administered i.m. prior to a one minute infusion of 0.52 mg/kg heroin. Rats were sacrificed one minute later and blood was collected following the procedure described above.

**2.3.3    Preparation of Brain Samples (Experiments C, E, and F, Table 1).** After sacrifice and collection of blood from the rat, the brain was removed, rinsed with ice-cold

10 mM formate pH 3.0 containing 4 mg/mL NaF, and placed in pre-weighed vials. Samples were weighed and then four parts (by mass) of ice-cold 10 mM formate buffer pH 3.0, or distilled water, with or without 4 mg/mL NaF was added. Samples were homogenized for 30-40 sec with a Brinkmann homogenizer and placed in a -20°C freezer. Samples were processed within one hour and then extracted. Before extraction, samples were thawed, swirled to mix (to prevent formation of bubbles), and then 400 µL of this matrix were used for extraction.

**2.3.4 Whole Blood Stability Experiment (A, B).** To determine the percent degradation occurring from the time of sacrifice of the rat and separation of the plasma, 1.0 mL of heparinized whole blood (containing 4 mg/mLNaF) from sacrificed rats was immediately added to tubes containing 10 µg of heroin. Tubes were then spun at 1200 g for 10 minutes at 4°C. Plasma was separated and samples were processed. The time from addition of whole blood to centrifugation was approximately two minutes. For plasma samples, 1 mL of rat plasma (containing NaF) was added to a tube containing 10 µg of heroin and processed. Various centrifuge speeds and times were also examined for optimal recovery of heroin. Samples were collected as described above and centrifuged at 1200 g for 3 minutes, 10 minutes, or at 3100 g for 3 minutes.

**2.3.5 Time Course Stability Experiment (Experiments D, E, F, and G, Table 1).** For time course stability experiments, all samples were prepared by addition of the specific analyte (heroin, 6-MAM, or morphine) directly into freshly collected rat brain homogenate or rat plasma, human plasma, or bovine serum for a final concentration of 100 ng/mL. Unless specified elsewhere, samples were diluted with NaF and formate buffer on ice. The time when an analyte was added to a matrix was considered time zero. Samples

were then incubated under different temperature conditions for specified periods of time (up to 30 minutes for short term experiments, and up to one week for long term stability).

**2.4      Sample Extraction.** Solutions and samples were kept on ice throughout the extraction to promote stability of the analytes.  Two hundred microliters of bovine serum were diluted 1:1 with 10 mM formate buffer, pH 3.0, containing 4 mg/mL NaF along with 100 µL of internal standard solution in a 1.5 mL centrifuge tube.  For standards and quality control samples, 10 µL of working stock solution were added to the tubes.  Four hundred microliters of rat plasma: buffer solution were added, along with 100 µL of internal standard solution.  Brain samples were prepared by adding 400 µL of rat brain homogenate to a 1.5 mL centrifuge tube along with 100 µL of internal standard solution.  Brain samples were then centrifuged for 10 min at 9,300 g and the supernatant was used for extraction.  All samples were agitated briefly by a vortex mixer before extraction.

Strata-X-RP 1 mL extraction cartridges (Phenomenex, Torrance, CA) were used for solid phase extraction.  Cartridges were conditioned with 1 mL methanol followed by 1 mL of water.  The matrix solution was then loaded onto cartridges and allowed to flow through the cartridge slowly via centrifugation at no more than 55 g for 30 seconds.  Cartridges were washed with 1 mL of cold 1% methanol in acidified water (acidified with formic acid to pH 3.0).  In a fresh tube, samples were eluted with 500 µL of cold 1% acetic acid in methanol twice.  Both elution volumes were collected in a single tube.  Samples were then dried on a Zymark Nitrogen Drier (Hopkinton, MA) at 17 psi and 27°C.  Samples were stored as the dry solid until reconstituted in 200 µL of 10 mM formic acid buffer pH

3.0, vortexed for 30 seconds, and centrifuged for 10 minutes at 9,300 g.  Sixty microliters of sample were then transferred into autosampler vials and analyzed immediately.

**2.5     LC-MS Conditions and Analysis.** Fifteen microliters of sample were injected onto a reversed phase Agilent (Santa Clara, CA) Zorbax XDB-C18 (2.1 mm X 50 mm i.d., 3.5 µm) column protected by a reversed phase 4 mm X 2 mm C18 guard column (Phenomenex, Torrance, CA).   The LC-MS system consisted of a 2010A Shimadzu (Tokyo, Japan) single-quadrupole LC-MS with a SCL-10Avp controller, dual LC-10ADvp pumps, SIL-10ADvp autoinjector, and DGU 14A degasser.  The samples were kept at ambient temperature during injection.

Gradient elution was performed with a mixture of 10 mM formate buffer pH 3.0 (mobile phase A) and acetonitrile (mobile phase B) as follows: 0-0.5 minutes 7% mobile phase B, 0.5-2.0 minutes 7→20% mobile phase B, 2.0-2.5 minutes 20% mobile phase B, 2.5-4.0 minutes 20→33% mobile phase B, 4.0-4.5 minutes 33→35% mobile phase B, 4.5-4.75 minutes 35→7% mobile phase B, 4.75-5.0 7% mobile phase B.  The flow rate was kept at a constant 0.21 mL/min and the total run time was 5 minutes.  The autosampler needle was washed with 100% methanol following each sample injection.

Ionization was achieved by assisted electrospray in the positive ion mode.  The heat block temperature was set at 200°C and the curved desolvation-line temperature was set at 250°C.  The detector voltage was 2.0 kV.  The nebulizing gas ($N_2$) was set at a flow rate of 1.5 mL/min.  Data acquisition and peak integration were interfaced to a computer workstation running LCSolutions™ (Tokyo, Japan).  LC-MS in the scan mode was used to identify the appropriate ions to monitor heroin, 6-MAM, and morphine.

Select ion monitoring for heroin, 6-MAM, and morphine were done at m/z 370 (heroin-$d_9$ m/z 379), m/z 328 (6-MAM-$d_6$ m/z 334), and m/z 286 (morphine-$d_6$ m/z 292), respectively.

**2.6    Validation and statistics.** Matrix effects were assessed in rat plasma, rat brain homogenate, bovine serum and human plasma.  The matrix effect is found to cause ion enhancement or suppression due to interfering compounds in the matrix that do not appear in blank matrix samples (Matuszewski et al., 2003; Srinivas, 2009).  Samples were analyzed in triplicate on three different days.  The matrix effect was determined by comparing spiked standard peak areas to unextracted standard peak areas and following the procedure of Matuszewski et. al (2003).

Peak area ratios (analyte/internal standard) were used for determination of concentration from extracted matrix.  The limit of quantitation (LOQ) was determined to be the lowest standard on the calibration curve that could be reliably measured ($\leq$ 20% coefficient of variation).  The limit of detection (LOD) had a signal to noise ratio of 5.  Validation of the assay was performed by preparing standard curves in triplicate (within-run) for each day on five different days (between run, N=15).  Quality control samples were prepared in blank bovine serum and were also run in triplicate.  The assay was validated before subsequent stability experiments were conducted. The percentage coefficient of variation (CV) was calculated from the average standard deviation of quality control samples at each level (N=15).  The estimated variability was determined from calculated concentrations of multiple triplicate weighted ($1/x^2$) standard curves.  Accuracy was calculated by dividing the grand mean by the target concentration, and multiplying by 100.

## 3.    RESULTS

**3.1    Assay validation.** The matrix effect was found to cause ion enhancement in all matrices for the analytes.  After correction for the matrix effect, the average extraction recovery for the analytes was found to be within ±20% of the target value.

Precision and accuracy for each analyte's quality controls are shown in Table 2.  The LOQ of the assay was determined to be 5 ng/mL for both heroin and 6-MAM and 10 ng/mL for morphine in all matrices.  The LOD was 2.5 ng/mL for heroin and 6-MAM, and 5 ng/mL for morphine in each matrix.  The within-day variability was found to be between 2.2% and 8.8%.  The between-day variability was found to be between 1.9% and 6.9%. This LC-MS assay is both accurate and precise with an LOQ of 5 ng/mL. Selectivity of the assay was confirmed by injection of diluted standards of other common opioids (methadone, codeine, hydrocodone, meperidine, and oxycodone). It was found that no interference occurred.

Fig. 2 shows a sample chromatogram of analytes and internal standards in rat plasma. Under the described chromatographic conditions, the retention times were 3.99 minutes (heroin), 3.00 minutes (6-MAM), and 1.27 minutes (morphine).  The retention times of the deuterated internal standards were 3.98 minutes (heroin-$d_9$), 3.02 minutes (6-MAM-$d_6$), and 1.26 minutes (morphine-$d_6$).

3.2    **Whole blood stability (Experiment A and B, Table 1).** Heroin levels decreased by 56.4% after being spiked into fresh rat whole blood with NaF present (Fig. 3) whereas, heroin concentrations decreased by only 5.9% in rat plasma containing NaF. 6-MAM concentrations increased concurrently with the decrease in heroin as a result of

esterase activity.  Whole blood and plasma samples to which 6-MAM had been added were also compared.  No significant difference was seen in 6-MAM concentrations between the two matrices (data not shown) and morphine was not detected. To investigate further the stability of heroin during sample processing, samples were centrifuged under varying conditions.  Samples centrifuged at 1200 g, for 3 minutes showed a 5.9 ± 5.3% increase in recovery compared to samples spun for 10 minutes at the same speed (data not shown).  Samples spun at 3100 g for 3 minutes allowed for a 12 ± 4.5% increase in recovery of heroin compared to 10 minutes at 1200 g.

**3.3     Live rat infusion (Experiment C).** Heroin, 6-MAM, and morphine concentrations were measured in rats dosed with 0.52 mg/kg heroin one minute before sacrifice (Fig. 4). Extracted concentrations of all analytes were within the range of the curve. Brain measurements were converted to ng/g. It was found that 6-MAM levels were highest in both plasma and brain, however, brain concentrations of all three analytes was more than 4 fold higher than plasma.

**3.4     Short term stability results (Experiments D and E, Table 1).** A ten-minute time course of heroin concentrations in rat plasma shows complete degradation of heroin in approximately two minutes when NaF is not present, whereas samples containing 4 mg/mL NaF showed a heroin degradation of 4.2% ten minutes after the first data point (data not shown).   When whole blood was collected with higher concentrations of NaF (9.6 or 20 mg/ml), hemolysis of the sample occurred and more heroin degradation was observed than with 4 mg/mL NaF (data not shown).

Rat brain homogenate (in formate) samples showed a slower degradation of heroin than seen in rat plasma. A 30-minute time course with 4 mg/mL NaF added to brain homogenate samples resulted in stable heroin concentrations whereas the absence of NaF resulted in a decline of heroin concentration in rat brain homogenate (Fig. 5, shown up to 10 minutes). A decline of 35% in final heroin concentrations in the samples containing formate buffer with no NaF was observed. Samples with NaF showed a decrease of only 5.3%. Brain samples homogenized with water containing NaF were equally stable with or without formate buffer. When comparing brain samples homogenized without NaF, it was found that addition of pH 3.0 formate buffer resulted in greater heroin stability compared to water (35% degradation compared to 87%). The instability of heroin in samples without NaF was confirmed by a concomitant increase of 6-MAM observed in samples run under the same conditions for 10 minutes.

**3.5    Storage Condition Optimization (Experiments F and G, Table 1).** The stability of heroin and 6-MAM in frozen plasma was examined by the addition of NaF to the 10 mM formate buffer (pH 3.0) used to dilute the plasma samples before storage at -80°C. Heroin degraded less over the 24-hour storage period when NaF was added with the formate buffer compared to formate buffer alone (13% versus 39% degradation) (data not shown). NaF similarly enhanced the stability of 6-MAM in frozen plasma. Most of the heroin degradation occurred during the freezing process (within the first three hours) at -80°C with no further degradation noted up to 24 hours. Samples spiked with 100 ng/mL of 6-MAM showed minimal degradation regardless of when NaF was added to the sample (4.1% versus 7.6%). Brain homogenate samples stored under similar conditions showed no degradation of any compounds. Post-extraction stability was assessed by storage of samples at -80°C as dried pellets. Heroin and 6-MAM samples remained

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within 10% of targeted concentrations in bovine serum and human plasma and within 20% in rat plasma when stored for up to one week, and within 8% of the first data point (5 minutes after addition of analyte). Morphine was stable in all three matrices for up to one week with minimal degradation (data not shown). Post-collection stability was also assessed by dosing of rats with heroin before sacrifice. Fig. 6 shows a time course comparison of samples stored in the 50:50 plasma:buffer solution (a) and in the pellet form after extraction (b) stabilities of these samples. Morphine levels are seen to increase throughout the time course in the samples stored pre-extraction whereas the samples stored as dried pellets show less variability.

## 4.    DISCUSSION

Heroin is susceptible to degradation at several points during sample processing.  Initial degradation can happen during the time necessary for separation of the plasma from the erythrocytes due to esterases present in erythrocyte membranes or in plasma. Degradation can also occur during the extraction process.  Optimization of all methods used in sample collection, processing, and analysis are critical in order to minimize degradation of this compound and to avoid underestimation of heroin in biological specimens.  When separating plasma from red blood cells the use of high centrifuge speeds for even a short amount of time (3 minutes) still allows for up to 40-50% degradation of heroin to occur.  Significant degradation of heroin also takes place in post-separation conditions during sample extraction and while in storage before analysis.  Differences in the time course of heroin degradation were seen across matrices with rat brain being the most stable, followed by bovine serum and human plasma, and rat plasma being the least stable.  Optimization of pH, NaF concentration, and temperature of collected samples for storage alone was found to be insufficient for stability of heroin in bovine serum, rat brain homogenate, and rat plasma.  Optimal stability for at least one week before analysis was attained by storing samples as post-extracted pellets at -80°C.

Stability of heroin in rat whole blood compared to rat plasma showed that plasma is a more stable environment for heroin than whole blood.  This supports previous studies that show various esterase-catalyzed compounds to be less stable in whole blood than in plasma (Minagawa et al., 1995; Skopp et al., 2001) and also supports the use of quantitative assays that use plasma instead of whole blood. Morphine was not observed in any of the samples incubated with heroin alone.  The absence of morphine could be

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attributed to the slower rate of degradation of 6-MAM into morphine, and the short amount of time 6-MAM was in whole blood (centrifugation occurred within 2 minutes of collection). In human blood, it was found that esterases catalyzing heroin act much more rapidly than esterases involved in 6-MAM degradation (Salmon et al., 1999).

It is normal practice to collect samples containing heroin into tubes containing NaF as a general esterase inhibitor (Rentsch et al., 2001; Rook et al., 2005; Karinen et al., 2009). When working with tissue samples, NaF can be diluted when buffers are added to the sample. It was found that keeping the NaF concentration constant by directly adding NaF to the 10 mM formate buffer (pH 3.0) aided in the stabilization of heroin.

Rat brain homogenate was a more stable matrix for heroin samples than the blood matrices. This could be due to greater esterase activity in blood matrices than in brain and the difference in the type of esterases present in brain (Salmon et al., 1999). These results are supported by the findings in the mouse by Karinen et. al (2009) that show brain samples are a more stable matrix than acidified whole blood samples for heroin.

Sample storage in solution, even when optimized for NaF, pH, and temperature conditions was not possible for longer than 24 h without significant degradation of heroin (13% degradation after 24 h). When it is difficult to coordinate schedules for instrument time or transport samples between laboratories, sample stability is essential. In order to find a stable stopping point in the sample preparation procedure, we examined the stability of heroin in samples that were extracted immediately after sample collection and stored in a dried pellet form. These analyte samples were more stable than when stored in dilute pre-extraction solutions containing NaF. Morphine showed minimal degradation

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when stored as dried a pellet.  Heroin and 6-MAM degradation occurred, < 1% for both heroin and 6-MAM at 24 h and 6.7% - 8.3% at 1 week.  Previous results have compared heroin degradation over a period of 3 hours to one week of storage and have shown a 50% degradation (Karinen et al., 2009).  Our results show a degradation of less than 1.5% between 3 h and 1 week after extraction for heroin.

Faster degradation rates (lower recoveries) of heroin were observed in rat plasma compared to bovine serum or human plasma and seem to occur primarily during the time between addition of plasma to NaF and dilution with formate buffer.  All rat plasma samples had a lower heroin level at the earliest time of measurement, perhaps due to greater esterase activity in rat plasma compared to other matrices (Minagawa et al., 1995; Welch et al., 1995; Liederer et al., 2005; Koitka et al., 2010) as well as the rapid half-life of heroin (approximately 5 minutes in human plasma) (Nakamura et al., 1975).

## 5.    CONCLUSION

Optimization of assay conditions that result in minimal degradation of heroin is imperative for pharmacokinetic studies involving quantification of heroin from blood. Conditions that resulted in the highest level of heroin recovery in blood were 1) adding 4 mg/ml sodium fluoride to blood immediately following tissue collection, 2) centrifugation of blood for 3 minutes at 3,100 g, 3) mixing plasma 1:1 with formate buffer containing 4 mg/ml sodium fluoride, and 4) drying samples down to a pellet post-extraction. This resulted in 68% recovery of heroin in heroin-spiked rat blood. In rat brain tissues, mixing brain tissues with 1:1 formate buffer containing 4 mg/ml sodium fluoride prior to homogenization resulted in a full recovery of heroin in heroin-spiked rat brain tissue. All samples and buffers were kept at ice-cold temperatures.

It is of particular importance to examine stability of samples and to identify points within the methodology that can provide the most stable environment for sample transport between laboratories.  In addition, knowledge of the extent and time course of heroin degradation is important when translating results from pharmacokinetic studies. Systematic evaluation of various techniques that are known to decrease heroin degradation were incorporated into this assay in order to provide a method that is sensitive, reliable, and allows for sample storage up to one week before analysis by LC-MS.

