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Title Page

Opioid dose- and route-dependent efficacy of oxycodone and heroin vaccines in rats

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Running Title Page

Efficacy of vaccines for opioid use disorders

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Abbreviations: 6-acetylmorphine (6-AM), arterial oxygen saturation (SaO_2), enzyme-linked immunosorbent assay (ELISA), keyhole limpet hemocyanin (KLH), Opioid use disorder (OUD), percent maximum possible effect (%MPE)

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Abstract

Heroin and oxycodone abuse occurs over a wide range of drug doses and by various routes of administration characterized by differing rates of drug absorption. The current study addressed the efficacy of a heroin vaccine (M-KLH) or oxycodone vaccine (OXY-KLH) for reducing drug distribution to brain after i.v. heroin or oxycodone, or s.c. oxycodone. Rats immunized with M-KLH or KLH control received an i.v. bolus dose of 0.26 or 2.6 mg/kg heroin. Vaccination with M-KLH increased retention of heroin and its active metabolites 6-acetylmorphine (6-AM) and morphine in plasma compared to KLH controls, and reduced total opioid (heroin + 6-AM + morphine) distribution to brain but only at the lower heroin dose. Immunization also protected against respiratory depression at the lower heroin dose. Rats immunized with OXY-KLH or KLH control received 0.22 or 2.2 mg/kg oxycodone i.v., the molar equivalent of the heroin doses. Immunization with OXY-KLH significantly reduced oxycodone distribution to brain after either oxycodone dose, although the magnitude of effect of immunization at the higher oxycodone dose was small (12%). By contrast, vaccination with OXY-KLH was more effective when oxycodone was administered s.c. rather than i.v., reducing oxycodone distribution to brain by 44% after an oxycodone dose of 2.3 mg/kg. Vaccination also reduced oxycodone-induced antinociception. These data suggest that the efficacy of OXY-KLH and M-KLH opioid vaccines is highly dependent upon opioid dose and route of administration.

Introduction

Opioid use disorders (OUD) are a public health burden affecting over 30 million people worldwide (UNODC, 2016). In the United States, over 2.5 million people are dependent on heroin and prescription opioids and OUD was associated with over 33,000 overdose deaths in 2015 (Paulozzi, 2012; NSDUH, 2014; Rudd et al., 2016; UNODC, 2016; Dowell et al., 2017). Medications to treat opioid abuse are available and effective, but less than 30% of individuals with OUD are receiving them (NSDUH, 2014). Treatment with the opioid receptor agonists methadone and buprenorphine is complicated by their abuse liability, potential diversion, and strict administrative regulations (Rosenberg and Phillips, 2003; Appel et al., 2004). Treatment with the opioid antagonist naltrexone can complicate pain management and requires detoxification prior to initiation of treatment. Immunization against abused opioids is being considered as an alternative or complementary option to pharmacotherapy. Opioid vaccines act by producing antibodies that bind the targeted opioid in blood and extracellular fluid, and reduce their distribution to brain. The potential advantages of vaccination over current pharmacotherapies include being long-acting, non-addictive, and devoid of the side effects associated with opioid receptor ligands.

Therapeutic vaccines for heroin and prescription opioid abuse have shown efficacy in a wide range of pre-clinical models, demonstrating that immunization can reduce opioid distribution to the brain and attenuate opioid-related behaviors including self-administration, locomotor activation, and analgesia in mice, rats, and non-human primates (Bonese et al., 1974; Anton and Leff, 2006; Li et al., 2011; Stowe et al., 2011; Pravetoni et al., 2012b; Pravetoni et al., 2012c; Kosten et al., 2013; Pravetoni et al., 2013; Raleigh et al., 2013; Schlosburg et al., 2013; Li et al., 2014; Pravetoni et al., 2014a; Raleigh et al., 2014; Laudenbach et al., 2015; Bremer et al., 2017). However, vaccine efficacy is often tested in animals using immunization protocols

involving routes of administration (e.g. intraperitoneal) and adjuvants (e.g., Freund's complete adjuvant) that are not used in humans, or at opioid doses that are suitable for the animal models chosen but are at the lower end of the range that may be abused by humans. In addition, many studies employ only a single opioid dose size so that the impact of opioid dose on vaccine efficacy is difficult to assess.

The primary goal of this study was to compare the efficacy of two vaccines directed against heroin (M-KLH; morphine hapten conjugated to keyhole limpet hemocyanin) or oxycodone (OXY-KLH; oxycodone hapten conjugated to KLH) in rats challenged with either a small or a large i.v. dose of the targeted opioid. The i.v. route for opioid dosing was examined because this is a common route of administration for abused heroin and occasionally for oxycodone as well. The i.v. route also represents the most rapid means of drug delivery and therefore the most rigorous test of vaccine efficacy. The highest opioid dose used (2.6 mg/kg) was chosen because it is within the range reportedly abused via the i.v. route by humans (Oviedo-Joekes et al., 2010), and this was contrasted with an opioid dose one tenth of that. For oxycodone, the s.c. route was also examined because oxycodone is most commonly abused by the oral route, which is characterized by slower drug absorption than the i.v. route. The oral route was not used to study oxycodone because its oral bioavailability in rats is low (Chan et al., 2008). The current study showed marked opioid dose-dependent efficacy for both M-KLH and OXY-KLH vaccines, as well as greater efficacy of OXY-KLH after s.c. than after i.v. oxycodone dosing.