**TABLES AND FIGURES**

**TABLES**

**Table 1.** Summary of stability experiments.

| Experiment | Matrix | NaF | Temp | Duration | Fig |
|---|---|---|---|---|---|
| **A** Whole Blood vs Plasma | Rat Whole Blood | Collected with NaF | 4 °C | Processed immediately | 3 |
| | Rat Plasma | 4 mg/mL | | | |
| **B** Centrifuge Speeds | Rat Whole Blood | Collected with NaF | 4 °C | Processed immediately | - |
| **C** NaF comparison | Rat Plasma | 4 mg/mL NaF Added in half samples | 4 °C | 10:00 min | 4 |
| **D** Heroin Degradation in Brain | Rat Brain Homogenate (in water) | 4 mg/mL | 4 °C | 30:00 min | - |
| | Rat Brain Homogenate (in water) | | | | |
| | Rat Brain Homogenate (in formate) | 4 mg/mL | | | |
| | Rat Brain Homogenate (in formate) | | | | |
| **E** 50:50 Storage | Rat Dosed with heroin | 4 mg/mL in formate and plasma | (-)80 °C | 24 hr | 5 |
| | Rat Plasma | 4 mg/mL in formate and plasma | | | |
| | Rat Plasma | 4 mg/mL in plasma | | | |
| | Rat Brain Homogenate (in formate) | 4 mg/mL | | | - |
| **F** Dried Pellet | Rat Dosed with heroin | 4 mg/mL in formate and plasma | (-)80 °C | 7 days | 6 |
| | Human Plasma | 4 mg/mL | | | |
| | Bovine Serum | 4 mg/mL | | | - |
| | Rat Plasma | 4 mg/mL | | | |

**Table 2.** Validation results for quality control samples (N = 15).

| Analyte | Concentration (ng/mL) | $CV_t$ (%) | Accuracy (%) |
|---------|----------------------|-----------|--------------|
| Heroin | 5.6 | 5.70 | 104.9 |
| | 40 | 7.71 | 100.3 |
| | 160 | 6.69 | 100.2 |
| 6-MAM | 5.6 | 7.98 | 101.0 |
| | 40 | 6.51 | 102.0 |
| | 160 | 4.06 | 94.9 |
| | 400 | 8.88[a] | 96.3 |
| Morphine | 16 | 8.59 | 94.6 |
| | 40 | 6.13 | 97.8 |
| | 160 | 7.71 | 98.2 |

[a]N = 8

**FIGURES**

**Figure 1.** Flowchart describing general method for collecting samples from rats. (I) Point in time where samples were spiked for whole blood experiments (A and B, Table 1). (II) Point in time where samples were spiked for stability experiments (C, E, and F, Table 1). (III) Point in time where samples were spiked for brain homogenate experiments (D and E, Table 1).



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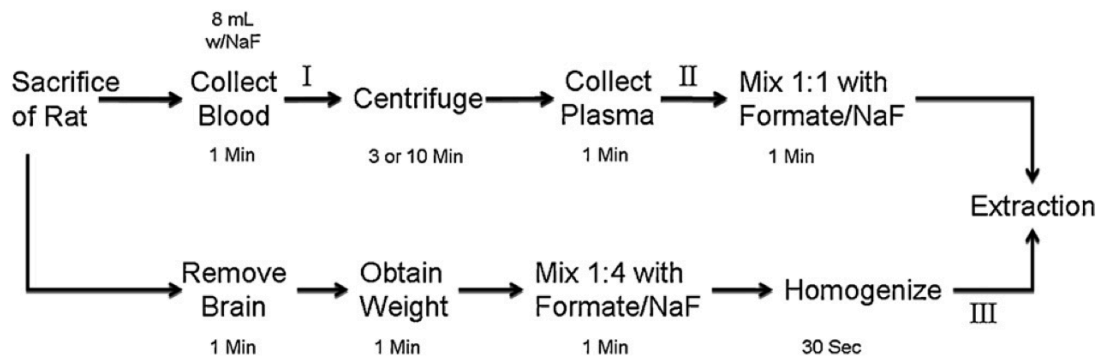
**FIGURE 2.** LC/MS chromatogram of heroin, 6-MAM, and morphine with internal standards. 80 ng/mL of each analyte was spiked into rat plasma before extraction and analysis.
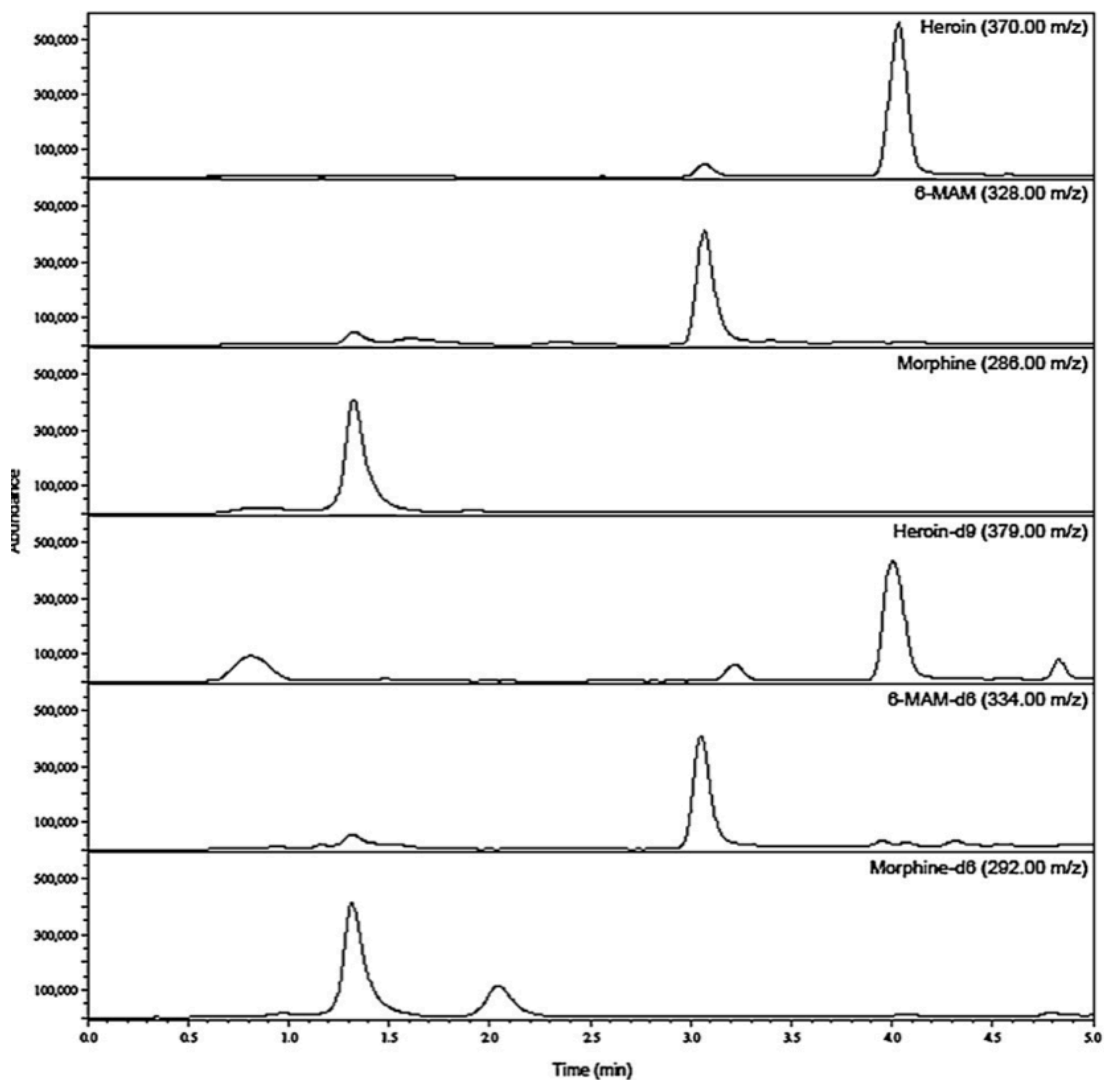
**Figure 3.** Experiment A. Mean ± SD, n = 3. Measured concentrations of heroin and 6-MAM in rat whole blood and plasma (containing NaF) after the addition of 10 µg/mL of heroin. Heroin degraded 56.4% in whole blood compared to 5.9% degradation in plasma.
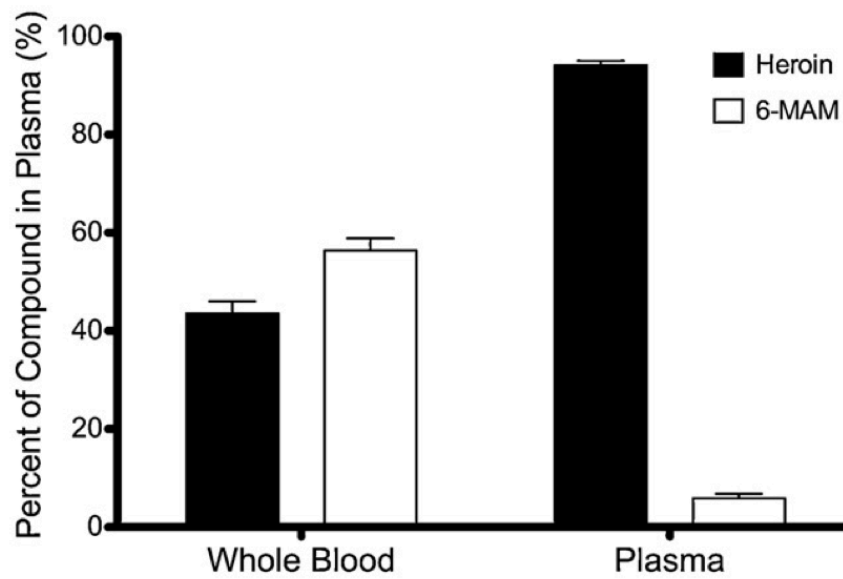
**Figure 4.** Experiment C. Mean ± SD (n = 6). Measured concentrations of heroin, 6-MAM, and morphine in plasma and brain from rats infused over the course of 1 min with 0.52 mg/kg heroin. Rats were sacrificed 1 min after infusion. Samples were collected with 4 mg/mL NaF, diluted with formate buffer containing 4 mg/mL NaF, and analyzed immediately after extraction.
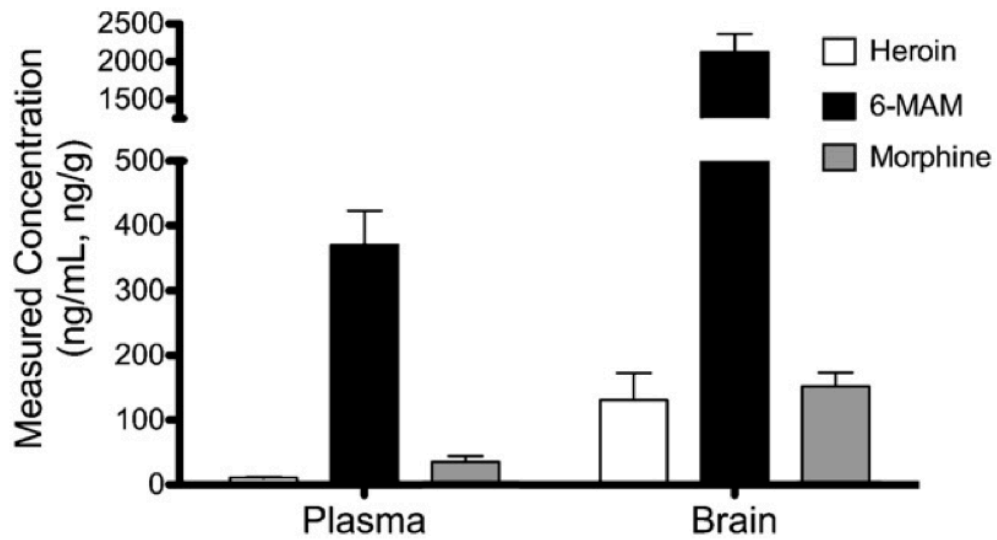
**Figure 5.** Experiment E. Ten-minute time course of heroin degradation after spiking of 100 ng/mL heroin into rat brain homogenate (brain homogenized with formate containing NaF (▲) or without NaF (○, run in duplicate) and brain homogenized with water containing NaF (■), or without NaF (□). For samples homogenized with formate buffer and no NaF, corresponding 6-MAM levels are observed (●, run in duplicate). Samples were stored on ice during experiment.
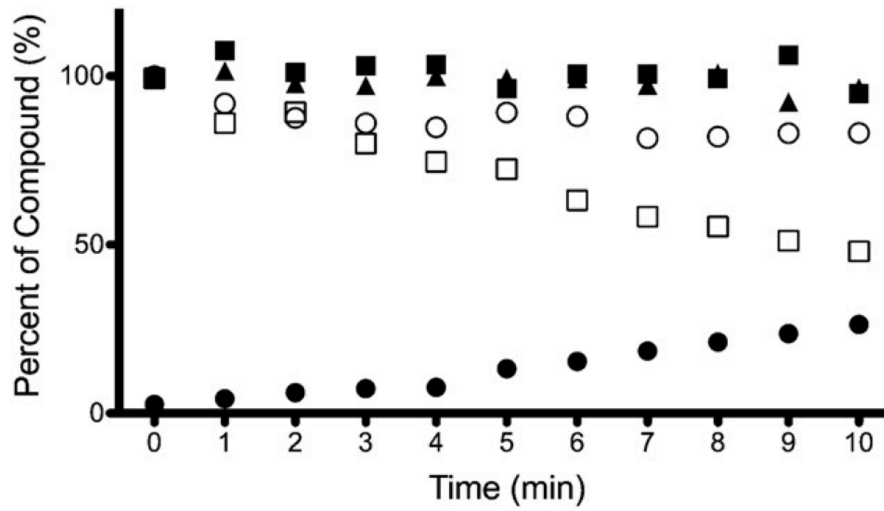
**Figure 6.** Experiments F and G. Mean ± SD (n=6). Comparison of heroin (△), 6-MAM (■), and morphine (●) stability in plasma post-collection from heroin dosed rats. Samples were stored either as pre-extracted plasma diluted with 10 mM formate buffer containing NaF (a) or in the post-extraction pellet state (b) at -80 °C for up to one week. Fluctuation of concentrations is greater in samples stored in the dilute plasma compared to post-extraction pellet samples (24% fluctuation compared to 16%).
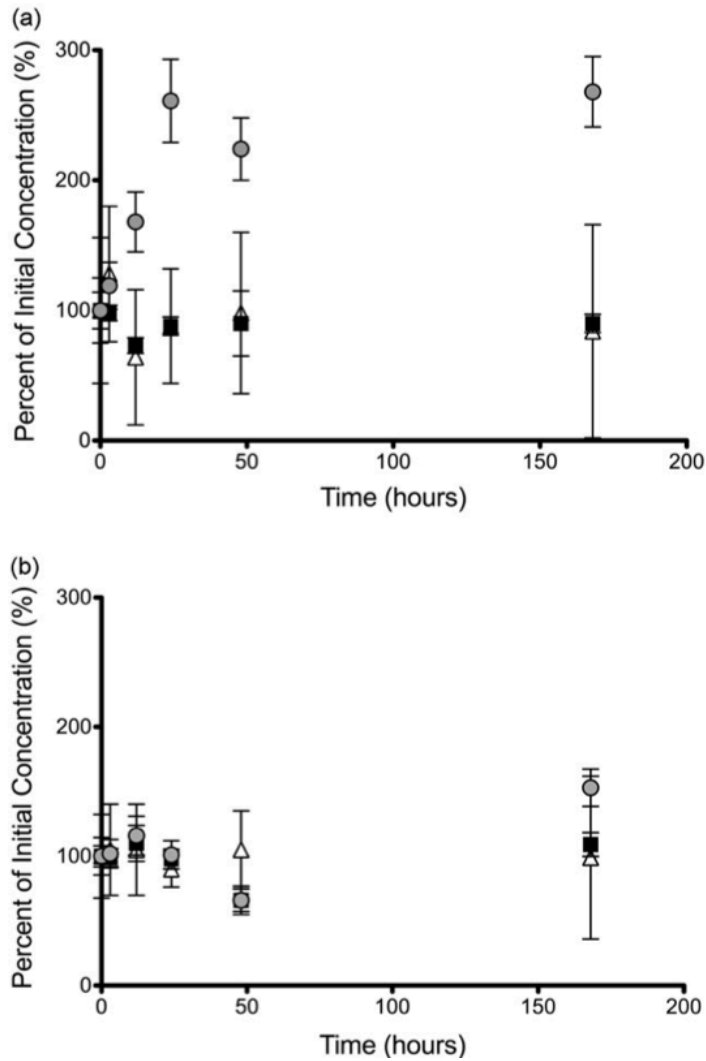
# Chapter 4


**Selective effects of a morphine conjugate vaccine on heroin and metabolite distribution and heroin-induced behaviors in rats**

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Morphine conjugate vaccines have effectively reduced behavioral effects of heroin in rodents and primates. To better understand how these effects are mediated, heroin and metabolite distribution studies were performed in rats in the presence and absence of vaccination. In non-vaccinated rats 6-monoacetylmorphine (6-MAM) was the predominant opioid in plasma and brain as early as 1 minute after i.v. administration of heroin and for up to 14 minutes. Vaccination with morphine conjugated to keyhole limpet hemocyanin (M-KLH) elicited high titers and concentrations of antibodies with high affinity for heroin, 6-MAM, and morphine. Four minutes after heroin administration vaccinated rats showed substantial retention of all three opioids in plasma compared to controls, and reduced 6-MAM and morphine, but not heroin, distribution to brain. Administration of 6-MAM rather than heroin in M-KLH vaccinated rats showed a similar drug distribution pattern. Vaccination reduced heroin-induced analgesia and blocked heroin-induced locomotor activity throughout 2 weeks of repeated testing. Higher serum opioid-specific antibody concentrations were associated with higher plasma opioid concentrations, lower brain 6-MAM and morphine concentrations, and lower heroin-induced locomotor activity. Serum antibody concentrations over 0.2 mg/ml were associated with substantial effects on these measures. These data support a critical role for 6-MAM in mediating the early effects of i.v. heroin and suggest that reducing 6-MAM concentration in brain is essential to the efficacy of morphine conjugate vaccines.

## 1. INTRODUCTION

An estimated 13 to 22 million people worldwide abuse opioids (UNODC, 2010). Intravenous heroin use is associated with crime, social disruption, and transmission of blood-borne pathogens such as HIV and Hepatitis C (Tang et al., 2006). Pharmacological treatments available for opioid addicts include opioid agonists (methadone and buprenorphine) and opioid antagonists (naltrexone), which bind to opioid receptors to either mimic or block the effects of heroin. Although these medications are safe, effective, and decrease the risk of HIV transmission (Fiellin et al., 2006), less than 20% of opioid addicts in the U.S. are treated with these medications and even fewer in many other countries (Tang and Hao, 2007; Mendelson et al., 2008; Krupitsky et al., 2010; Lobmaier et al., 2010). Compliance with antagonist use is low (O'Malley et al., 2000) and agonists have a high abuse potential, risk of diversion, and relatively short duration of efficacy limiting their appeal and necessitating tight regulation. These issues suggest that longer acting treatments that do not elicit their own pharmacological effects could benefit addicts unwilling to use currently available treatments.

Opioid vaccines are being studied as an alternative or complementary treatment for heroin addiction because they are long acting, highly selective, and have few side effects. Although opioids are too small to be recognized by the immune system, they can be rendered immunogenic by conjugating them to foreign carrier proteins. Vaccination with heroin or morphine immunogens can elicit a robust immune response and reduce heroin self-administration and other opioid-related behavioral effects in animals (Bonese et al., 1974; Anton and Leff, 2006; Li et al., 2011; Stowe et al., 2011). These effects are presumably due to the binding of opioids by drug-specific antibodies in blood and

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reduction of opioid distribution to brain (Pravetoni et al., 2012b). Opioid vaccines have only been studied in animals but vaccines for cocaine and nicotine have reached phase II-III clinical trials and have shown efficacy in subjects with high antibody responses (Martell et al., 2005; Haney et al., 2010; Hatsukami et al., 2011).

The potential use of morphine vaccines presents a number of challenges. Nicotine and cocaine vaccines have simpler targets than morphine conjugate vaccines because only the parent drug is active. In contrast, heroin is sequentially metabolized both peripherally and in the central nervous system (Fig. 1A) as: *heroin → 6-monoacetylmorphine (6-MAM) → morphine → morphine-6-glucuronide* (M-6-G, Antonilli et al., 2005), which are all active in humans. Heroin enters brain but is presumed to be a pro-drug because it is rapidly metabolized, has considerably lower affinity for opioid receptors than 6-MAM or morphine (Inturrisi et al., 1983), and is generally found at low concentrations in brain (Andersen et al., 2009). 6-MAM is more likely to be the primary mediator of heroin's early behavioral effects because it is found at high levels in plasma and brain after heroin administration in mice (Way et al., 1960; Andersen et al., 2009) and administration of equimolar heroin or 6-MAM doses results in similar behavioral effects (Andersen et al., 2009). The contributions of morphine and M-6-G to the early effects of heroin are assumed to be smaller due to their lower levels and slower accumulation in brain (Andersen et al., 2009). With regard to the mechanism of action of an opioid vaccine, it is unclear whether the antibodies it generates must bind heroin, its downstream metabolites, or both to prevent opioid distribution from plasma to brain and reduce heroin's behavioral effects.

Heroin and metabolite distribution after i.v. heroin administration has not been well characterized in non-vaccinated rodents. Distribution studies have primarily focused on the s.c. route (Umans and Inturrisi, 1982; Pacifici et al., 2000; Andersen et al., 2009), which may subject heroin to increased peripheral degradation and slower distribution to brain compared to the i.v. route used by humans (Klous et al., 2005). Heroin distribution after i.v. administration has been studied but an extremely high dose was used to compensate for less sensitive analytical methods and these studies were performed in mice (Way et al., 1960). The use of rats could allow a wider range of pharmacokinetic and behavioral studies to be performed.

In the current study heroin and metabolite distribution was studied after i.v. dosing in both non-vaccinated and vaccinated rats to determine the relative levels of heroin and metabolites in plasma and brain. Effects of vaccination on opioid-induced behaviors were also examined. Results support a predominant role for 6-MAM in mediating heroin effects and the importance of binding of 6-MAM by vaccine-generated antibodies in reducing those effects.

## 2.    MATERIALS AND METHODS

**2.1    Reagents.** [Leu$^5$]-enkephalin was purchased from Tocris Biosciences (Ellisville, MO). All other drugs were obtained through the NIH NIDA Drug Supply Program or Sigma Chemical Co. (St. Louis, MO). Drug doses and concentrations are expressed as the weight of the base.

**2.2    Synthesis of the M-KLH vaccine.** Morphine hapten was synthesized as previously reported (Pravetoni et al., 2012b). Briefly, the C3 position of morphine sulfate was protected with di-tert-butyl carbonate and a tetraglycine $(Gly)_4$ linker was attached at the C6 to create the hapten $M(Gly)_4$ (Fig. 1B). $M(Gly)_4$ was conjugated to bovine serum albumin (BSA) for use as a coating antigen for ELISA and to keyhole limpet hemocyanin (KLH) for immunization of rats (Pravetoni et al., 2012a; Pravetoni et al., 2012b).

**2.3    Vaccinations.** Animal protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee. Male Holtzman rats (Harlan Laboratories, Madison, WI) weighing 350 grams at arrival were single housed under a 12/12 hr standard light/dark cycle. All testing occurred during the light (inactive) phase. Rats were immunized with M-KLH or unconjugated KLH at an immunogen dose of 25-100 μg. Immunogen was injected in a volume of 0.4 ml using either Freund's adjuvant i.p. or 2.5 mg/ml alum s.c. (Alhydrogel Brenntag Biosector) (Pravetoni et al., 2011b). Freund's complete adjuvant (EMD Millipore, Billerica, MA) was used for the first immunization while Freund's incomplete adjuvant (Sigma) was used for subsequent vaccine boosts. For initial experiments on heroin and 6-MAM distribution, rats were immunized on days 0, 21, 42, 64, and 86.  Because titers were maximal after 3 vaccinations (Fig. 4A), subsequent behavioral experiments omitted the last two vaccine

doses. Blood was obtained one week after the final vaccination for antibody characterization.

**2.4    Antibody characterization.** ELISA plates were coated with 5 ng/well M-BSA in carbonate buffer at 9.6 pH and blocked with 1% gelatin. Goat anti-rat and anti-mouse antibodies conjugated to horseradish peroxidase were used as secondary antibodies. Antibody specificity was determined by competitive binding ELISA to calculate $IC_{50}$ values. Morphine-specific antibody concentrations were measured as previously described (Pravetoni et al., 2012a; Pravetoni et al., 2012b) using mouse monoclonal anti-morphine IgG (Qed Biosciences, San Diego, CA).

**2.5    Drug level analysis.** Plasma and brain heroin, 6-MAM, and morphine concentrations were measured by LC/MS within three days of extraction under conditions that minimized their degradation (Jones et al., 2013). The assay measured total (bound and unbound) drug. M-6-G was not measured because it is not appreciably formed in rats (Antonilli et al., 2005). Morphine-3-glucuronide was not measured because it lacks reinforcing effects (Vindenes et al., 2009) and appreciable levels were not expected in plasma and brain shortly after heroin or 6-MAM administration. Briefly, 8 ml of trunk blood was collected in a syringe containing 4 mg/ml of ice-cold NaF and heparin (100 IU/ml) and centrifuged immediately at 2400 RPM for 10 min at +4°C. Plasma was transferred to a 5 ml vial and diluted 1:1 with ice-cold 10mM formate buffer (pH 3.0) prior to extraction.  Brains were rinsed with 10mM formate buffer pH 3.0, and placed in pre-weighed vials; 4 parts (by weight) of 10 mM formate buffer pH 3.0 was added to each sample. Samples were homogenized for 30-40 sec and stored at -20°C until extraction. Plasma and brain drug concentrations that were below the limit of

quantitation (5 ng/ml for heroin and 6-MAM and 10 ng/ml for morphine) were estimated when above the detection limit and are indicated as such.

**2.6     Protein binding.** The protein binding of heroin in serum could not be measured accurately because of its rapid degradation. Instead, morphine 2 µg was added to 1 ml of drug-free serum from M-KLH vaccinated and KLH control rats. Equilibrium dialysis of the spiked serum was carried out in Sorenson's buffer at pH 7.35 for 4 hr at 37°C using 1 ml Teflon cells.  Protein binding determined in this manner represents binding to all serum proteins including opioid-specific IgG. The percent of unbound morphine was calculated as the buffer morphine concentration (unbound drug) divided by the total concentration of morphine on the serum side.

The percent unbound morphine calculated in this manner was then used to estimate the unbound plasma morphine concentrations in experimental animals that had received heroin.   This was estimated from the product of the measured plasma morphine concentrations in the rats that had received heroin and the mean percent unbound morphine from spiked ex-vivo samples.

**2.7     Stoichiometric relationships.** The total number of moles/kg of morphine-specific IgG in rats vaccinated with M-KLH was calculated as the product of the reported IgG volume of distribution (131 ml/kg) in rats and the plasma antibody concentration and converted to moles/kg using a molecular weight of 150 kDa for IgG (Bazin-Redureau et al., 1997; Pravetoni et al., 2012b). The corresponding number of drug-binding sites of IgG was twice that number because there are 2 binding sites per IgG.

**2.8     Experimental design overview.** Heroin and metabolite distribution was studied in non-vaccinated rats after i.v. infusion of heroin to assess the early time-course of opioid concentrations in plasma and brain. Opioid distribution after i.v. infusion of heroin or 6-MAM was then studied in vaccinated rats to determine the effect of vaccination on plasma and brain opioid levels. Finally, drug-induced anti-nociception and locomotor activity were studied in vaccinated rats to determine if vaccination could block behavioral effects of targeted opioids.

**2.9     Distribution of drug in non-vaccinated rats.** Rats (n = 12) were anesthetized with ketamine/xylazine i.m. and an indwelling catheter was placed in the right external jugular vein. Distribution of heroin and its metabolites in plasma and to brain was studied after a 1 min i.v. infusion of 0.703 µmol/kg (0.26 mg/kg) heroin, dissolved in 2% (v/v) dimethyl sulfoxide (DMSO) in physiological saline immediately prior to infusion. Rats were decapitated for collection of trunk blood and brain 1, 4, and 14 min (n = 4 per time point) after heroin infusion. This heroin dose was chosen because it is within the range of reinforcing doses in heroin-dependent individuals (Comer et al., 1999), the resulting 6-MAM concentrations in plasma were similar to those found in humans receiving comparable heroin doses (Comer et al., 1999), and because it did not suppress respiration in anesthetized rats.

**2.10    Effect of vaccination on drug distribution.** The effect of immunization with M-KLH was compared to that of KLH control vaccine. Rats (n = 6 per group) were anesthetized one week after the 5th vaccination and 0.703 µmol/kg heroin or 6-MAM was infused i.v. as described above. Blood was withdrawn from catheters for antibody

characterization prior to drug administration. Rats were decapitated for collection of trunk blood and brain 4 min after drug infusion.

**2.11    Behavioral studies.** Rats were vaccinated with M-KLH (n = 14) or KLH (n = 8) 3 times (days 0, 21, and 42) and a series of behavioral studies was performed (See Fig. 2 for timeline). Two subsequent vaccine boosts were given on days 118 and 168 to maintain antibody levels. The thermal nociceptive tests were performed on days 49 and 50 (heroin and methadone), days 56 and 57 (heroin and oxycodone), and day 203 (nicotine). Locomotor activity was tested on days 75 – 93 (heroin), 180 – 184 (cocaine), and 194 – 198 (repeat heroin challenge). Blood collection for antibody characterization was performed on days 49, 99, and 189, when rats had not received opioids recently. Fentanyl anti-nociception was tested in a separate group of rats (n = 10).

**2.12    Effect of vaccination on drug-induced anti-nociception.** Two weeks after the 3$^{rd}$ vaccination, rats were habituated for two hours to the testing environment and nociception measured on a hot plate (Columbus Instruments, Columbus, OH) set to a temperature of 54 ± 0.2º C. A nociceptive response of hindpaw lick or jumping was measured as the latency to respond. Baseline hot-plate responses were obtained 2 hours prior to drug dosing. A maximum cutoff of 60 seconds was used to avoid tissue damage. Percent maximum possible effect was calculated as (postdrug latency – predrug latency) / (maximum latency – predrug latency) x 100.

Hot plate response latency was measured 30 min after s.c. injection of heroin (1 mg/kg), methadone (2.25 mg/kg), oxycodone (2.25 mg/kg), or fentanyl (0.05 mg/kg). Rats were tested using a crossover design such that half of the animals in the M-KLH and KLH

control groups received heroin on the first day and methadone, oxycodone, or fentanyl on the second day, and half received these drugs in the reverse order. These opioid doses were chosen because they elicited near-maximal effects in pilot studies. Rats were tested with nicotine to study vaccine specificity towards opioids. Habituation and pre-test procedures used for nicotine anti-nociception were the same as for the opioid studies except that rats were placed on the hot-plate (52 ± 0.2º C) 5 min after an injection of 0.35 mg/kg nicotine s.c. (McCallum et al., 1999). Because vaccine blocked heroin-induced locomotor activity the previous week, a crossover design was not used with nicotine anti-nociception.

**2.13    Effect of vaccination on locomotor activity.** Baseline locomotor activity was obtained on three consecutive days (days -3 through -1) by administering 1 ml/kg saline s.c. to M-KLH vaccinated and KLH control rats and placing them in open field activity chambers for 90 min immediately following injection (Roiko et al., 2008). The final saline day was used as baseline locomotor activity. On days 1, 2, 5, 7, 9, 12, 14, and 16 rats received 0.25 mg/kg heroin s.c. (Swerdlow et al., 1985) and were tested for activity for 90 min (Li et al., 2011). To confirm specificity of the assay for opioids, rats received 15 mg/kg cocaine i.p. This cocaine dose was used because it induces locomotor activity that can be blocked by immunization with a cocaine vaccine (Carrera et al., 2001). Two weeks later a final heroin challenge of 0.25 mg/kg heroin s.c. was administered to confirm that vaccination blockade of this response had been maintained. Baseline locomotor activity was re-established prior to cocaine and the final heroin challenge.

**2.14    Statistical analysis.** Effects of vaccination on drug distribution and thermal nociception between M-KLH and KLH control groups, as well as effect of drug

administration order on %MPE using the crossover design, were analyzed using unpaired $t$ tests. Differences in antibody titers and concentrations between groups were compared using 1-way ANOVA. Relationships between antibody levels, opioid distribution, and locomotor activity were analyzed by linear regression. The effect of treatment on heroin-induced locomotor activity (measured as total horizontal distance traveled in meters) was analyzed by 2-way ANOVA with group as a between-subjects factor and day as a within-subject factor, followed by Bonferroni post hoc tests. When group variances were unequal, $t$ tests were performed on log-transformed data.

## 3. RESULTS

**3.1 Heroin time-course and distribution in non-vaccinated rats.** After i.v. infusion of heroin, 6-MAM was the predominant analyte at 1 and 4 min in plasma (Fig. 3A) and at all measured times in brain (Fig. 3B). Heroin concentration in plasma and brain was measurable at 1 minute, but was below the limit of quantitation (5 ng/ml) at 4 min, and below the limit of detection at 14 min. 6-MAM concentration in plasma and brain was highest at 1 minute. Morphine concentration in plasma was highest at 4 min. Morphine concentration in brain increased over time, then decreased.

**3.2 Serum antibody characterization.** Immunization with M-KLH either i.p. in Freund's adjuvant or s.c. in alum elicited substantial antibody titers (Fig. 4A) and concentrations (Fig. 4B), though antibody levels were higher after i.p. vaccination with Freund's. Titers did not increase appreciably after the 4th or 5th dose. Immunization using 25 or 100 µg M-KLH in Freund's elicited generally similar titers and antibody concentrations so the 25 µg dose was used for distribution and behavior studies. Averaging all experiments, not just those shown in figure 4, rats receiving 25 µg M-KLH i.p. in Freund's elicited serum antibody titers of $460 \pm 400 \times 10^3$ and antibody concentrations of $0.50 \pm 0.38$ mg/ml (mean ± SD). There was considerable individual variability in both antibody titers and concentrations (Fig. 4). Vaccination with M-KLH resulted in antibodies that were specific for heroin, 6-MAM, morphine, and morphine-6-glucuronide as measured by $IC_{50}$ values of $\leq 0.1$ µM (Table 1) compared to those of the off-target opioids (where binding was undesirable due to potential effects on their clinical use or the endogenous opioid system) methadone, buprenorphine, naloxone, naltrexone, oxycodone, and the endogenous opioid enkephalin, which had $IC_{50}$ values two to three orders of magnitude higher.

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**3.3    Vaccine effects on drug distribution after i.v. administration of heroin.**

Plasma heroin concentrations in KLH controls were below the limit of quantitation but were still detectable at 4 min and were estimated at $3.7 \pm 0.6$ ng/ml (mean $\pm$ SD). Using this estimate heroin retention in plasma was increased 280 fold in M-KLH vaccinated rats compared to KLH controls at 4 min (see Fig. 5). 6-MAM and morphine concentrations in plasma were increased 8 fold in vaccinated rats compared to controls at 4 min.

Vaccination with M-KLH did not reduce heroin distribution to brain compared to controls (Fig. 5, inset), but brain heroin concentrations in both groups were low compared to 6-MAM and morphine concentrations. Vaccination reduced the distribution of 6-MAM to brain by 44% ($p < 0.05$). Morphine distribution to brain in vaccinated rats was decreased by 40% compared to controls, but the difference was not quite significant ($p = 0.052$). However, both 6-MAM and morphine distribution to brain were reduced by 70% ($p < 0.01$) if data from two rats with very low serum antibody concentrations ($< 0.08$ mg/ml) were removed. Non-vaccinated (Fig. 3) and KLH control (Fig. 5) rats, although not directly compared, had similar plasma and brain opioid distribution at 4 min, showing that KLH vaccination did not alter drug distribution.

Serum antibody concentrations were positively correlated with plasma heroin and 6-MAM concentrations, but not with plasma morphine concentrations (Fig. 6A, C, and E). No significant correlation was found between heroin brain concentrations and serum antibody concentrations, but heroin concentrations in both groups were low (Fig. 6B). Higher antibody concentrations were associated with reduced brain 6-MAM and morphine concentrations (Fig. 6D and F).

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**3.4    Vaccine effects on drug distribution after i.v. administration of 6-MAM.** In rats vaccinated i.p. with M-KLH in Freund's, 6-MAM retention in plasma was increased 51 fold compared to KLH controls (Fig. 7) and morphine concentrations were 6 fold higher. Vaccination reduced 6-MAM concentration in brain by 76% compared to controls. Morphine concentrations in brain were too low to compare between groups. In rats vaccinated s.c. with M-KLH in alum effects were similar but smaller (Fig. 7). Serum antibody concentrations were directly correlated with plasma 6-MAM but not morphine concentrations (Fig. 8A and 8B). Serum antibody concentrations were inversely correlated with 6-MAM brain concentrations (Fig. 8C). Because effects on drug distribution were similar in rats vaccinated i.p. with either 25 and 100 µg M-KLH in Freund's, the 100 µg group was not included in Figure 7 for clarity but was added to Figure 8 to provide more data for linear regression.

**3.5    Protein binding.** Protein binding of morphine in spiked serum (of rats that had not received opioid in vivo) was substantially higher in the M-KLH serum (99.7%) than the KLH control serum (15.8%). Estimates of unbound morphine concentrations in plasma of experimental rats that had received heroin (see Fig. 5) were markedly lower in M-KLH vaccinated rats than KLH controls  (0.7 ± 0.3 ng/ml vs. 24.3 ± 7.3 ng/ml, mean ± SD, respectively).

**3.6    Thermal nociception test.** Vaccination with M-KLH significantly reduced response latency to heroin ($p < 0.001$), methadone ($p < 0.01$), and oxycodone ($p < 0.05$) compared to KLH controls (Fig. 9A), although the effect on heroin was greatest.  There was no effect of drug administration order on %MPE using the crossover design. Vaccination was highly effective at reducing opioid anti-nociception at all measured

antibody concentrations so no correlation was apparent. Vaccination did not alter nicotine or fentanyl-induced anti-nociception showing that vaccine effects were specific to certain opioids (Fig 9A).

**3.7**     **Heroin-induced locomotor activity.** Rats vaccinated with M-KLH or KLH had comparable baseline locomotor activity (Fig. 9B). There was a significant effect of vaccination ($p < 0.001$), day ($p < 0.001$), and interaction ($p < 0.001$) on heroin-induced locomotor activity. M-KLH vaccinated rats had substantially lower heroin-induced locomotor activity than controls on all days (Bonferroni $t = 4.6 - 8.0$, $p < 0.001$). Vaccination had no effect on cocaine-induced locomotor activity. On the final day of testing, higher antibody concentrations were associated with lower heroin-induced locomotor activity (Fig. 9C). Morphine-specific antibody titers were still elevated after completion of locomotor activity testing ($180 \pm 100 \times 10^3$, mean ± SD), but were 62% lower than after the final vaccination two months earlier.