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Materials and Methods

Animals

Male Holtzman rats (Harlan Laboratories, Madison, WI) weighing between 325-350 g at arrival were double housed under a 12/12-hr standard light/dark cycle and free-fed. Testing occurred during the light phase. These studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee. Surgery was performed under ketamine (75 mg/kg) and dexmedetomidine (0.05 mg/kg) anesthesia, animals were euthanized by CO_2 inhalation using AAALAC approved chambers, and all efforts were made to minimize suffering.

Drugs

Oxycodone and heroin were obtained through the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD) or Sigma-Aldrich (St. Louis, MO). Drug doses and concentrations are expressed as the weight of the base.

Vaccines against heroin and oxycodone

The study tested the efficacy of two analogous vaccines consisting of morphine or oxycodone-based haptens conjugated via a tetraglycine linker to keyhole limpet hemocyanin (KLH) and adsorbed to alum (Pravetoni et al., 2012b; Pravetoni et al., 2012c; Raleigh et al., 2013). *Experiments 1 and 2* used vaccines consisting of opioid haptens conjugated to native (didecamer) KLH (Thermo Fisher Scientific, Waltham, MA), *Experiment 3* used a vaccine consisting of an oxycodone hapten conjugated to KLH subunit dimer (Stellar Biotechnologies, Port Hueneme, CA) because of greater ease of formulation. We have found that native and dimer KLH conjugates of oxycodone elicit equivalent antibody responses and comparable effects on

opioid distribution (Pravetoni et al., 2014b). M-KLH generates antibodies specific for heroin, 6-AM, morphine, and morphine-6-glucuronide, but do not appreciably cross-react with methadone, buprenorphine, naloxone, naltrexone, oxycodone, or leu-enkephalin (Raleigh et al., 2013). OXY-KLH generates antibodies specific for oxycodone and oxymorphone, but do not appreciably cross-react with naloxone, naltrexone, methadone, buprenorphine, or leu-enkephalin (Pravetoni et al., 2012b).

Experimental design

Experiment 1: M-KLH vaccine efficacy following i.v. heroin dosing

Groups of 8 rats each were vaccinated i.m. with 25 μg of M-KLH (groups 1-3) or unconjugated KLH (groups 4-6) with 0.5 mg aluminum as aluminum hydroxide (Alhydrogel, Brenntag Biosector, Denmark) in a volume of 0.3 mL on days 0, 21, and 42 (Table 1). One week after the final vaccine dose, all groups were anesthetized and cannulated via the jugular vein, and blood removed for antibody characterization. To prevent potentially fatal heroin-induced respiratory depression, groups 2, 3, 5, and 6 then received naloxone 0.1 mg/kg i.v. One min later groups 2 and 5 received 0.26 mg/kg (0.7 μmol/kg) heroin and groups 3 and 6 received 2.6 mg/kg (7.0 μmol/kg) heroin i.v. We have previously shown that antibodies elicited by vaccination with M-KLH do not bind naloxone (Raleigh et al., 2013). However, to ensure that naloxone did not affect heroin and metabolite distribution, groups 1 and 4 receiving the lower heroin dose of 0.26 mg/kg heroin were pre-treated with saline rather than naloxone prior to administration of heroin. Pretreatment with saline in groups 1 and 4 also allowed assessment of vaccine effects on arterial oxygen saturation (SaO_2), which was measured every minute after saline administration by percutaneous oximetry (Nellcor OxiMax N-65, Medtronic, Minneapolis, MN). Rats were sacrificed 4 minutes after heroin administration, decapitated, and trunk blood and brain obtained to quantitate heroin, 6-AM, and morphine levels.

Experiment 2: OXY-KLH vaccine efficacy following i.v. oxycodone dosing

This experiment was identical to that of *Experiment 1* except as follows. 1) Groups of 8 rats were immunized i.m. with 25 µg of OXY-KLH (Groups 1 and 2) or unconjugated KLH (Groups 3 and 4) on days 0, 21, 42, and 63. The additional vaccine booster dose was used, based on our prior experience with this vaccine, to achieve satisfactory antibody levels. 2) Rats received 0.22 or 2.2 mg/kg oxycodone i.v., the molar equivalents of the heroin doses used in *Experiment 1.* Because naloxone had no effect on vaccine efficacy or opioid distribution in M-KLH treated animals, naloxone was administered to only the high oxycodone dose groups to prevent respiratory compromise.

Experiment 3: OXY-KLH efficacy following s.c. oxycodone dosing

Two groups of 6 rats each were vaccinated with OXY-KLH or KLH as in *Experiment 2*. On day 70, blood was collected via tail vein for antibody characterization. On day 77, both groups received 2.3 mg/kg oxycodone subcutaneously and were tested for hot plate nociception 30 min later. Immediately following testing, blood and brain were collected to measure oxycodone concentrations.