**3.8**     **Stoichiometry.** In all experiments the dose of heroin or 6-MAM administered was nearly equal to or above the antibody binding capacity (Table 2), except for one experiment in which M-KLH vaccinated rats had very high antibody concentrations.

## 4.    DISCUSSION

Distribution of heroin and its metabolites was studied in rats in order to understand how morphine conjugate vaccines alter the behavioral effects of heroin. The main findings of this study were that 1) in non-vaccinated rats 6-MAM was the predominant metabolite in brain shortly after i.v. heroin administration; 2) vaccination with M-KLH led to a reduction of brain 6-MAM but not heroin concentration after i.v. heroin administration; 3) vaccination reduced heroin's behavioral effects; and 4) higher levels of opioid-specific antibodies were associated with greater effects on heroin pharmacokinetics and heroin-induced behavioral effects. These findings suggest that the binding of 6-MAM by vaccine-generated antibodies is critical for blockade of heroin's behavioral effects, and that relatively high serum levels of opioid-specific antibodies are needed to produce substantial blockade.

The finding that 6-MAM was the predominant opioid in plasma and brain in non-vaccinated rats for up to 14 min after i.v. heroin administration is consistent with several prior preclinical studies (Way et al., 1960; Andersen et al., 2009), despite differences in species and route of heroin administration. Since 6-MAM also has a higher affinity for the mu-opioid receptor than heroin (Inturrisi et al., 1983), these observations support a critical role of 6-MAM in mediating the early effects of heroin. These preclinical data contrast with several human studies that found heroin to be the predominant opioid in plasma shortly (2 to 10 min) after similar i.v. doses of heroin (Comer et al., 1999; Girardin et al., 2003). The metabolism of heroin to 6-MAM and morphine in plasma is attributable to esterases in blood as well as non-enzymatic degradation (Salmon et al., 1999; Selley et al., 2001; Rook et al., 2006). Rats have higher plasma esterase activity than humans (Minagawa et al., 1995; Bahar et al., 2012), which complicates

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extrapolation of opioid data across species but may explain why higher heroin and lower 6-MAM levels were found in humans.

The current data suggest that morphine conjugate vaccines act mainly by reducing 6-MAM concentrations in brain. Accumulation of 6-MAM in brain after heroin administration may result from either heroin distribution to brain and subsequent hydrolysis to 6-MAM, or conversion of heroin to 6-MAM in plasma and subsequent 6-MAM distribution to brain. Vaccine-generated antibodies could potentially target these pathways at several points (Fig. 10). 1) Vaccine-generated antibodies might bind heroin in plasma and prevent its distribution to brain. This is unlikely because brain heroin concentrations were not reduced in vaccinated rats compared to controls. 2) Vaccine-generated antibodies might reduce conversion of heroin to 6-MAM in brain. This is unlikely because drug-specific antibody is largely excluded from the central nervous system by the blood brain barrier (Satoskar et al., 2003). 3) Vaccine-generated antibodies might reduce the conversion of heroin to 6-MAM in plasma, reducing its availability for distribution to brain. This did not appear to be the case because plasma 6-MAM concentrations in vaccinated rats were substantial, indicating that conversion of heroin to 6-MAM in plasma was not prevented by vaccination. 4) Vaccine-generated antibodies might bind 6-MAM in plasma and impair its distribution to brain. This mechanism is consistent with the very high serum 6-MAM concentrations measured in vaccinated rats, reflecting its extensive binding and retention in serum. In support of this mechanism being the predominant explanation for reduced 6-MAM levels in brain, vaccine-generated antibodies also reduced 6-MAM levels in brain when 6-MAM was administered in place of heroin.

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Unbound heroin and 6-MAM concentrations could not be directly measured in plasma because rapid degradation complicated their measurement. However, estimates of unbound morphine concentrations in plasma based on in vitro protein binding data suggest that M-KLH rats had much lower unbound morphine concentrations than controls despite an overall increase in total plasma morphine concentrations. If this were also true for 6-MAM, it would account for the lower 6-MAM levels in brain in vaccinated rats.

Further support for the importance of 6-MAM binding is provided by a heroin vaccine composed of hapten conjugated to protein through the bridge nitrogen. This vaccine generated antibodies with a higher affinity for 6-MAM than heroin yet blunted heroin analgesia in rats (Stowe et al., 2011). Another immunogen, which had a lower affinity for 6-MAM than heroin, was not as effective (Stowe et al., 2011).

Higher plasma antibody concentrations in this study were associated with higher plasma and lower brain drug levels, and reduced behavioral effects of heroin. While a distinct threshold for effective antibody concentrations was not apparent, the combined distribution and behavioral studies suggest that antibody concentrations above 0.2 to 0.3 mg/ml produced substantial effects. No other such estimates are available for morphine conjugate vaccines, but this range is generally consistent with previous data for nicotine or cocaine vaccines in rodents (Kantak et al., 2000; Cornish et al., 2011).

The reduction of oxycodone- and methadone-induced anti-nociception found in vaccinated rats was unexpected because the $IC_{50}$ data showed relatively low antibody specificity for oxycodone and methadone compared to heroin, 6-MAM, or morphine

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(Table 1). This discrepancy may be due to the nature of the competitive ELISA method. The morphine conjugate used as a solid phase in the ELISA has many haptens available allowing antibody, which has two binding sites per molecule of IgG, to bind to two haptens at a time. In contrast the soluble competing opioid can bind only to one site. The ELISA may therefore reflect antibody avidity (binding to 2 sites on the ELISA coating conjugate), which is higher than its affinity (binding to one site on the competing antigen), and underestimate the ability of antibodies to bind some opioids in vivo. Nonetheless, vaccine did not block fentanyl or nicotine analgesia or reduce cocaine-induced locomotor activity showing that the effect of vaccination are specific to certain opioids. Vaccination with M-KLH has previously been shown to reduce brain oxycodone levels in rats by 33% after a 0.1 mg/kg i.v. dose (Pravetoni et al., 2012b). The ability to reduce oxycodone effects could be a desirable feature of a morphine conjugate vaccine, broadening its effects to another commonly abused opioid.  Alternatively, if this is not desired, positioning the linker at the nitrogen bridge of the hapten has been shown to minimize cross-reactivity with oxycodone (Stowe et al., 2011). A similar approach could perhaps be explored to minimize methadone cross-reactivity if needed.

Vaccination with M-KLH was effective even after administration of large, clinically relevant heroin doses that matched or exceeded the estimated drug-binding capacity of the available antibody. Efficacy in the face of large heroin doses encourages further study of this and related morphine-conjugate vaccines.  On the other hand, efficacy was associated with relatively high serum antibody levels, which, at least for analogous nicotine or cocaine vaccines, have been more difficult to achieve in humans than in rodents.  Achieving high serum antibody levels will be important in translating this strategy to the treatment of humans.

Vaccination i.p. using Freund's adjuvant produced a robust antibody response but it is not suitable for human use. The primary purpose of this study was to better understand vaccine effects on heroin and metabolite distribution so a potent adjuvant was desirable. A clinically acceptable adjuvant, such as alum, will be necessary for human use. Vaccination s.c. with alum did alter plasma drug levels compared to controls, but effects were not as large as those seen with Freund's i.p. However the immunogen dose in the s.c. alum formulation was not optimized and, by analogy with another morphine conjugate vaccine (Anton and Leff, 2006), higher immunogen doses may improve response.

A limitation of this study is that vaccine effects on heroin pharmacokinetics were only studied for single doses. Nevertheless, the heroin doses used were within the range abused by addicts, and vaccination remained effective in blocking heroin-induced locomotor activity over 2 weeks of repeated dosing. Other morphine conjugate vaccines of similar design have also blocked heroin self-administration (Bonese et al., 1974; Anton and Leff, 2006), which involves even more frequent heroin dosing. A similar morphine vaccine blocked heroin-induced motor activity and these effects are predictive of efficacy in heroin self-administration in rats (Li et al., 2011). The current data contribute to understanding how these behavioral effects of morphine conjugate vaccines are mediated. Further studies under different heroin dosing conditions should be helpful in determining the extent to which vaccine effects can be sustained over time, and the antibody levels needed to do so.

# TABLES AND FIGURES

## TABLES

**Table 1.** Competitive binding ELISA $IC_{50}$ values ($\mu M$).

| Drug | $IC_{50}$ ($\mu M$) |
|---|---|
| **Target** | |
| Heroin | 0.03 |
| 6-MAM | 0.04 |
| Morphine | 0.05 |
| M-6-G | 0.10 |
| **Off-target** | |
| Methadone | 177.50 |
| Buprenorphine | 574.00 |
| Naloxone | 38.70 |
| Naltrexone | 41.90 |
| Oxycodone | 8.98 |
| Enkephalin | 16.07 |

**Table 2.** Molar ratios of opioid-specific binding sites and opioid dose.

| Opioid administered | Opioid dose (µmol/kg) | Vaccine adjuvant and route | Total opioid-specific IgG binding sites (µmol/kg) | Molar ratio of drug dose: IgG binding sites |
|---|---|---|---|---|
| Heroin i.v. | 0.703 | Freund's, i.p. | 0.42 | 1.7 |
| 6-MAM i.v. | 0.703 | Freund's, i.p. | 1.09 | 0.6 |
| | | Alum, s.c. | 0.22 | 3.2 |
| Heroin s.c. | $2.70^{1}$ | Freund's, i.p. | 0.95 | 2.8 |
| | $0.68^{2}$ | Freund's, i.p. | 0.42 | 1.6* |

[1]Heroin-induced anti-nociception study
[2]Heroin-induced locomotor activity study
*Molar ratios reflect single doses though repeated doses were used to test behavioral responses in these protocols.

**FIGURES**

**Figure 1. (A)** Heroin degradation pathway and **(B)** M-KLH hapten.
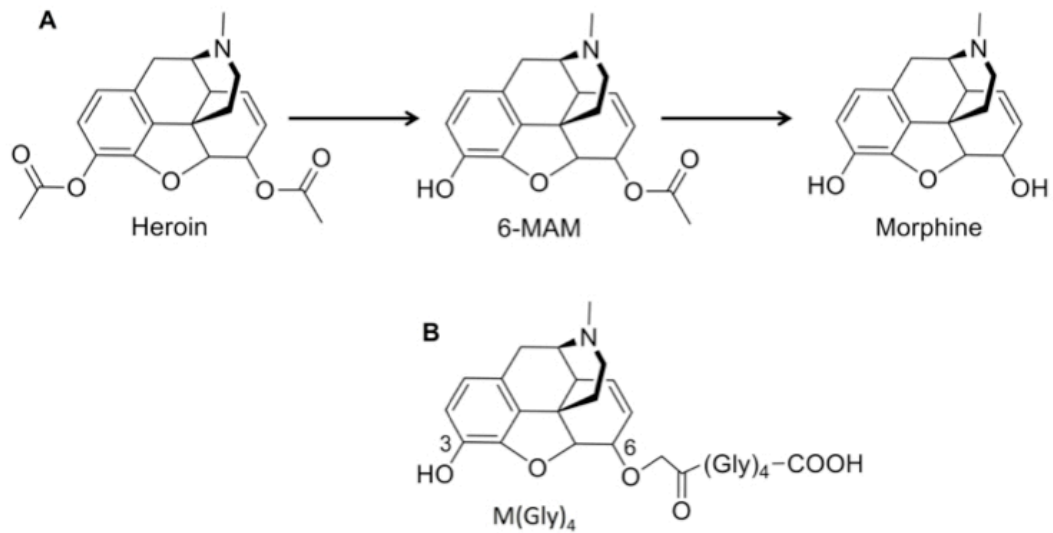
**Figure 2.** Timeline for the thermal nociceptive and locomotor activity behavioral experiments. Drug abbreviations: H, heroin; M, methadone; O, oxycodone; Coc, cocaine; Nic, nicotine. The 'x' denotes a crossover design.

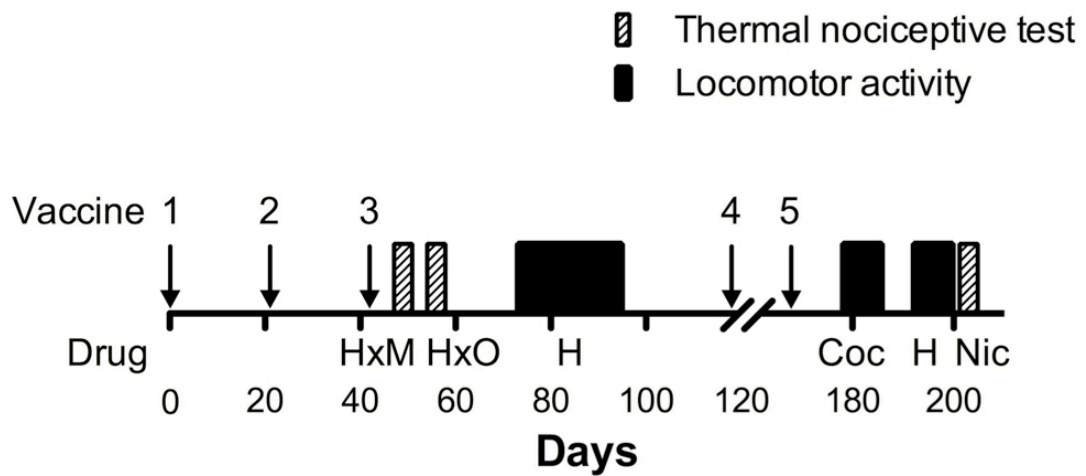**Figure 3. (A)** Plasma and **(B)** brain concentrations of opioids (mean ± SD) after 1 minute infusion of heroin 0.26 mg/kg i.v (n = 4 per time point). Heroin concentrations were above the limit of quantitation (5 ng/ml) in plasma and brain at one-minute post-infusion and estimated at 4 min. ***, $p < 0.001$ compared to heroin concentrations; †, $p < 0.05$ and †††, $p < 0.001$ compared to morphine concentrations.
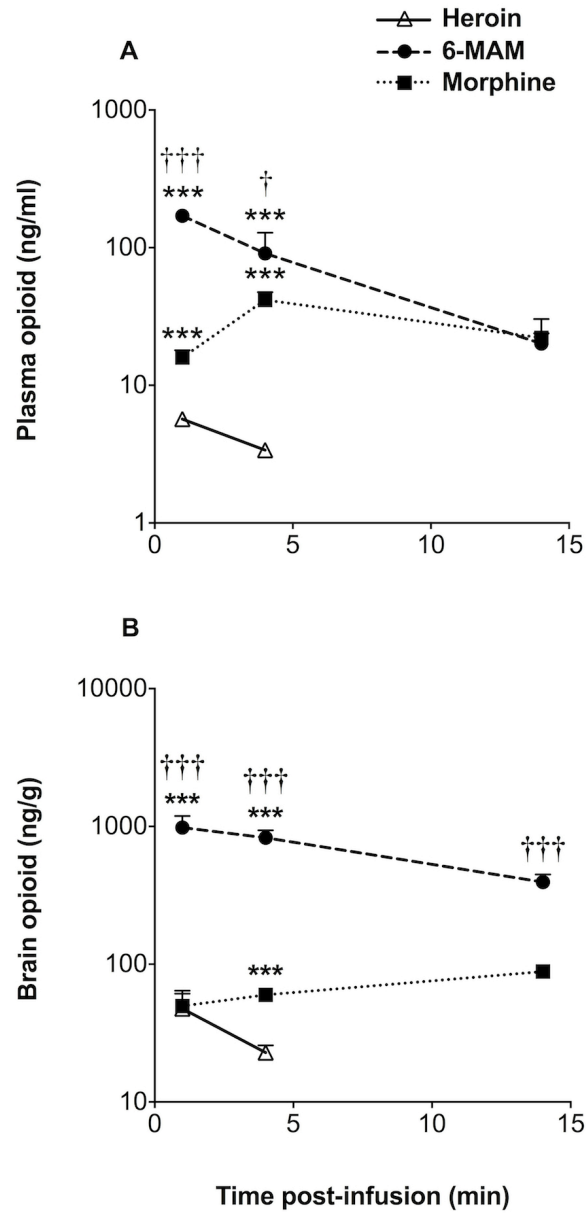
**Figure 4. (A)** Morphine-specific antibody titers and **(B)** concentrations after vaccination

i.p. with either 25 or 100 µg M-KLH with Freund's or s.c. with 25 µg M-KLH with alum (n

= 5 per group). Rats were vaccinated once every 3 weeks (arrows indicate times of

vaccinations). Antibody concentrations were measured after the 5th vaccination only.
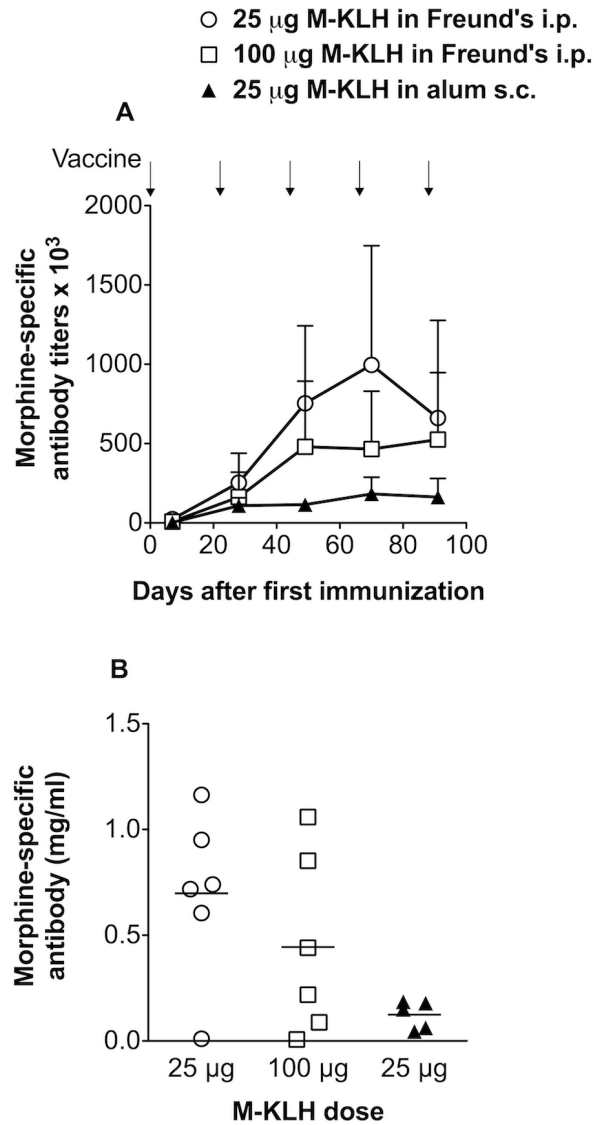
**Figure 5.** Effect of vaccination on distribution of heroin and its metabolites (mean ± SD) 4 min after a 1 min infusion of heroin 0.26 mg/kg i.v. in M-KLH and KLH rats (n = 6 per group). Rats were tested after the 5th vaccination i.p. with 25 µg M-KLH or KLH in Freund's adjuvant. Inset shows heroin brain concentrations in M-KLH and KLH rats on a reduced scale. *, p < 0.05 and ***, p < 0.001 compared to KLH controls and †††, p < 0.001 compared to estimated heroin concentrations in KLH controls. Numbers in parentheses are the percent decrease compared to controls.
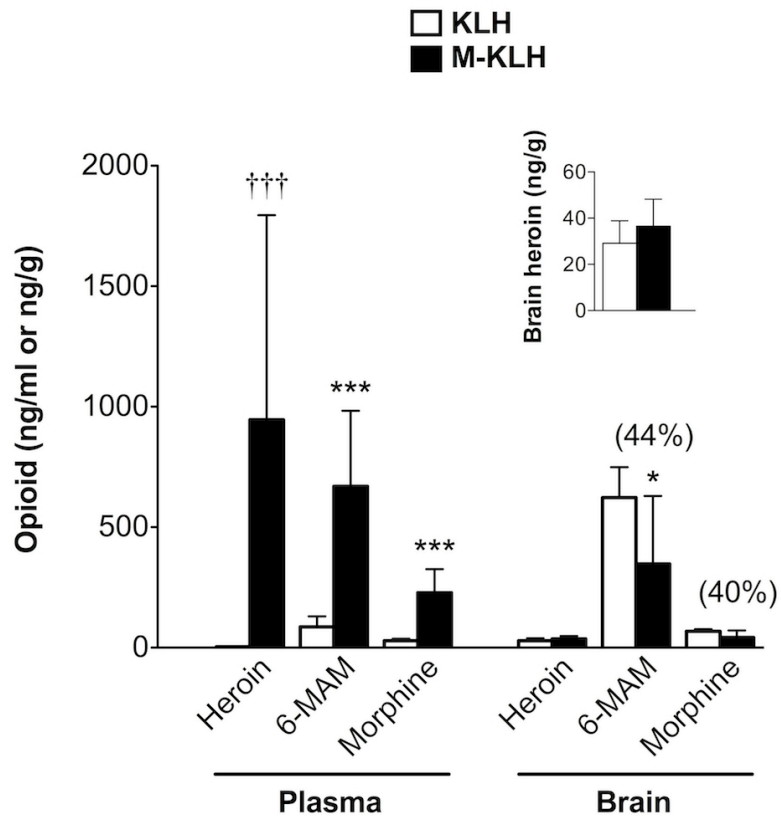
**Figure 6.** Relationship between morphine-specific antibody concentrations and opioid concentrations in plasma **(A, C, and E)** and brain **(B, D, and F)** 4 min after i.v. infusion of 0.26 mg/kg heroin in M-KLH vaccinated rats.
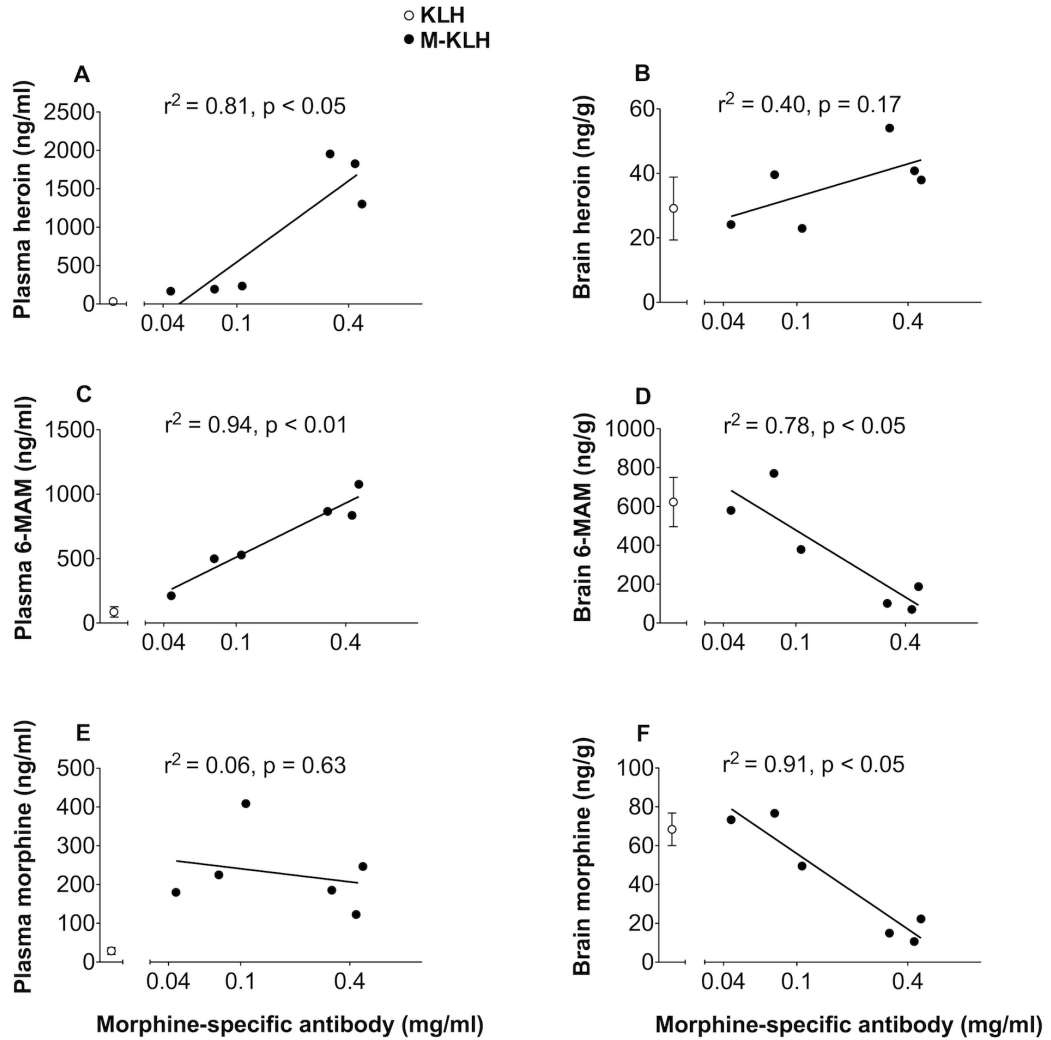
**Figure 7.** Plasma and brain concentrations of 6-MAM and morphine (mean ± SD) 4 min after a 1 min infusion of 0.23 mg/kg 6-MAM in M-KLH and KLH rats (n = 5 per group). Rats were tested after the 5th vaccination i.p. with 25 μg M-KLH + Freund's, s.c. with M-KLH + alum, and i.p. with 25 μg KLH + Freund's (n = 5 per group). Brain concentrations of morphine were below detection in all groups. **, $p < 0.01$ and ***, $p < 0.001$ compared to KLH controls; ††, $p < 0.01$ and †††, $p < 0.001$ compared to M-KLH + alum s.c. Numbers in parentheses are the percent decrease in concentrations compared to controls.
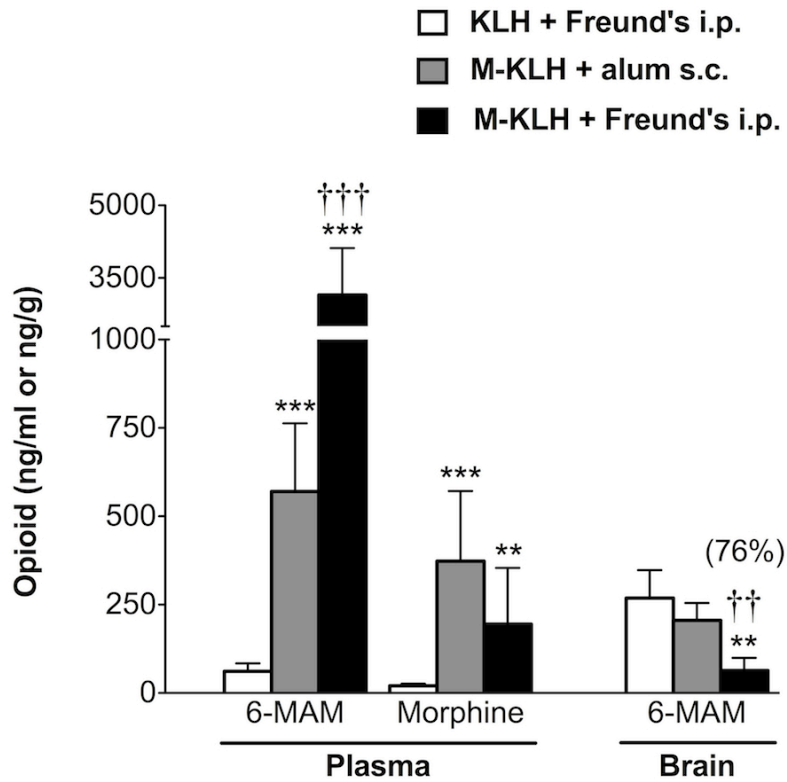
**Figure 8. (A)** Relationship between morphine-specific antibody concentrations and 6-MAM concentrations in plasma, **(B)** morphine concentrations in plasma, and **(C)** 6-MAM concentrations in brain 4 min after i.v. infusion of 0.23 mg/kg 6-MAM in M-KLH vaccinated rats. Morphine was not detected in brain 4 min after 6-MAM infusion.
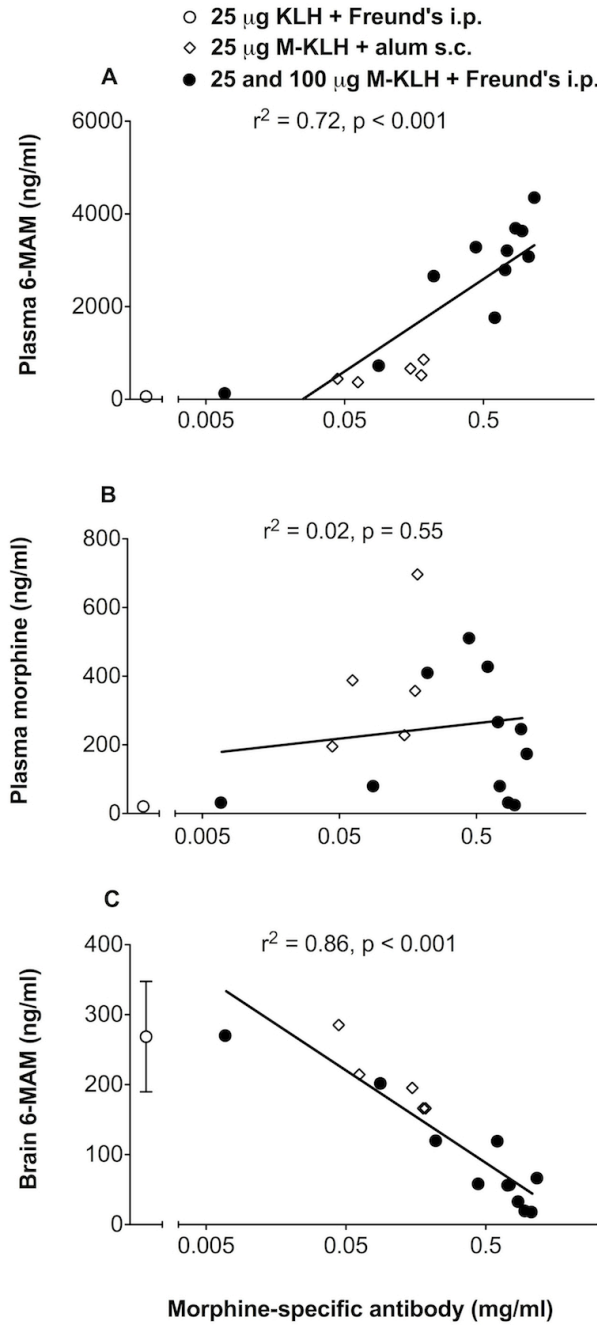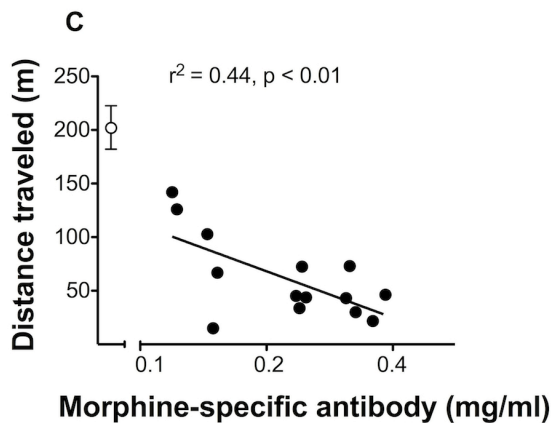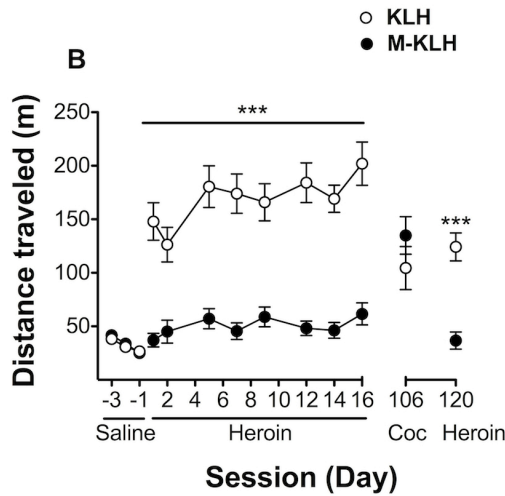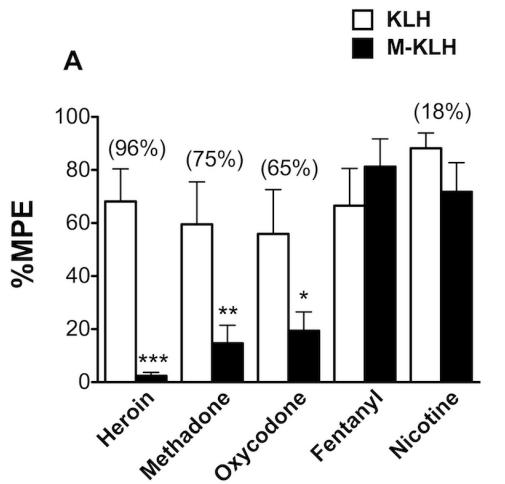
**Figure 9. (A)** Vaccine effect on drug-induced anti-nociception in M-KLH (n = 14) and KLH (n = 8) rats after s.c. administration of heroin (1.0 mg/kg), methadone (2.25 mg/kg), oxycodone (2.25 mg/kg), and nicotine (0.35 mg/kg) and in M-KLH (n = 10) and KLH (n = 10) rats after s.c. administration of fentanyl (0.05 mg/kg). Data are the mean percent maximum possible effect (%MPE) ± SEM. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared to KLH controls. Numbers in parentheses are the percent decrease of %MPE in M-KLH compared to KLH controls. **(B)** Vaccine effect on heroin-induced locomotor activity in M-KLH (n = 14) and KLH (n = 8) rats. Total horizontal distance traveled (mean ± SEM) was measured for 90 min after s.c. administration of 0.25 mg/kg heroin. ***, $p < 0.001$ heroin-induced locomotor activity compared to KLH controls. **(C)** Relationship of log morphine-specific IgG concentrations and locomotor activity in M-KLH (n=14) and KLH (n=8) rats on day 16.

**Figure 10.** Steps involved in the accumulation of 6-MAM in brain after heroin administration. Vaccination could potentially interfere by reducing 1) heroin distribution from plasma to brain, 2) hydrolysis of heroin to 6-MAM in brain, 3) hydrolysis of heroin to 6-MAM in plasma, or 4) distribution of 6-MAM from plasma to brain. The data generated in this study suggest that M-KLH acted primarily by binding 6-MAM in plasma and reducing its distribution to brain (step 4).
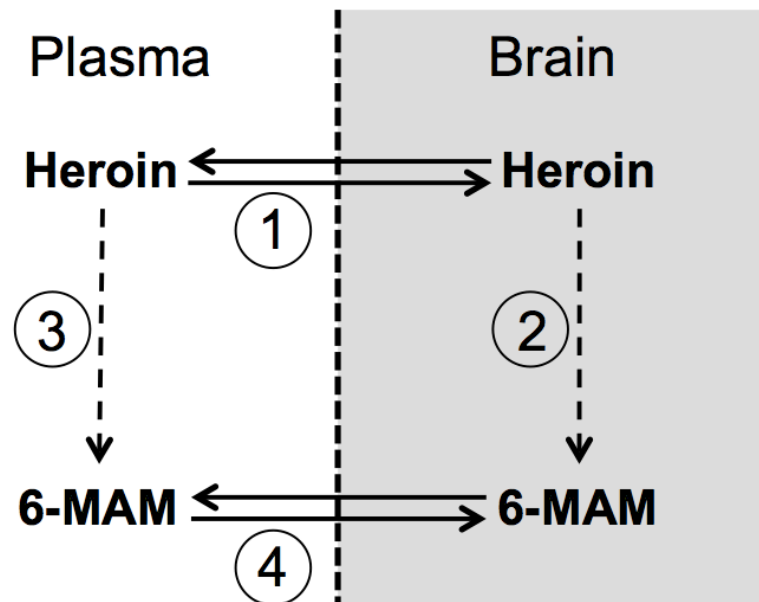
# Chapter 5

**A bivalent morphine and oxycodone vaccine reduced the distribution of 6-monoacetylmorphine and oxycodone to brain in rats**

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**Preface to Chapter 5: Note on authorship contribution**

The manuscript resulting from this work was contributed to equally by Michael D. Raleigh and by Dr. Marco Pravetoni. Michael Raleigh contributed to this manuscript by: vaccinating all animals, running titer and competitive ELISAs for all M-KLH and bivalent vaccinated animals, designing and running the drug distribution study, performing data analysis, and writing and editing portions of this manuscript. Marco Pravetoni contributed to this manuscript by: conjugating all immunogens (M-KLH and OXY-KLH), running titer and competitive ELISAs for all OXY-KLH and bivalent vaccinated animals, designing the drug distribution study, performing data analysis, and writing and editing portions of this manuscript.

Opioid conjugate vaccines have shown promise in animal models as a potential treatment for opioid addiction. Individual vaccines are quite specific and each targets only a limited number of structurally similar opioids. Since opioid users can switch or transition between opioids, we studied a bivalent immunization strategy of combining 2 vaccines that could target several of the most commonly abused opioids; heroin, oxycodone and their active metabolites. Morphine (M) and oxycodone (OXY) haptens were conjugated to keyhole limpet hemocyanin (KLH) through tetraglycine $(Gly)_4$ linkers at the C6 position. Immunization of rats with M-KLH alone produced high titers of antibodies directed against heroin, 6-monoacetylmorphine (6-MAM) and morphine. Immunization with OXY-KLH produced high titers of antibodies against oxycodone and oxymorphone. Immunization with the bivalent vaccine produced consistently high antibody titers against both immunogens. Bivalent vaccine antibody titers against the individual immunogens were higher than with the monovalent vaccines alone owing, at least in part, to cross-reactivity of the antibodies. Administration of a single concurrent intravenous dose of 6-MAM and oxycodone to rats immunized with the bivalent vaccine increased 6-MAM, morphine and oxycodone retention in serum and reduced the distribution of 6-MAM and oxycodone to brain. Vaccine efficacy correlated with serum antibody titers for both monovalent vaccines, alone or in combination. Efficacy of the individual vaccines was not compromised by their combined use. Consistent with the enhanced titers in the bivalent group, a trend toward enhanced pharmacokinetic efficacy with the bivalent vaccine was observed. These data support the possibility of co-administering two or more opioid vaccines concurrently to target multiple abusable opioids without compromising the immunogenicity or efficacy of the individual components.