Drug level analysis

Heroin, 6-AM, and morphine concentrations were measured by liquid chromatography/mass spectrometry (Jones et al., 2013). Samples were analyzed within 3 days of extraction under conditions that minimized their degradation (Jones et al., 2013). In brief, trunk blood was collected in a syringe containing 4 mg/mL of ice-cold sodium fluoride and 100 IU/mL heparin (for ease of sample handling) and centrifuged immediately at 3100 x *g* for 3 minutes at 4 °C. Plasma was diluted 1:1 with ice-cold 10 mM formate buffer (pH 3.0) prior to extraction. Brains were rinsed with 10 mM formate buffer (pH 3.0) and four 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 no longer than 60 min at -20 °C until extraction.

Oxycodone concentrations were measured by gas chromatography/mass spectrometry (Pravetoni et al., 2013). Trunk blood was centrifuged at 3100 x *g* for 3 minutes at 4 °C and serum collected. Brain was rinsed with distilled H_2O and patted dry. Serum and brain were stored at -20 °C until analysis.

Antibody characterization

Antibody titers

To measure drug-specific serum IgG antibody titers, morphine or oxycodone haptens conjugated to bovine serum albumin were coated on ELISA plates at 5 ng/well in carbonate buffer pH 9.6 and blocked with 1% gelatin (Pravetoni et al., 2012c; Raleigh et al., 2013; Raleigh et al., 2017). Goat anti-rat IgG conjugated to horseradish peroxidase was used as secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Antibody affinity and concentration in serum

Serum antibody affinity for oxycodone was measured by ultrafiltration. A 20-fold dilution of serum was mixed 1:1 (v:v) with oxycodone at various concentrations in 0.9% NaCl. Samples were mixed by gentle rotation (5-10 RPMs) for 4 hr at room temperature and then placed in 10 kDa MWCO ultrafiltration tubes (PALL Nanosep with Omega membrane) and spun at 10,000 x g for 15 min. The total oxycodone concentration was measured from an aliquot obtained prior to ultrafiltration, and the unbound concentration measured from the ultrafiltrate. Bound oxycodone was calculated as the total minus unbound drug. The same procedure was used to measure morphine-specific antibody affinity. Because heroin is not stable in serum (Jones et al., 2013), morphine was used instead. Antibody affinity (Kd) was measured as half-maximum binding at

equilibrium and converted to molar concentrations using Prism v7.0 (GraphPad Software, Inc., La Jolla, CA). Opioid-specific antibody concentrations were calculated from the Bmax using a molecular weight of 150 kDa for IgG (Bazin-Redureau et al., 1997; Pravetoni et al., 2012b) and 2 binding sites per IgG.

Stoichiometry

The total number of moles per kilogram of opioid-specific IgG in rats vaccinated with M-KLH or OXY-KLH was estimated as the product of the measured antibody concentration in serum and the reported IgG volume of distribution (131 mL/kg) in rats (Bazin-Redureau et al., 1997; Pravetoni et al., 2012b).

Protein binding

The percent of oxycodone bound to protein in serum or morphine bound to protein in plasma (because blood collected for analysis had to be heparinized) in *Experiments 1* and *2* was measured by ultrafiltration. Serum or plasma from vaccinated animals was placed in 10 kDa MWCO ultrafiltration tubes and spun at 10,000 x *g* for 1 hr. The total drug concentration was measured from an aliquot of serum or plasma before ultrafiltration, and the unbound concentration measured as the ultrafiltrate. Bound opioid was determined as the total opioid minus unbound opioid concentrations. Because of technical issues, samples from two rats in the KLH group in *Experiment 2* were removed from analysis.

Saturation of serum opioid-specific antibody

The percent of serum morphine-specific antibody that was occupied by total opioid (heroin + 6-AM + morphine) was calculated from the ratio of the molar bound opioid concentration in serum (as determined by the protein binding assay described immediately above) divided by the antibody binding site concentration in serum (measured by the affinity assay). This measure

was obtained in *Experiment 1* for group 3 (M-KLH vaccinated rats receiving 2.6 mg/kg heroin i.v.). The same measure was obtained in *Experiment 2* for group 2 (OXY-KLH vaccinated rats receiving 2.2 mg/kg oxycodone i.v.).

Thermal nociception

Rats were habituated for 1 hr to the testing environment and nociception assessed on a hot plate (Columbus Instruments, Columbus, OH) set to 54°C ± 0.2 °C (Raleigh et al., 2013). A nociceptive response of hindpaw lick or jumping was measured as the latency to respond. Baseline hot plate responses were obtained 2 hr prior to drug dosing. A maximum cutoff of 60 sec was used to avoid injury. Vaccine efficacy in blocking opioid-induced antinociception was expressed as percent maximum possible effect (%MPE) calculated as (postdrug latency – predrug latency / maximum latency – predrug latency) x 100.

Statistics

All statistical analyses were performed using Prism v7.0. Mean antibody titers, opioid concentrations, and %MPE were compared using unpaired t tests. When variances were significantly different, Welsh's correction was applied. One-site specific binding was used to calculate Bmax and Kd from ultrafiltration data. For protein binding, log transformed unbound oxycodone concentrations were used to compare controls versus vaccine that received the low oxycodone dose due to the large difference in variance between groups. SaO_2 levels were compared using two-way ANOVA and Sidak's multiple comparison test.

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Results

Experiment 1: M-KLH vaccine efficacy following i.v. heroin dosing

Because the two groups pretreated with naloxone v. saline prior to receiving heroin 0.26 mg/kg did not differ with respect to any measures of plasma or brain opioid concentrations (Supplemental Figure 1), only the group pretreated with saline was used for further analyses. Morphine-specific antibody titers were comparable between the low and high heroin dose groups (180 ± 100 versus 160 ± 80 x 10^3, mean ± SD, respectively; $t = 0.441$, $p > 0.1$). The morphine-specific antibody concentration in pooled serum was 787 µg/mL (10.5 µM) and affinity for morphine was 13.9 nM (Supplemental Figure 2). The molar ratio of the low dose of heroin (0.26 mg/kg) to estimated antibody binding sites in vaccinated rats was 0.51 while the ratio of the high dose of heroin (2.6 mg/kg) to estimated antibody binding sites was 5.1.

M-KLH vaccinated rats from both the low and high heroin dose groups had significantly higher plasma concentrations of heroin and active metabolites compared to KLH controls (Fig. 1A-C). M-KLH vaccinated rats that received the low heroin dose had significantly lower 6-AM ($t = 4.232$, $p < 0.01$), but not heroin or morphine, concentrations in brain compared to controls (Fig. 1D-F). Vaccination with M-KLH did not alter heroin or metabolite brain concentrations in rats that received the high heroin dose compared to KLH controls. Total plasma opioid concentrations (heroin + 6-AM + morphine) were significantly increased in rats vaccinated with M-KLH following either the low or high heroin doses compared to KLH controls (Fig. 2A). Brain total opioid concentration was significantly decreased in rats vaccinated with M-KLH after the low heroin dose compared to KLH controls, but did not significantly differ from KLH controls after the high heroin dose (Fig. 2B, $t = 3.429$, $p < 0.01$).

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Vaccination with M-KLH increased the plasma protein binding of total opioid (Table 2). The unbound total opioid concentration in both low or high heroin dose groups was not changed by vaccination with M-KLH but the associated variances were high. The calculated saturation of opioid-specific antibody by total opioid (heroin + 6-AM + morphine) in serum was 68.8% in M-KLH vaccinated rats given the high heroin dose of 2.6 mg/kg.

Experiment 2: OXY-KLH vaccine efficacy following i.v. oxycodone dosing

Oxycodone-specific antibody titers were comparable between the low and high oxycodone dose groups (190 ± 50 and 145 ± 52 x 10^3, mean ± SD, respectively; t = 1.795 p = 0.094). The oxycodone-specific antibody concentration from pooled sera was 757 µg/mL (10 µM) and affinity for oxycodone was 14.5 nM (Supplemental Figure 2). The molar ratio of low dose of oxycodone (0.22 mg/kg) to estimated antibody binding sites in OXY-KLH vaccinated rats was 0.53 while the ratio of the high dose of oxycodone (2.2 mg/kg) to estimated antibody binding sites was 5.3.

Serum oxycodone concentrations were significantly increased in OXY-KLH vaccinated rats following i.v. dosing with 0.22 or 2.2 mg/kg oxycodone compared to KLH controls (Fig. 2C). Oxycodone concentrations in brain were significantly reduced in OXY-KLH vaccinated rats (by 52%) following the 0.22 mg/kg oxycodone dose compared to KLH controls (t = 8.531, p < 0.001) and, to a lesser extent (12%), following the 2.2 mg/kg oxycodone dose (Fig. 2D, t = 2.22, p < 0.05).

Vaccination with OXY-KLH increased the serum protein binding of oxycodone (Table 2). The unbound oxycodone concentration was significantly reduced by vaccination with OXY-KLH in the low oxycodone dose group compared to KLH controls, but was not altered by vaccination in the high oxycodone dose group. The calculated saturation of oxycodone-specific antibody by

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oxycodone in serum was 70.6% in OXY-KLH vaccinated rats given the high oxycodone dose of 2.2 mg/kg.

Experiments 1 and 2 opioid-induced respiratory depression

Vaccination with M-KLH prevented the decrease in arterial SaO_2 produced by the 0.26 mg/kg heroin dose (Fig. 3). Effects of vaccination on SaO_2 were not analyzed in the low dose oxycodone group because this oxycodone dose did not produce significant respiratory depression in KLH controls (data not shown). SaO_2 values in the high dose oxycodone or heroin groups were not compared because respiratory depression was prevented by pretreatment with naloxone.

Experiment 3: OXY-KLH vaccine efficacy following s.c. oxycodone dosing

Oxycodone-specific antibody titers ($210 \pm 51 \times 10^3$, mean ± SD) were comparable to those elicited in *Experiment 2* which examined vaccine effects on oxycodone administered i.v. Serum oxycodone concentrations were increased (by 620%) and brain oxycodone concentrations decreased (by 44%) compared to KLH controls (Fig. 4A; serum, $t = 10.06$, $p < 0.001$; brain, $t = 3.934$, $p < 0.01$). Vaccination with OXY-KLH also significantly reduced oxycodone-induced antinociception compared to controls by 60% (Fig. 4B; $t = 4.19$, $p < 0.01$).