## 1. INTRODUCTION

Opioid abuse and addiction in the USA encompasses a wide variety of opioids (Maxwell, 2011). Prior to the 1990s, heroin abuse was predominant and was the focus of treatment strategies. Over the past 15 years prescription opioid abuse has increased dramatically and is now substantially more common than that of heroin (Compton and Volkow, 2006a; Compton and Volkow, 2006b). Oxycodone and to a lesser extent hydrocodone or oxymorphone abuse have been increasingly reported in various populations, including teens and USA military personnel (SAMHSA, 2010). Patterns of opioid abuse are also diverse. Daily use by intravenous injection or smoke inhalation is common with heroin, while more occasional oral or intravenous use is more common with prescription opioids (Butler et al., 2011).

Existing medications for the treatment of opioid addiction are effective and helpful, yet are taken advantage of by only a small fraction of opioid abusers (Dodrill et al., 2011). Agonist therapies including methadone and buprenorphine are themselves addictive. Their use requires substantial oversight, and is legally restricted to established daily opioid users. Despite the potential benefits, some opioid addicts object to taking an addictive treatment medication. Because abuse of prescription opioids is often episodic, continuous agonist therapy is a less attractive option to treat this pattern of abuse. The opioid antagonist naltrexone is effective for heroin addiction, but compliance is generally poor. Additional treatment options are needed which address the diversity of both the opioids abused and their different routes of administration and patterns of use (Wu et al., 2011).

Vaccines targeting drugs of abuse are being developed as an alternative or supplemental approach to addressing addictions (Chi, 2011). These vaccines stimulate production of antibodies which bind the target drug, alter its distribution to brain, and reduce drug-related behaviors in animals. Vaccines against cocaine and nicotine have reached clinical trials (Martell et al., 2009; Hatsukami et al., 2011). A number of vaccines have been developed which elicit antibodies that bind heroin and its sequentially produced active metabolites 6-monoacetylmorphine (6-MAM), morphine, and morphine-6-glucuronide. Some of these vaccines have been shown to reduce heroin- or morphine-induced behaviors, including self-administration in animals (Berkowitz and Spector, 1972; Berkowitz et al., 1974; Bonese et al., 1974; Anton and Leff, 2006; Anton et al., 2009; Stowe et al., 2011). These vaccines show structural specificity and little binding of other opioids such as methadone, buprenorphine, or naltrexone. This structural specificity is advantageous in that use of such vaccines should not preclude the concurrent use of agonist therapies, but high specificity also means that these vaccines do not appreciably bind other abusable opioids such as oxycodone. An oxycodone vaccine was recently described which binds oxycodone and its active metabolite oxymorphone but has a much lower affinity for heroin and its metabolites, reduces oxycodone distribution to brain, and reduces oxycodone-induced hot plate analgesia in rats (Pravetoni et al., 2012a). The availability of this vaccine presents the possibility of combining heroin and oxycodone vaccines in order to achieve broader opioid binding activity.

In the current study rats received an oxycodone-KLH conjugate vaccine (OXY-KLH) targeting oxycodone and its active metabolite oxymorphone, or a morphine-KLH conjugate vaccine (M-KLH) targeting heroin, 6-MAM and morphine. The two vaccines

were administered alone or in combination to determine whether their combined use would preserve their individual efficacies. Because heroin is rapidly converted in vivo to 6-MAM, which is considered largely responsible for its acute effects, and resulting heroin levels are quite low (Andersen et al., 2009), 6-MAM was used as a model opioid in this study rather than heroin. Rats vaccinated with M-KLH and OXY-KLH vaccines concurrently developed high titers of antibodies to all of the targeted drugs, and showed substantial reductions in the distribution of 6-MAM and oxycodone to brain.

## 2.  MATERIAL AND METHODS

**2.1  Drugs and reagents**. 6-monoacetylmorphine (6-MAM), morphine, oxycodone and opioids used for competitive binding studies were obtained through the NIDA Drug Supply Program and Sigma (St. Louis, MO). [Leu5]-enkephalin was purchased from Tocris Biosciences (Ellisville, MO). All drug doses and concentrations are expressed as the weight of the base.

**2.2  Synthesis of haptens.** OXY(Gly)$_4$ hapten consisting of oxycodone with a tetraglycine (Gly)$_4$ linker at the C6 position was synthesized as previously described (Figure 1) (Pravetoni et al., 2012a). The analogous morphine M(Gly)$_4$ hapten with C6 linker position was synthesized as detailed below (see Supplemental Figure 1). This linker position was selected because of its efficacy in previously reported immunogens for eliciting antibodies against heroin, 6-MAM, morphine and morphine-6-glucoronide (Bonese et al., 1974; Anton and Leff, 2006), and its efficacy for the OXY-KLH vaccine (Pravetoni et al., 2012a).  The (Gly)$_4$ linker length was found to be more effective than shorter linkers for the OXY-KLH vaccine, and is similar to the linker length of an heroin vaccine shown to be highly immunogenic and effective in blocking heroin-induced behaviors  (Anton and Leff, 2006).

For characterization of synthetic intermediates and haptens, $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were taken on a Bruker Avance 400 MHz instrument (Brucker, Billerica, MA) and calibrated using an internal reference. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ). Analytical thin-layer chromatography (TLC) was performed on EM Science (Gibbstown, NJ) silica gel 60 F254 (0.25 mm) and plates visualized by UV light, iodine vapor, or ninhydrine solution.

**3-Boc-M (1).** Morphine sulfate (5 mmol) was dissolved in a dimethylformamide (DMF)/H$_2$O mixture (9/1: v/v) and catalytic 4-dimethylaminopyridine (DMAP) (0.025 mmol) was added. Tert-Butyloxycarbonyl (Boc) anhydride (1.1 equivalent) dissolved in DMF was added over 30 min and the mixture stirred at room temperature overnight. Solvents were evaporated under reduced pressure and the residue was purified by flash chromatography (3% MeOH/DCM/1%NH$_4$OH) on silica gel (230 – 400 mesh, Fisher Scientific, Pittsburgh, PA) to afford **1** (84% yield) (See Supplemental Figure 1 for synthesis scheme).

**3-BOC-M-6-*O-tert*Butylglycolate (2).** A suspension of sodium hydride (60% dispersion in mineral oil) (2.9 mmol) in dry tetrahydrofuran (5 ml) at 0°C under nitrogen was gradually added to **1** (1.9 mmol) and the reaction mixture was allowed to stir at room temperature for 30 min, followed by the addition of tert-butylbromoacetate (2.9 mmol). The reaction mixture was stirred at room temperature for 8 hr. The reaction was concentrated under vacuum, diluted with water and then extracted with dichloromethane (DCM). The organic layers were combined, dried over sodium sulfate, filtered and concentrated under vacuum to furnish a final crude residue. This residue was subjected to flash column chromatography using a DCM/MeOH/NH$_4$OH mixture (97/2/1) to afford **2** as an oil (67% yield).

**M-6-*O*-glycolic acid (3).** Trifluoroacetic acid (TFA) (20% v/v) was added to a solution of the ester **2** (2 mmol) in DCM (20 ml). The resultant solution was stirred at room temperature. Upon complete disappearance of starting material, the solvent was removed under vacuum. The crude reaction mixture was subjected to azeotropic drying

using toluene. The residue was taken up in a methanol/ diethyl ether ($Et_2O$) mixture and the precipitate was filtered and washed with $Et_2O$ to afford **3** as a white solid (85% yield).

**M(Gly)$_4$**. The M-6-*O*-glycolic acid **(3)** was coupled to tetraglycine tertbutyl esters (Gly$_4$tBu) using Dicyclohexylcarbodiimide (DCC)/ hydroxybenzotriazole (HOBt) followed by acid hydrolysis to complete the synthesis of M(Gly)$_4$ as described before for OXY(Gly)$_4$ (Pravetoni et al., 2012a). M-6-*O*-glycolic acid **(3)** (0.15-0.4 mmoles), DCC (1.3 equivalent), and HOBt (1.2 equivalent) were dissolved in 5 ml of anhydrous DMF. The solution was cooled to 0°C, placed under a nitrogen atmosphere, and after 15 minutes Gly$_4$tBu (0.15-0.4 mmoles) was added. The solution was sealed under a nitrogen atmosphere and was allowed to reach room temperature and then stirred overnight. The reaction mixture was filtered to remove dicyclohexylurea into water (10x initial volume of DMF) and extracted with ethylacetate. The combined organic layers were dried on magnesium sulfate, and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM/MeOH/NH$_4$OH: 94/5/1) and to a solution of this protected intermediate (0.15-0.4 mmol scale) in DCM (5 ml) was added TFA (20% v/v). The resultant solution was stirred at room temperature. Upon complete disappearance of starting material, the solvent was removed under vacuum. The crude reaction mixture was subjected to azeotropic drying using toluene. The residue was taken up again in a methanol/$Et_2O$ mixture and the precipitate was filtered and washed with $Et_2O$. The final crude was purified by reverse phase high pressure liquid chromatography using an acetonitrile/$H_2O$/TFA (80/20/0.1%) mixture to provide M(Gly)$_4$ as a slightly yellow solid (43% over 2 steps) (see Supplemental Figure 1 for synthesis scheme). M(Gly)$_4$ was converted to its TFA salt for conjugation to carrier proteins.

**2.3    Conjugation and purification of conjugates.** Haptens for use in the vaccines were conjugated to keyhole limpet hemocyanin (KLH) because this protein is highly immunogenic and acceptable for use in humans.  For use as coating antigen in ELISA assays, haptens were conjugated to bovine serum albumin (BSA) or chicken albumin (OVA).   Hapten 5 mM and ethyl-N′-(3 dimethylaminopropyl)carbodiimide hydrochloride 50 mM were dissolved in 0.1M MES buffer at pH 4.5.. BSA, OVA or KLH (Thermo Fisher Rockford, IL) were added and reactions were stirred for 3 hr at room temperature followed by dialysis and storage at $+4^{o}$ C as previously described (Pravetoni et al., 2012a).  Molar hapten:protein conjugation ratios (moles of hapten conjugated per mole of protein) for the BSA and OVA conjugates were quantitated by MALDI-TOF (Reflex III, Bruker).

**2.4    Vaccination.** All animal studies have been carried out in accordance with EU Directive 2010/63/EU and approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee. Male Holtzman rats weighing 350 grams (Harlan Laboratories, Madison, WI) were housed with a 12/12 hrs standard light/dark cycle. Conjugates were injected i.p. in a final volume of 0.4 ml in complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for 2 subsequent booster injections at 3 and 6 weeks. Experiments were conducted 7-10 days after the 3$^{rd}$ immunization.  Rats (n=12 group) were immunized as follows, with unconjugated KLH added as needed so that all groups received a total KLH protein dose of 50 µg: Group (1) KLH 50 µg; Group (2) Monovalent M-KLH 25 µg + KLH 25 µg; Group (3) Monovalent OXY-KLH 25 µg + 25 µg KLH; Group (4) Bivalent M-KLH 25 µg + OXY-KLH 25 µg.

To compare vaccine route and adjuvant male BALB/c mice (n=5 per group) (Harlan Laboratories, Madison, WI) were vaccinated on days 0, 14 and 28 with 25 µg of

monovalent OXY-KLH.  Vaccine was administered either i.p. in a final volume of 0.2 ml in complete Freund's adjuvant for the first dose and incomplete Freund's for the remaining doses, or s.c. in a final volume of 0.2 ml containing alum adjuvant (Thermo Fisher) at a final concentration of 5 mg/ml.  Blood was obtained on day 35 for serum antibody titer measurement.

**2.5    Antibody cross-reactivity and specificity**. ELISA plates were coated with 5 ng/well of M-BSA or OXY-OVA conjugate or unconjugated protein control in carbonate buffer at pH 9.6 and blocked with 1% gelatin. Rat primary antibodies were incubated with goat anti-IgG antibodies conjugated to horseradish peroxidase, while rabbit anti-mouse IgG antibodies were used to measure mouse immunized serum.  The extent of cross-reactivity to immunogen between antibodies generated by each monovalent vaccine was assessed by measuring titers against both the M-BSA and OXY-OVA coating immunogens.  Cross-reactivity for the M-KLH vaccine was calculated as the ratio of its ELISA titer to OXY-OVA divided by its ELISA titer to M-BSA, and vice versa for the OXY-KLH vaccine.  Antibody specificity was characterized by competitive ELISA and $IC_{50}$ values for each inhibitor were obtained as described before (Pravetoni et al., 2012a).

**2.6    Morphine-specific    antibody    concentration.**    Morphine-specific    IgG concentrations from immunized rats were measured by ELISA using a standard curve constructed using commercially available murine monoclonal anti-morphine IgG. The morphine-conjugate used to generate the monoclonal anti-morphine IgG utilized the same C6 linker position for conjugation to carrier protein (BSA) as the $M(Gly)_4$ immunogen used in the current study (Qed Biosciences, San Diego, CA).    A

corresponding oxycodone monoclonal antibody with C6 linker position was not available so this approach could not be used to measure anti-oxycodone IgG concentrations.

Because the serum morphine-specific antibody being measured was from rats while the monoclonal IgG used for the standard curve was from mice, preliminary experiments were performed to determine dilutions of anti-mouse IgG-horseradish peroxidase (HRP) or anti-rat IgG-HRP which produced the same optical density reading when added to wells containing 5 ng of commercially obtained purified mouse or rat IgG, respectively. These secondary antibody dilutions were then used for the standard curve or serum ELISAs to quantitate morphine-specific IgG levels in vaccinated rats. For analysis of rat serum, wells were coated with 5 ng of M-BSA and serum dilutions were added followed by goat anti-rat IgG-HRP at a 1:50,000 dilution. The corresponding standard curve was obtained by coating ELISA wells similarly and adding various concentrations of anti-morphine monoclonal antibody followed by rabbit anti-mouse IgG-HRP at a 1:10,000 dilution. Controls consisting of wells coated with BSA alone showed no binding of either the monoclonal antibody or immune serum to this protein.

**2.7    Effects of vaccination on 6-MAM, morphine and oxycodone distribution.**
The effects of immunization with the bivalent M-KLH + OXY-KLH vaccine on drug distribution were measured in rats receiving 0.1 mg/kg (0.32 µmol/kg) 6-MAM and an equimolar dose of oxycodone administered concurrently i.v.. 6-MAM was dissolved in 2% (v/v) DMSO in physiological saline while oxycodone was dissolved directly in saline on the day of the experiment. 6-MAM and oxycodone were mixed 1:1 (vol: vol) in the same syringe prior to intravenous infusion. One week after the final vaccine dose rats were anesthetized with ketamine/xylazine (respectively 100 mg/kg and 10mg/kg) and an

indwelling catheter was placed in their right external jugular vein. Blood was withdrawn for ELISA assays, and 6-MAM + oxycodone were administered as a 60 sec infusion. Rats were decapitated 4 minutes later and trunk blood and brain collected. Oxycodone and 6-MAM doses were chosen as the largest doses that, when combined, would avoid respiratory depression or overdose.

**2.8    Oxycodone assay.** Serum and brain oxycodone or oxymorphone concentrations were measured by gas chromatography coupled to mass spectrometry as previously described (Pravetoni et al., 2012a). The reported oxycodone concentrations represent the total drug (protein or antibody-bound as well as free) in each sample.

**2.9    6-MAM and morphine assay**. All solutions and samples were kept on ice. Whole blood samples were collected into a syringe containing ice-cold sodium fluoride (4 mg/ml) and heparin (100 IU/ml). Plasma was separated and 0.5 ml of plasma was diluted 1:1 with formate buffer (pH 3.0, 10mM). Brain halves were rinsed with 10 mM formate (pH 3.0), placed into pre-weighed vials, then weighed and 10 mM formate pH 3.0 was added (4 parts by weight). Samples were homogenized 30-40 sec and stored at -20°C until extraction.

Samples underwent solid-phase extraction using Strata-X-RP 1 ml extraction cartridges and were quantified by liquid-chromatography coupled to mass spectrometry (LC-MS). All samples were extracted immediately after sample collection to avoid degradation of heroin and 6-MAM. Standard curves were prepared using bovine serum and were linear in the range of 5 ng/ml to 200 ng/ml for 6-MAM and 10 ng/ml to 200 ng/ml for morphine. The internal standards were the deuterated analogues of 6-MAM and morphine

(Cerilliant Analytical Reference Standards, Round Rock, TX). Samples were analyzed on a 2010A Shimadzu (Tokyo, Japan) single-quadrupole LC-MS with a reversed phase Agilent (Santa Clara, CA) Zorbax XDB-C18 (2.1 mm X 50 mm i.d., 3.5 µm) column, a reversed phase 4 mm X 2 mm C18 guard column (Phenomenex, Torrance, CA), and a gradient mobile phase mixture of 10 mM formate buffer pH 3.0 and acetonitrile. Total run time was 5 minutes. Validation was based on a six point standard curve prepared in triplicate (within-run) for each day on five different days (between run, N=15). The within day variability was between 2.2% and 7.9%, the between day variability 1.9% and 6.9%, and the limit of quantitation 5 ng/ml.

**2.10  Stoichiometric relationships.** The total number of moles/kg of anti-morphine IgG elicited in rats vaccinated with M-KLH was calculated as the product of the mean serum anti-morphine IgG concentration and its reported volume of distribution of 131 ml/kg in rats (Bazin-Redureau et al., 1997). The corresponding number of drug-binding sites of IgG was twice that number since there are 2 binding sites per IgG. The % saturation of anti-morphine IgG in serum was calculated as the molar ratio of the antibody-bound serum ligand concentration (6-MAM + morphine) to the serum anti-morphine IgG binding site concentration. Antibody-bound drug concentration in serum was calculated as (total 6-MAM + morphine concentration in the M-KLH group) minus (mean 6-MAM + morphine concentration in the control KLH group). The drug assay used measured total (bound and unbound) drug in each sample so that the total concentration in the M-KLH group represented both bound and unbound drug, while the concentrations in the control group represented only unbound drug since there are not antibodies present. The difference between these therefore provided an estimate of the bound drug concentration.

**2.11  Statistical analysis and calculations**. Group differences were analyzed by one-way analysis of variance followed by the Bonferroni post hoc test.  Relationships between serum antibody titers or concentrations and drug concentrations in serum and brain were analyzed by linear regression within groups and analysis of covariance was used to compare their linear regression slopes across groups.