Discussion

The key findings from this study were that: 1) both OXY-KLH and M-KLH reduced drug distribution to the brain after i.v. challenges with low opioid doses but vaccines were only minimally effective (OXY-KLH) or ineffective (M-KLH) against the higher i.v. opioid doses, 2) OXY-KLH was effective in blocking oxycodone distribution to brain, even with a high oxycodone dose, when immunized rats were challenged with oxycodone s.c. rather than i.v., and 3) M-KLH had a protective effect against heroin-induced respiratory depression. The data highlight the dose- and route-dependent efficacy of vaccination against opioids.

M-KLH and OXY-KLH produced very high serum antibody concentrations of 0.75 µg/mL, consistent with other reports of heroin and oxycodone vaccine immunogenicity in rats (Anton and Leff, 2006; Pravetoni et al., 2012c; Kosten et al., 2013; Raleigh et al., 2013; Pravetoni et al., 2014a; Jalah et al., 2015), although one report found even higher concentrations of up to 2.8 mg/mL (Stowe et al., 2011). These concentrations are higher than have generally been reported with nicotine or cocaine vaccines using similar linkers and carrier proteins suggesting that opioid-based haptens are particularly immunogenic, perhaps due to their larger size (Kantak et al., 2000; Keyler et al., 2008; Roiko et al., 2008; Pravetoni et al., 2012a). In clinical trials of nicotine or cocaine vaccines, serum antibody concentrations have been considerably lower than those elicited in animals and this likely contributed to their limited efficacy in reducing smoking or cocaine use (Martell et al., 2009; Hatsukami et al., 2011; Esterlis et al., 2013). If opioid vaccines prove more immunogenic than nicotine or cocaine vaccines in humans, this could substantially improve their chances of showing clinical efficacy against opioid abuse but this has to be addressed in humans.

In the current study the molar ratio of the oxycodone or heroin doses to antibody binding sites in vaccinated rats was approximately 0.5:1 at the lower opioid dose and 5:1 at the higher dose. The large excess of drug relative to antibody drug-binding capacity readily explains the lesser efficacy of the OXY-KLH and M-KLH vaccines at high drug doses. However, OXY-KLH was effective in reducing oxycodone distribution to brain when 2.3 mg/kg oxycodone was administered s.c., a dose similar to the high dose administered i.v. OXY-KLH also reduced opioid-induced antinociception in the hotplate assay at this same oxycodone dose given s.c., as previously reported (Pravetoni et al., 2014b). The expected slower absorption of oxycodone into blood after s.c. compared to i.v. administration was likely responsible for the increased vaccine efficacy despite the high oxycodone dose. In addition, the bioavailability of oxycodone administered s.c. to rats is 57% (Huang et al., 2005; Chan et al., 2008), similar to the bioavailability of oral oxycodone in humans (Poyhia et al., 1992), which would reduce the amount of drug presented to serum antibody compared to i.v. dosing. Because oxycodone is most commonly abused by the oral or intranasal routes (Jones et al., 2011), vaccination with OXY-KLH might be clinically useful for blocking oxycodone effects. On the other hand, heroin is most often abused by the i.v. route. Efficacy of M-KLH against heroin will likely depend heavily upon the heroin dose used. Extrapolation of rat data to humans is made more difficult for heroin because the main active components are its metabolites 6-AM and morphine. These metabolites are formed at different rates in humans compared to rodents (Comer et al., 1999; Rentsch et al., 2001; Gottas et al., 2013) and their rates of distribution to brain may also differ. These considerations underscore the need to advance opioid vaccines to human studies in order to properly assess their therapeutic potential.

Antibody affinity towards morphine (13.9 nM) or oxycodone (14.5 nM) was within the range of affinities measured by other groups (0.4 – 24 nM) using similar immunogens (Anton and Leff, 2006; Stowe et al., 2011; Torres et al., 2016; Kimishima et al., 2017). It is not clear whether

higher affinity antibodies would have performed substantially better. The calculated saturation of serum morphine-specific antibody with total opioid in rats receiving the higher heroin dose was already quite high (68.8%), as was the saturation of oxycodone-specific antibody with oxycodone (70.6%), so that enhancing this percentage by generating higher affinity antibodies would have contributed relatively little to the amount of drug bound in serum and the ability of these vaccines to reduce drug distribution to brain. By contrast, the total opioid or oxycodone concentrations in brain varied inversely with the drug-specific antibody titers, a surrogate for antibody concentration (Supplemental Figure 3). Enhancing the efficacy of vaccination would seem to require generating higher serum antibody concentrations more so than enhancing antibody affinity.

Heroin doses used in humans vary considerably. Heroin abusers report significant drug-liking effects in a clinical laboratory setting after single i.v. heroin doses as low as 0.09 – 0.36 mg/kg (Comer et al., 1999), which is similar to the low heroin dose used in the current study. In support of heroin's reinforcing effects at relatively low doses, subjects in a clinical laboratory study consumed 0.3-0.57 mg/kg/session when each heroin dose of 10 mg (approximately 0.14 mg/kg) was administered by pressing a lever at a fixed ratio of 300 (Mello and Mendelson, 1980; Mello et al., 1981). These doses are at the lower end of the range examined in the current study. On the other hand the typical i.v. heroin dose range used in heroin maintenance programs, presumably reflecting typical abuse rates, is 150-600 mg divided into 2 or 3 i.v. injections (Oviedo-Joekes et al., 2010; Strang et al., 2010). This wide range of doses from different settings may reflect individual differences in opioid tolerance. Thus M-KLH could be effective in reducing heroin effects in some individuals with heroin use disorder but is likely to be less effective in those abusing very high doses.

Abuse patterns for oxycodone differ from those of heroin. In a clinical laboratory study of nondependent opioid users, 20 mg (0.29 mg/kg) oxycodone administered i.v. (Backonja et al., 2016) or 30 mg (0.43 mg/kg) administered as intranasal powder (Harris et al., 2014) produced significant “drug liking” effects. These doses are at the lower end of the range administered to rats i.v. in the current study. Self-report of orally abused oxycodone doses in one survey were somewhat higher at 0.3 to 1.2 mg/kg, most often taken orally (Jones et al., 2011) but still within the range at which OXY-KLH showed efficacy in the current study against oxycodone administered s.c. Drug absorption following s.c. oxycodone, as in the current study, is likely more rapid than with oral dosing, suggesting that OXY-KLH would be at least as effective against orally dosed oxycodone. Overall, the current data suggest that OXY-KLH could be effective in reducing the effects of oxycodone at the doses and routes typically used by those with oxycodone use disorder.

Vaccination with M-KLH protected against heroin-induced respiratory depression in the low dose heroin group. M-KLH effects on respiratory depression were not studied in the high dose heroin group because they had received naloxone to prevent respiratory arrest and OXY-KLH effects were not reported for the low dose oxycodone group because controls did not show significant respiratory depression. However, OXY-KLH has been shown to reduce oxycodone-induced respiratory depression following a cumulative s.c. oxycodone dose of 9 mg/kg (Raleigh et al., 2017). Protection against opioid-induced respiratory depression is of note because respiratory depression is the principal mechanism of death from opioid overdose. While the opioid vaccines studied are not likely to protect against respiratory depression from very large opioid doses, vaccination might provide some protection to occasional or infrequent heroin users who have little or no opioid tolerance and use lower doses.

It should be noted that slightly different vaccine formulations were used in the different experiments, with oxycodone or heroin haptens conjugated to either native KLH (a didecamer) or dimer KLH, the latter used because it is available as a cGMP product. However, we have previously shown that nKLH or dKLH are equally effective as carrier proteins for the development of oxycodone vaccines (Pravetoni et al., 2014b) and in the current study both vaccine formulations elicited similar serum opioid-specific antibody concentrations.

In summary, these data highlight the potential efficacy of OXY-KLH or M-KLH for reducing the effects of oxycodone or heroin but also point out the dose and route-dependence of their efficacy. Because oxycodone is often abused at doses within the range examined in this study, and by the oral or intranasal routes which are characterized by slower absorption than i.v. dosing, OXY-KLH could be helpful in treating oxycodone use disorder and merits further study in clinical trials. The very high heroin doses that are often abused, and the predominance of the i.v. route of abuse, make the treatment of heroin abuse disorder with a vaccine more challenging. However, there is a very wide range of abused heroin doses, and heroin pharmacokinetics in humans differ from those of rodents, so further study of M-KLH and similar heroin vaccines remains of interest.

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Authorship Contributions

Participated in research design: Raleigh, Winston, Pentel, Pravetoni

Conducted experiments: Raleigh, Laudenbach, Baruffaldi, Peterson, Roslawski

Contributed new reagents or analytical tools: Roslawski, Birnbaum, Carroll, Runyon

Performed data analysis: Raleigh, Roslawski, Birnbaum, Pentel, Pravetoni

Wrote or contributed to the writing of the manuscript: Raleigh, Laudenbach, Baruffaldi, Peterson, Birnbaum, Carroll, Winston, Pentel, Pravetoni

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Footnotes

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Figure Legends

Figure 1. Effect of vaccination with M-KLH on heroin, 6-AM, and morphine concentrations after i.v. administration of a low or high heroin dose. Plasma (A - C) and brain (D - F) were collected 4 min following the end of the 1 min infusion of heroin. ** $p < 0.01$, *** $p < 0.001$ for the difference between M-KLH and KLH control groups. Numbers above bars represent the percent difference from controls. Unpaired t tests with Welch's correction were used to compare groups. Mean ± SD, n = 8/group.

Figure 2. Effect of vaccination with M-KLH or OXY-KLH on opioid concentrations after i.v. administration of low or high heroin or oxycodone doses. Plasma (A) and brain (B) were collected 4 min following the end of the 1 min infusion of heroin and serum (C) and brain (D) were collected 4 min following the end of the 1 min infusion of oxycodone. Plasma and brain drug concentrations after heroin administration represent total opioid (heroin + 6-AM + morphine), converted data from Figure 1 and presented here for comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the difference between vaccinated rats and KLH controls. Numbers above bars represent the percent difference from controls. Unpaired t tests with Welch's correction were used to compare groups. Mean ± SD, n = 8/group.

Figure 3. Effect of vaccination with M-KLH on arterial oxygen saturation (SaO_2) after i.v. administration of 0.26 mg/kg heroin. Heroin was infused i.v. for 1-min following saline pretreatment. Vaccination with M-KLH significantly blunted the decrease in SaO_2 seen in the KLH control group. Both groups had significantly lower $\%SaO_2$ levels following heroin administration ($p < 0.01$). ** $p < 0.01$ for the difference between M-KLH and KLH control groups. Two-way ANOVA with Sidak's multiple comparison test was used to compare groups. Mean ± SEM, n = 8/group.