## 3.    RESULTS

**3.1    Synthesis and conjugation of M-KLH and OXY-KLH vaccines.** Haptenation ratios for $M(Gly)_4$ or $OXY(Gly)_4$ conjugated to BSA or OVA ranged from 17 to 21 moles of hapten per mole of carrier protein. Haptenation ratios for the M-KLH and OXY-KLH conjugates could not be measured by mass spectrometry due to the larger size of KLH.

**3.2    Antibody response in rats.** <u>Monovalent M-KLH</u>:  Immunization with M-KLH alone elicited serum antibodies that were highly specific for heroin, 6-MAM and morphine. Serum from rats vaccinated with M-KLH showed 20% cross-reactivity with the OXY-OVA coating antigen on ELISA (Table 1), and negligible cross-reactivity with unconjugated OVA alone (not shown).  Competition ELISA showed high relative affinities of antiserum (low $IC_{50}$ values) for heroin, 6-MAM, morphine, hydrocodone, and hydromorphone, and a low relative affinity for oxycodone and oxymorphone (See Supplemental Table 1).   Cross-reactivity with the $(Gly)_4$ linker alone was negligible (not shown).   Serum anti-morphine IgG concentrations were consistently high, with a mean ± SD of 558 ± 62 $\mu$g/ml and no values below 452 $\mu$g/ml.

<u>Monovalent OXY-KLH</u>:  Immunization with OXY-KLH alone elicited serum antibodies that were highly specific for oxycodone and oxymorphone.  Serum from rats vaccinated with OXY-KLH showed 18% cross-reactivity with the M-BSA coating antigen on ELISA (Table 1), and negligible cross-reactivity with unconjugated BSA alone (not shown) Competition ELISA values showed high relative affinities for oxycodone and oxymorphone, low relative affinity for heroin, 6-MAM or morphine, and negligible cross-reactivity with the $(Gly)_4$ linker alone or the endogenous opioid Leu-enkephalin similarly to our previous report (data not shown, Pravetoni et al., 2012a).

Bivalent M-KLH and OXY-KLH:  Rats immunized with the bivalent vaccine developed serum antibody titers against each immunogen that were higher than in rats vaccinated with monovalent vaccines alone (Table 1).   These titers were greater than could be accounted for simply by adding the increase in titers attributable to cross-reactivity of the antibodies as determined by ELISA.  For example, the anti-morphine titer in the bivalent group of $325 \times 10^3$ was greater than the anti-morphine titer of $140 \times 10^3$ in the group vaccinated with monovalent M-KLH even allowing for an additional titer of $29 \times 10^3$ which would be expected in the bivalent group due to cross-reactivity from immunization with OXY-KLH.  Antibody specificities in the bivalent group, as determined by $IC_{50}$ values, were similar to those obtained with the monovalent vaccines.   While there was considerable individual variability in serum antibody titers in the bivalent group, there was a high correlation between anti-morphine and anti-oxycodone antibody titers measured in individual animals (Figure 2).

**3.3** **Antibody response in mice**. Monovalent OXY-KLH vaccine produced comparable antibody titers in BALB/c mice whether injected i.p in Freund's adjuvant ($43\pm14 \times 10^3$, mean$\pm$SE) or s.c. in alum adjuvant ($44\pm16 \times 10^3$) ($p> 0.05$).

**3.4** **Effects of vaccination on drug distribution.** Monovalent M-KLH:  Vaccination with M-KLH substantially increased the retention of 6-MAM and morphine in serum (Figure 3).  Distribution of 6-MAM to brain was reduced by 69% compared to controls. Morphine concentrations in brain in all groups were below assay sensitivity ($<50$ ng/ml). Serum anti-morphine antibody concentration was inversely correlated with the brain 6-MAM concentration (See Supplemental Figure 2).   Vaccination with M-KLH also produced a small (33%) but significant decrease in distribution of oxycodone to brain

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(Figure 4), consistent with ELISA titers showing some cross-reactivity of anti-morphine antibodies with the oxycodone immunogen. The mean total number of IgG drug-binding sites in rats vaccinated with M-KLH was 0.98 µmol/kg, nearly 3-fold greater than the administered 6-MAM dose of 0.32 µmol/kg. The calculated saturation of serum anti-morphine IgG with its ligands (6-MAM + morphine) in the monovalent M-KLH group was 54 ± 15% (mean±SD).

Monovalent OXY-KLH:  Vaccination with OXY-KLH increased retention of oxycodone in serum and reduced oxycodone distribution to brain by 66% (Figure 4).  Oxymorphone concentrations in all groups were too low to quantitate (<5 ng/ml).  Vaccination with OXY-KLH produced a small increase in serum retention of morphine (Figure 3) but had no significant effect on serum or brain 6-MAM concentrations.

Bivalent M-KLH and OXY-KLH:  Vaccination with the bivalent vaccine preserved the effects of the monovalent vaccines, significantly increasing 6-MAM and oxycodone retention in serum and decreasing their distribution to brain (Fig 3 and 4).  The effects of the bivalent vaccine were generally similar to those of the monovalent vaccines alone. The bivalent vaccine increased serum retention of 6-MAM significantly more than the monovalent M-KLH vaccine.  However the bivalent vaccine produced less retention of morphine in serum than the monovalent M-KLH vaccine, possibly due to increased binding of the precursor 6-MAM by the bivalent vaccine resulting in less conversion to morphine.  Brain ($p = 0.12$) or serum ($p = 0.06$) oxycodone concentrations did not significantly differ between the bivalent and monovalent groups, although the difference in serum concentrations approached significance.

Vaccine effects on serum and brain 6-MAM or oxycodone concentrations were highly correlated with the corresponding serum antibody titers (Figure 5). The relationships between drug-specific titers and drug concentrations regression slopes were not significantly different between the monovalent and bivalent groups (all p >0.2).

## 4.    DISCUSSION

Opioid vaccines are of interest as a potential adjunct to the treatment of opioid abuse or addiction but the wide variety of commonly abused opioids makes this challenging. Individual opioid vaccines reflect the high specificity of humoral antibody responses and each targets only a limited range of opioids.  Vaccines based on morphine conjugates produce antibodies, which bind heroin and its active metabolites 6-MAM and morphine but have substantially lower affinity for oxycodone (Anton and Leff, 2006; Stowe et al., 2011).  A vaccine based on an oxycodone conjugate produces antibodies which bind oxycodone and its active metabolite oxymorphone but have substantially lower affinity for heroin and its metabolites (Pravetoni et al., 2012a).  In the current study combining M-KLH and OXY-KLH vaccines allowed their concurrent use while fully retaining their individual immunogenicity and effect on opioid pharmacokinetics.  These observations provide proof of concept that a broad range of opioid coverage can be obtained through the use of multivalent vaccines.

The use of multivalent vaccines is well established for infectious diseases.  Unrelated vaccines (e.g. DPT = diphtheria, pertussis, tetanus; MMR = measles, mumps, rubella) can be combined for convenience, or administered as separate injections at the same time, with little or no loss of efficacy.  In principle the same should be true of conjugate vaccines constructed from small molecule haptens.  This has been shown to be feasible for nicotine vaccines through the use of different linker positions to create immunologically distinct haptens (Pravetoni et al., 2011a).  Each of these haptens activated different populations of B cells to produce distinct populations of antibodies to nicotine.   The current study extends this approach to opioids with the goal of targeting a wider range of opioids.

The efficacy of the M-KLH vaccine was studied using 6-MAM which has a higher affinity for the mu opioid receptor than heroin and is present in brain at higher concentrations. 6-MAM is considered the principal mediator or the early reinforcing and rewarding effects of heroin (Andersen et al., 2009). A number of morphine-conjugate vaccines similar to M-KLH, which also utilize the C6 linker position, have been shown to block heroin self-administration in animals (Bonese et al., 1974; Anton and Leff, 2006; Anton et al., 2009). While the influence of M-KLH on heroin pharmacokinetics needs to be specifically studied, the high serum concentration of antibodies produced is comparable to the highest reported with other morphine-conjugate vaccines (Anton et al., 2009).

The OXY-KLH vaccine targeted oxycodone but the resulting antibodies also had a high affinity for oxymorphone. Oxymorphone is only a minor metabolite of oxycodone in humans but it is marketed separately as an analgesic medication. Tighter regulation of oxycodone prescribing and reformulation of oxycodone into tablets that are more difficult to dissolve for intravenous injection has led to increased abuse of alternative opioids including oxymorphone (SAMHSA, 2010). The potential use of the OXY-KLH vaccine to block oxymorphone distribution and effects is therefore of interest. Similarly, the relatively low $IC_{50}$ values of both the M-KLH and OXY-KLH vaccines for hydrocodone suggest their efficacy might extend to this drug as well.

Antibodies generated by both vaccines showed some cross-reactivity as measured by binding to immunogen conjugates on ELISA plates. Cross-reactivity in the setting of a bivalent vaccine is beneficial, as it allows both immunogens to contribute to binding either 6-MAM or oxycodone. Antibody titers to each immunogen in the bivalent group were however higher than could be accounted for by ELISA cross-reactivity alone. Since

antibodies may bind differentially to the drug hapten alone compared to the corresponding hapten-linker immunogen (Tars et al., 2012), it is possible that there was additional cross reactivity of antibodies that was not detected by ELISA. The ELISA used immunogens with linkers attached, which may cover some epitopes that free drug in serum could nevertheless bind to. It is also possible that antibody affinity was enhanced in the bivalent group, but the generally similar $IC_{50}$ values for bivalent and monovalent vaccines suggest this was not the case.

Consistent with enhanced titers in the bivalent group, 6-MAM retention in serum was greater and distribution to brain lower compared to the monovalent M-KLH group. Morphine retention in serum was less in the bivalent group, perhaps because increased binding of 6-MAM led to less conversion to morphine. Oxycodone concentrations showed a similar but non-significant trend toward greater efficacy in the bivalent group. Nevertheless, the most important result from the drug level measurements is confirmation that the two vaccines maintained their efficacy and did not interfere with each other when combined.

A limitation with available nicotine and cocaine vaccines has been the high variability in antibody titers and concentrations they produce in both animals and humans, with some subjects having levels too low to produce their desired effect (Kantak et al., 2001; Martell et al., 2009; Haney et al., 2010; Hatsukami et al., 2011; Pravetoni et al., 2011b). The absence of such non-responders to M-KLH or OXY-KLH vaccines, and the very high serum antibody concentrations produced, suggests that their efficacy may be more uniform than that of nicotine or cocaine vaccines. Morphine-conjugate vaccines studied by others have also reported uniformly high antibody titers or concentrations and it may

be that opioids in general are particularly effective haptens compared to nicotine or cocaine (Anton et al., 2009; Stowe et al., 2011).

A cautionary note regarding the high serum antibody concentrations produced by opioid vaccines is that it is often more difficult to achieve very high serum antibody concentration in humans than it is in experimental animals. The reasons for this are not entirely clear. The current study used the i.p. route and Freund's adjuvant in rats, which are not appropriate for use in humans. However, vaccination of mice by the s.c. route with alum adjuvant, which is acceptable in humans, generated anti-oxycodone antibody titers as high as vaccination i.p. with Freund's. Other heroin or morphine vaccine studies have also successfully used the s.c. route and alum adjuvant. Whatever the reason, it may be challenging to produce serum antibody concentrations in humans as high as those measured in the current study. Because antibody concentrations were uniformly high, our data do not directly comment on whether such high concentrations were necessary to achieve substantial efficacy. However magnitude of effect did correlate with antibody titer or concentration within the range of values measured, and this will be an important question to address.

A limitation of this study is that animals received only a single dose of opioid rather than repeated or chronic administration, and only one dose size. Because serum antibody concentrations were quite high, the calculated number of antibody drug-binding sites provided by M-KLH vaccination (0.98 µmol/kg) exceeded the dose of 6-MAM administered (0.32 µmol/kg). It may be more challenging to block the effects of higher or repeated 6-MAM doses. Also, effects of vaccination on opioid pharmacokinetics were evaluated but vaccine effects on opioid-induced behaviors were not. However the OXY-

KLH vaccine, used alone, has already been shown to reduce oxycodone distribution to brain at a 5-fold higher i.v. oxycodone dose and to block hot plate analgesia induced by a 20-fold higher s.c. oxycodone dose (Pravetoni et al., 2012a). In addition, several morphine-conjugate vaccines have been shown to block heroin or morphine self-administration in rats, which involves repeated drug dosing (Anton and Leff, 2006; Stowe et al., 2011). These questions need to be addressed for the bivalent vaccine, but similar efficacy seems likely.

The possible role of vaccines in addiction treatment is unclear because these vaccines are in early stages of development, and the vaccines that have reached clinical trials to date have not had satisfactory immunogenicity (Hatsukami et al., 2011). It is unlikely that addiction vaccines will replace current therapies but they could serve as useful adjuncts. An opioid vaccine could be useful for opioid abusers who refuse agonist or antagonist treatment or who do not qualify for it because their use is occasional. Opioid vaccines may also be usable in combination with agonist therapy to provide efficacy during periods of agonist medication noncompliance or for those who persistently abuse opioids even while on agonist therapy. Opioid vaccines like M-KLH and OXY-KLH have been designed so that they target the desired opioids but not opioids which may be needed for therapeutic use such as methadone, buprenorphine or naltrexone. The utility of this approach is not known, but highly immunogenic vaccines provide a tool for studying its potential. A bivalent vaccine which blocks a wide range of abusable opioids may have distinct advantages in areas of the world, like the USA, in which many different opioids are available and abused.

## TABLES AND FIGURES

### TABLES

**Table 1.** Serum antibody titers (x $10^3$) and cross-reactivity (%)[#]

| Coating immunogen | Vaccine | | |
| --- | --- | --- | --- |
| | M-KLH | OXY-KLH | M-KLH + OXY-KLH |
| M-BSA | 140 ± 47 | 18 ± 14 (18%) | 325 ± 220* |
| OXY-OVA | 29 ± 17 (21%) | 102 ± 78 | 239 ± 172* |

# Cross-reactivity for each monovalent vaccine was calculated as the ratio of titer against the non-target immunogen/target immunogen.

* $p < 0.05$ v. monovalent control

**Supplemental Table 1.**  Competitive ELISA IC$_{50}$ values (µM)

| | Vaccine | | | |
|---|---|---|---|---|
| | **M-KLH** | **M-KLH + OXY-KLH** | **OXY-KLH** | **M-KLH + OXY-KLH** |
| | Coating immunogen M-BSA | | Coating immunogen OXY-OVA | |
| Heroin | 1.7 | 0.15 | 1.8 | 6.8 |
| 6-MAM | 0.3 | 0.08 | 12.7 | 11.5 |
| Morphine | 0.06 | 0.11 | 9.08 | 13.8 |
| Oxycodone | 16.0 | 6.4 | 0.03 | 0.07 |
| Oxymorphone | 12.3 | 7.2 | 0.002 | 0.01 |
| Hydrocodone | 0.04 | 0.11 | 0.13 | 0.03 |
| Hydromorphone | 0.8 | 0.17 | 2.5 | 2.2 |

**FIGURES**

**Figure 1**. Morphine, oxycodone, their immunogens and linker structures.



M

M(Gly)$_4$

(Gly)$_4$

OXY

OXY(Gly)$_4$
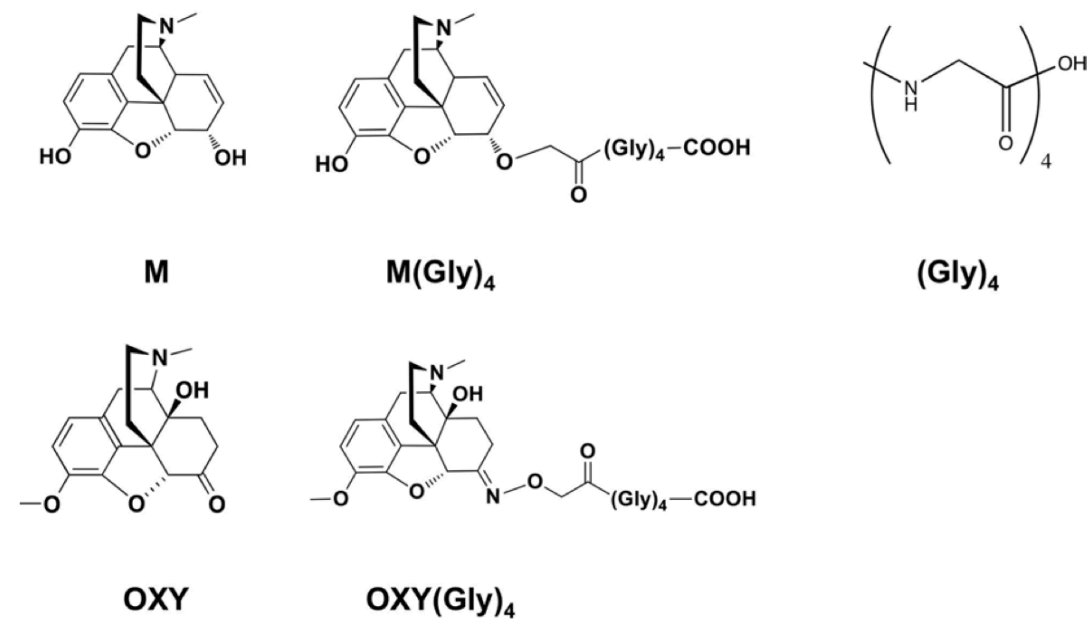
**Figure 2**. (Upper panel) Relationship of log serum morphine-specific and oxycodone-specific antibody titers in the bivalent vaccine group showing a significant positive correlation. (Lower panel) Serum 6-MAM and oxycodone concentrations in the bivalent group were also correlated.
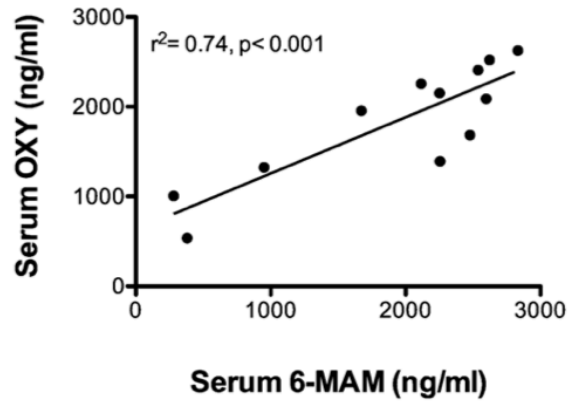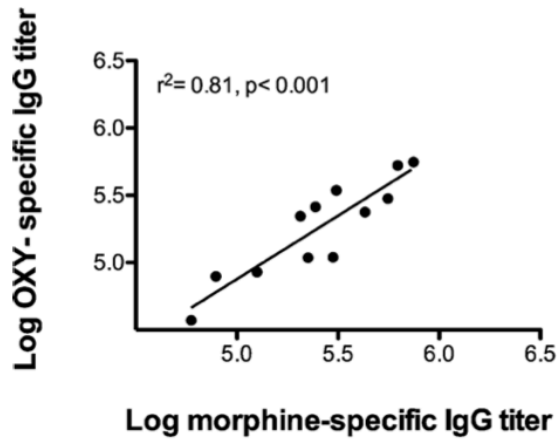
**Figure 3**. Vaccine effects on (upper panel) 6-MAM retention in serum, (middle panel) morphine retention in serum, and (lower panel) 6-MAM distribution to brain. Note 10-fold different vertical axis scales for 6-MAM and morphine serum concentrations. Numbers show the % decrease compared to the KLH control group brain concentrations. Morphine distribution to brain is not shown because concentrations were below the assay detection limit. Data are the mean±SD. * p < 0.05 compared to KLH control, # p< 0.05 bivalent compared to monovalent vaccine.
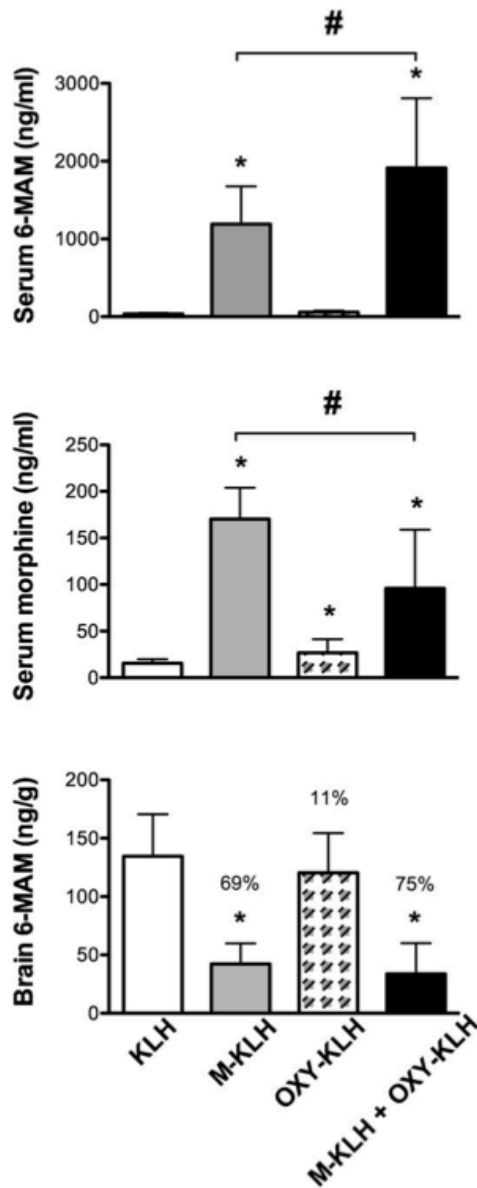
**Figure 4**. Vaccine effects on oxycodone concentrations in serum (upper panel) and brain (lower panel). Numbers show the % decrease compared to the KLH control group. Data are the mean±SD, * p < 0.05 compared to KLH control.
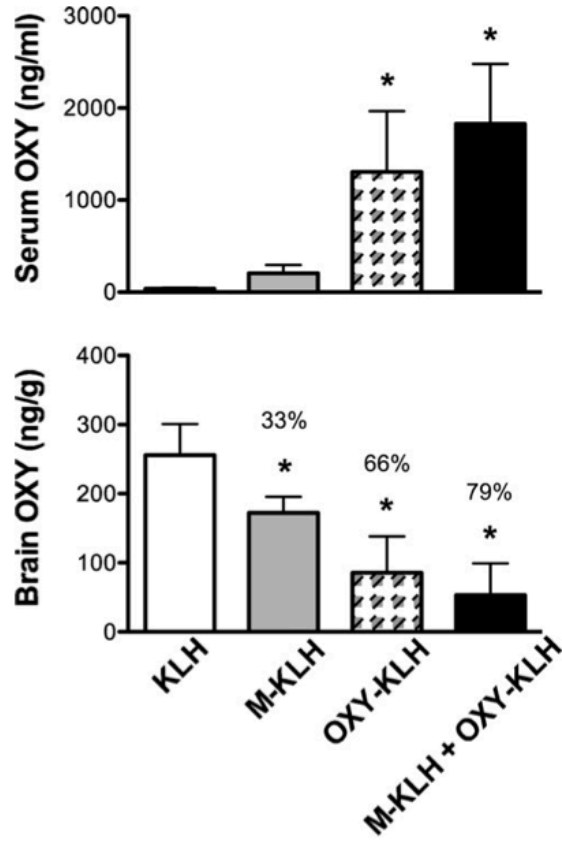
**Figure 5**. Relationships between log serum antibody titers and serum or brain 6-MAM

(A) or oxycodone (B) concentrations. Brain concentrations of drug for the KLH control

group are also shown (white open circle). Separate regression lines are shown for

monovalent and bivalent vaccines in each panel, and their slopes did not differ

significantly between groups.
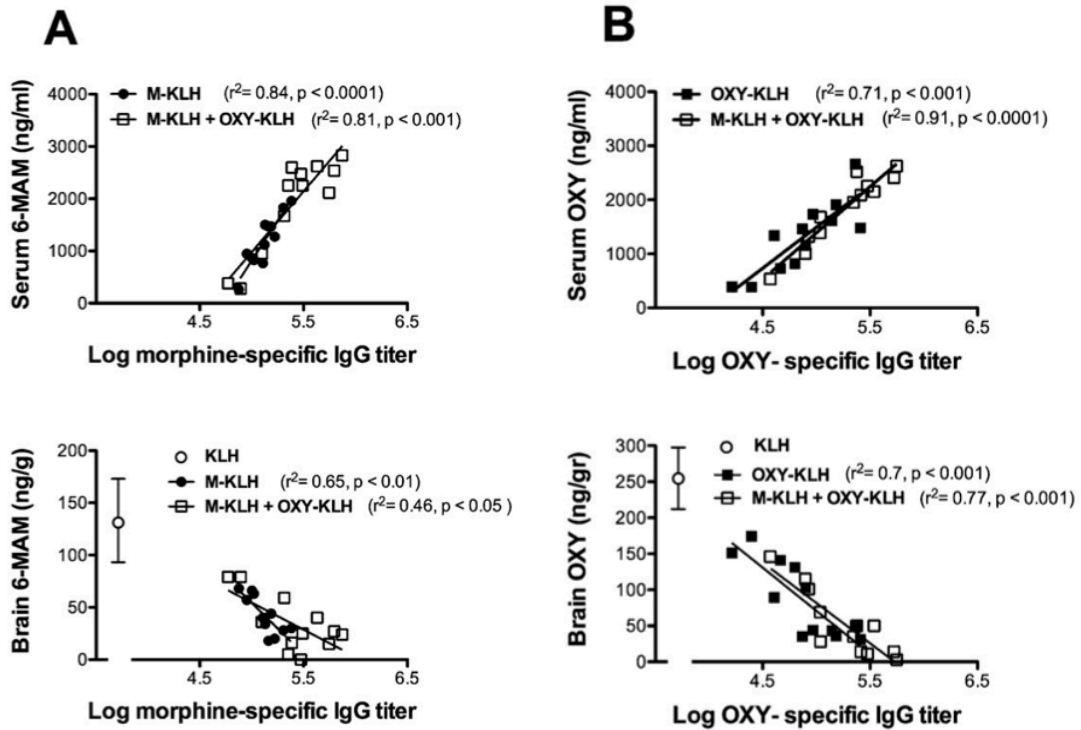
**Supplemental figure 1.** Synthesis of morphine hapten.
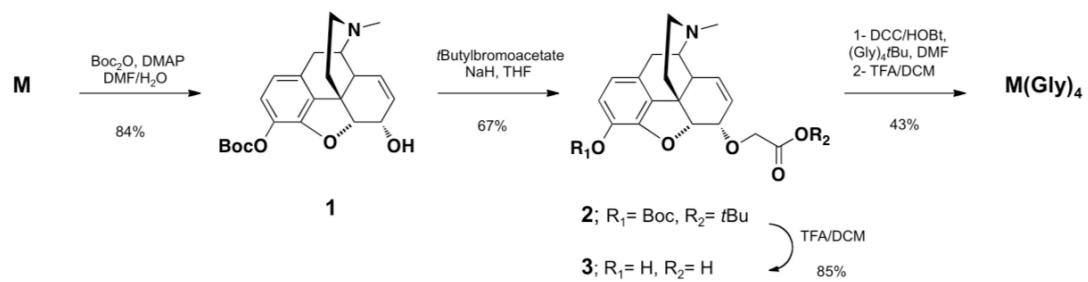
**Supplemental figure 2.** Relationship between morphine-specific serum IgG antibody concentrations and brain 6-MAM concentrations in the monovalent M-KLH group. Open circle is the mean ± SD brain 6-MAM concentrations in KLH controls
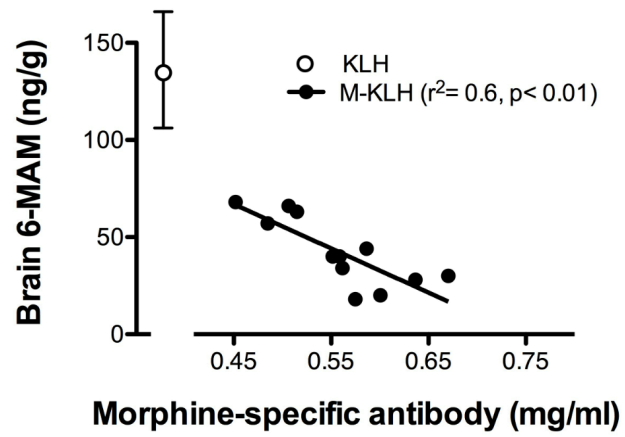
# CHAPTER 6

## Conclusions

This chapter provides conclusions for this thesis.

**1.    Summary/overview**

Immunotherapy as a treatment for drug addiction was first studied using a morphine-conjugate vaccine but little is known about how these vaccines elicit their effects on heroin-induced behaviors. Published data on nicotine and cocaine vaccines show that they reduce drug-induced behaviors by 1) retaining drug in serum after acute administration and preventing distribution to brain, 2) slowing distribution of drug to brain after chronic administration, and 3) slowing elimination of drug (nicotine vaccines). Nicotine and cocaine vaccines have simpler targets than morphine conjugate vaccines because only the parent compound is active. Studying the mechanism of action of morphine-conjugate vaccines, however, is much more difficult because heroin's distribution and metabolism is complex, heroin breaks down rapidly in tissues, and i.v. distribution studies in naïve animals had not previously been performed.

The overall goal of this thesis was to study how morphine-conjugate vaccines reduce the behavioral effects of heroin and to extend their use to other abused opioids. The specific aims of this thesis addressed questions related to 1) the stability of heroin in rat tissues, 2) acute distribution of heroin and its metabolites in both non-vaccinated and vaccinated rats, 3) the effect of vaccination on heroin-induced behaviors, and 4) the effect of combining M-KLH with Oxy-KLH on the efficacy of the individual vaccines.

**2.    Implications of findings**

2.1    Aim 1:

A highly reproducible and validated assay to measure heroin and its metabolites was developed in this dissertation in order to have confidence in the drug levels reported. Ensuring accurate drug levels makes it easier to interpret data from drug distribution

studies and subsequently determine the mechanism of action of morphine-conjugate vaccines. Maintaining NaF at 4 mg/ml, processing samples through solid phase extraction, and storing samples as dry pellets at -80°C, produced the most stable storage conditions for heroin in rat tissues, with a maximum recovery in heroin-spiked blood and brain tissues at 68% and 100%, respectively. Although 100% of heroin could not be recovered in blood, all groups of animals (M-KLH and controls) that receive heroin should be affected equally. Also, because drug levels in brain may provide more insight into vaccine efficacy, and because heroin and its metabolites appear to be more stable in brain tissues, heroin stability in blood may not be as critical. These studies provided conditions that would allow for more accurate measurements of heroin and its metabolites in subsequent drug distribution studies.

## 2.2    Aim 2:

Understanding the relative levels of heroin and its metabolites in rat tissues after intravenous heroin administration may provide insight into the important mediators of heroin's behavioral effects and the important targets for heroin/morphine vaccines. 6-MAM was the predominant opioid in plasma and brain in non-vaccinated rats for up to 14 minutes after i.v. administration of 0.26 mg/kg heroin. These data are consistent with previous studies (Way et al., 1960; Andersen et al., 2009), despite differences in route of administration and species, and support a critical role of 6-MAM in mediating the early effects of heroin. This suggests that targeting and reducing 6-MAM distribution to brain is important for a heroin vaccine to be effective.

## 2.3     Aim 3:

To our knowledge this is the first study that demonstrates altered distribution of heroin and its metabolites in the presence of vaccine-generated antibodies. The key finding of this study is that vaccine-generated antibodies likely reduce the effects of heroin by binding 6-MAM, not heroin, in plasma and prevent 6-MAM distribution to brain. This suggests that effective heroin/morphine vaccines should generate antibodies that are selective for 6-MAM. Further support for the importance of 6-MAM binding was shown using a heroin vaccine composed of hapten conjugated to protein through the bridge nitrogen. This vaccine generated antibodies with a higher affinity for 6-MAM than heroin yet blunted heroin analgesia in rats (Stowe et al., 2011). Another immunogen, which had a lower affinity for 6-MAM than heroin, was not as effective (Stowe et al., 2011). Understanding the importance of binding and retaining 6-MAM in plasma to reduce heroin's effects may lead to better heroin/morphine vaccine design if the current vaccines are not effective when translated to humans.

## 2.4     Aim 4:

Vaccination with M-KLH reduced anti-nociception from heroin, oxycodone, or methadone, but not fentanyl or nicotine showing that M-KLH binds to certain opioids, but leaves some opioids available for pain management (such as fentanyl). Vaccination also reduced heroin-induced locomotor activity for over 2 weeks, supporting the long-term efficacy of immunotherapy for the treatment of heroin addiction. Vaccination was still effective even after administration of heroin doses that equaled or exceeded the estimated binding capacity of antibodies. Although a distinct threshold for effective antibody concentrations was not apparent, the combined distribution (above, from 2.3) and behavioral studies suggest that antibody concentrations above 0.2 to 0.3 mg/ml

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produced substantial effects. No other such estimates are available for morphine conjugate vaccines, but this range is generally consistent with previous data for nicotine or cocaine vaccines in rodents (Kantak et al., 2000; Cornish et al., 2011). This provides a target antibody concentration for effective heroin/morphine vaccines when translated to humans.

## 2.5    Aim 5:

Efficacy of M-KLH and Oxy-KLH was retained in animals vaccinated with both immunogens, suggesting that opioid vaccines may be combined in addicts that may switch to other opioids. There were significantly higher titers in the combination group compared to the individual groups and a trend for greater effects on opioid distribution, which suggests that combining opioid vaccines may improve vaccine immunogenicity. This may result in a greater number of individuals that respond to the vaccine when translated to humans, where generating effective antibody levels have been difficult to achieve.

## 3.    Translation of morphine-conjugate vaccines to humans

Morphine-conjugate vaccines have been shown to reduce heroin-self-administration in monkeys and rats and reduce heroin-induced locomotor activity and anti-nociception in rodents, but no mechanistic requirements have been shown. A better understanding of how morphine-conjugate vaccines mediate their behavioral effects will be beneficial when translating to humans. Establishing antibody concentrations that are required to reduce the reinforcing effects of heroin across a range of doses will provide useful parameters to guide effective vaccines in humans. Results generated in this thesis act as a basis for understanding how morphine vaccines function. However, some barriers

regarding their translation to human use exist and will be described in the following section.

### 3.1    Improving vaccine efficacy

Data presented in this thesis showed that antibody concentrations of 0.2 – 0.3 mg/ml were associated with reduced 6-MAM distribution to brain and reduced behavioral effects of heroin. Achieving antibody concentrations this high in humans may be challenging. In one nicotine vaccine clinical trial only 30% of 301 patients achieved significant antibody levels (Hatsukami et al., 2011). However, this group did have a higher smoking cessation rate than low responders, suggesting that it is possible to achieve sufficient antibody concentrations that block drug-reinforcing effects in humans. Improving vaccine efficacy is still under investigation, but a number of strategies have been examined.

First, monoclonal antibodies have been administered i.v. to abruptly increase antibody concentrations to a target level that will block drug effects, which may be useful in boosting antibody protection in those who develop low antibody levels (Cornish et al., 2011). However, this method is currently far too expensive to implement. Second, using different adjuvants may increase vaccine immunogenicity and has shown greater efficacy using morphine-conjugate vaccines (Bremer and Janda, 2012; Matyas et al., 2013). Third, combining immunization with pharmacotherapies might reduce the need to achieve high antibody concentrations and has been shown to enhance efficacy in rodents (LeSage et al., 2012). This is currently being studied in a phase II clinical trial using a nicotine vaccine as well (Hoogsteder et al., 2012). Finally, combining structurally distinct vaccines towards similar targets (as in Aim 5) may improve immunogenicity of

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individual vaccines, suggesting that this might be a useful strategy to increase antibody concentrations in humans.

3.2    Abused drug doses and multiple injections

This thesis explored vaccine effects using either i.v. heroin doses that were rewarding in heroin-dependent individuals (Comer et al., 1999) or s.c. heroin doses that produced behavioral effects in rats. However, the i.v. doses given to rats were lower than doses that might be abused by addicts (0.26 mg/kg vs 2.5 mg/kg, respectively) and doses given s.c. may subject heroin to increased peripheral degradation and slower distribution to brain compared to the i.v. route used by humans. Also, heroin was only administered as acute, single doses. It remains unclear how effective morphine vaccines will be in the context of chronic heroin dosing. However, other similar morphine-conjugate vaccines have been shown to reduce heroin self-administration, which involves more frequent heroin dosing (Bonese et al., 1974; Anton and Leff, 2006). A similar morphine vaccine blocked heroin-induced motor activity, and these effects are predictive of efficacy in heroin self-administration in rats (Li et al., 2011). It will be important to understand how effective morphine vaccines will be in the face of chronic and large bolus doses used by heroin addicts.

**4    Concluding remarks**

Heroin addiction is a complex disease with environmental, social, and pharmacological factors that contribute to the continued abuse of heroin. Current pharmacotherapies are effective at reducing cravings and reducing the reinforcing efficacy of heroin but are inconvenient, have abuse potential, and side effects that lead to less than 20% of heroin addicts benefiting from these therapies. Alternative treatment options should be

explored. Immunotherapy as a means of altering drug pharmacokinetics has been explored for a number of drugs of abuse and has demonstrated some efficacy in humans that achieve high antibody concentrations. Morphine vaccines have shown great promise in animal models despite the complex pharmacokinetic profile of heroin and may eventually benefit those seeking an alternative treatment for heroin addiction. This thesis provides a mechanism of action through which morphine vaccines reduce the behavioral effects of heroin, provides critical parameters (such as antibody concentrations) required to achieve these effects, and explored the use of combining opioid vaccines to broaden their efficacy to other abused opioids. Future studies examining M-KLH vaccine efficacy in a heroin self-administration model, after repeated doses, and after large heroin doses may build on the current findings to evaluate the feasibility of using vaccines targeting heroin in humans.

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