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Figure 4. Effect of vaccination with OXY-KLH on oxycodone concentrations following subcutaneous administration of a high oxycodone dose. Serum and brain oxycodone concentrations (A) and oxycodone-induced antinociception (B) 30 minutes following s.c. administration of 2.3 mg/kg oxycodone. ** $p < 0.01$, *** $p < 0.001$ for the difference between OXY-KLH and KLH control groups. Numbers above bars represent the percent difference from controls. Unpaired t tests with Welch's correction were used to compare groups. Mean ± SD, n = 6/group.

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Tables

Table 1. *Experiment 1* treatment groups.

Group	Vaccine	Pretreatment	Heroin dose (mg/kg)
1	M-KLH	Saline	0.26
2	M-KLH	0.1 mg/kg naloxone	0.26
3	M-KLH	0.1 mg/kg naloxone	2.6
4	KLH	Saline	0.26
5	KLH	0.1 mg/kg naloxone	0.26
6	KLH	0.1 mg/kg naloxone	2.6

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Table 2. Protein binding data for *Experiments 1* and *2.* Drug concentration in plasma (total opioid; heroin + 6-AM + morphine) or serum (oxycodone) 4 min after i.v. administration of a low or high opioid dose. * $p < 0.05$ for the difference between vaccine and control groups given the same dose. Unpaired t tests were used to compare groups. Mean ± SD, n = 6 – 8/group.

		Drug concentration in plasma or serum		
	Heroin dose	**Total**	**Unbound**	**%Bound**
	μmol/kg (mg/kg)	*μM*	*μM*	
KLH			*plasma*	
	0.7 (0.26)	0.26 ± 0.09	0.12 ± 0.06	52.9 ± 11.7
	7.0 (2.6)	2.75 ± 0.33	0.82 ± 0.17	70.0 ± 6.7
M-KLH				
	0.7 (0.26)	4.03 ± 1.20*	0.08 ± 0.13	97.0 ± 5.8*
	7.0 (2.6)	8.17 ± 1.94*	0.87 ± 0.23	88.5 ± 5.1*
	Oxycodone dose			
	μmol/kg (mg/kg)			
KLH			*serum*	
	0.7 (0.22)	0.21 ± 0.04	0.17 ± 0.03	18.8 ± 2.5
	7.0 (2.2)	2.85 ± 0.43	2.37 ± 0.26	15.1 ± 15.2
OXY-KLH				
	0.7 (0.22)	5.97 ± 1.97*	0.10 ± 0.10*	97.9 ± 2.1*
	7.0 (2.2)	9.63 ± 2.48*	2.34 ± 0.51	74.1 ± 9.8*

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Figure 1

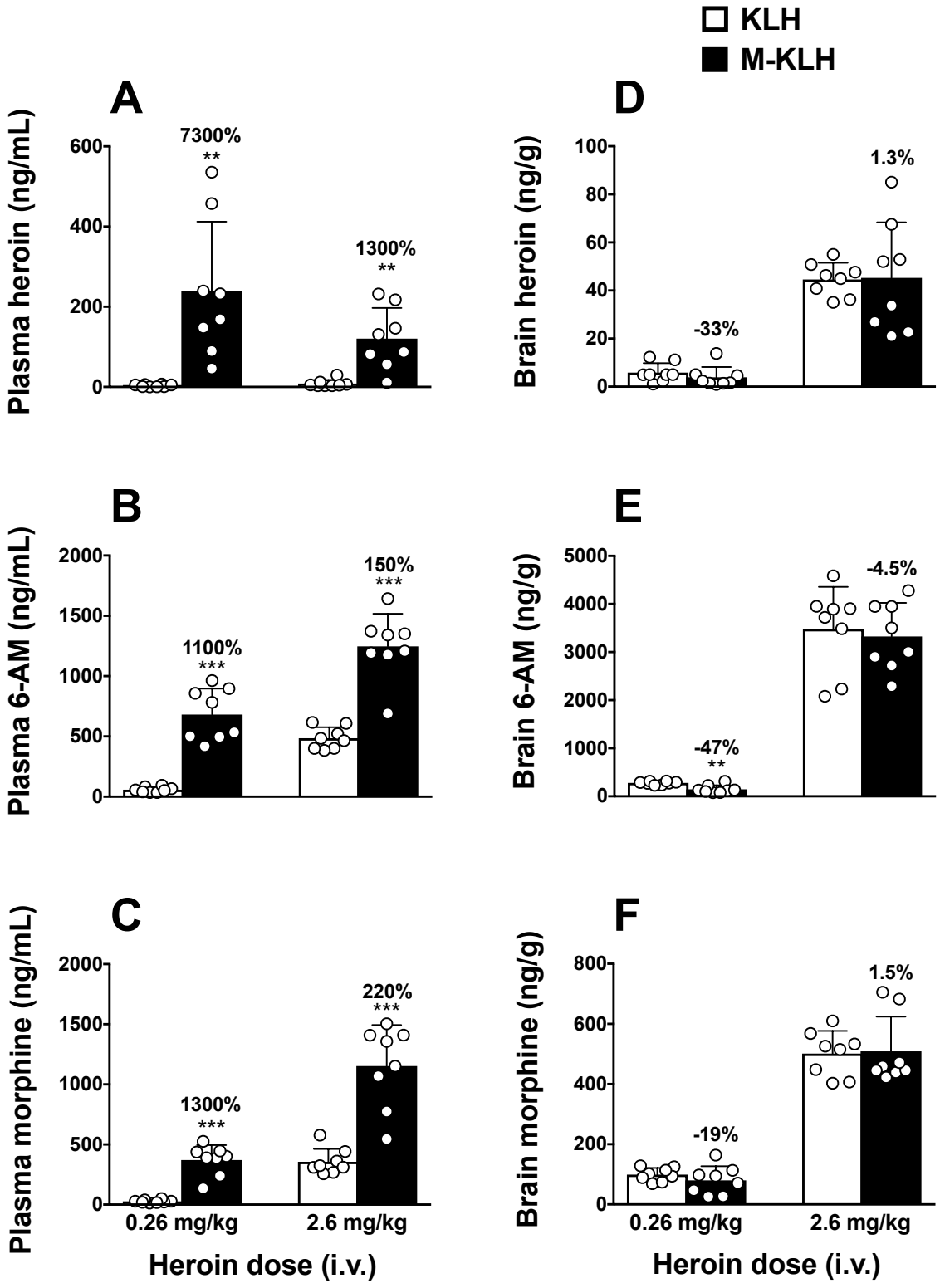

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Figure 2

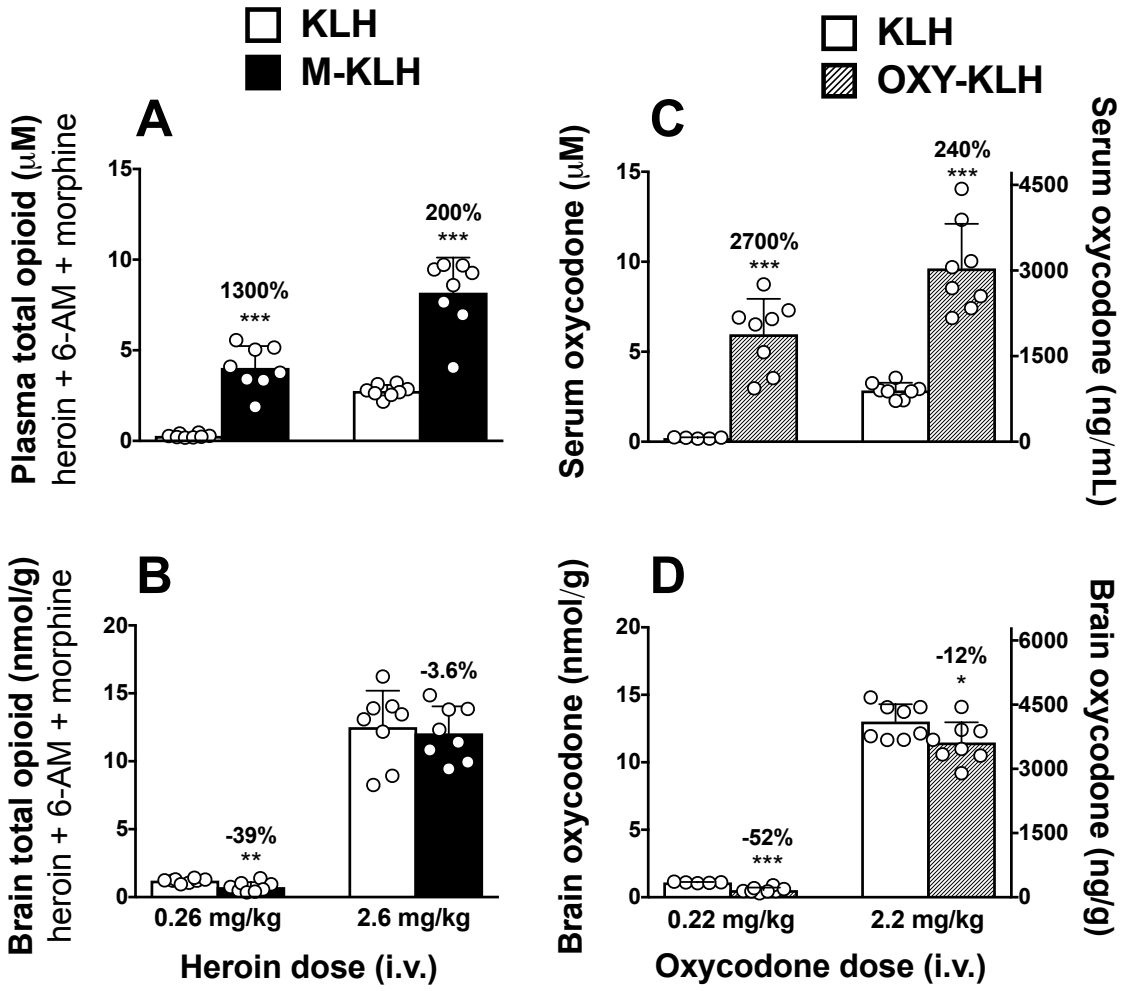

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Figure 3

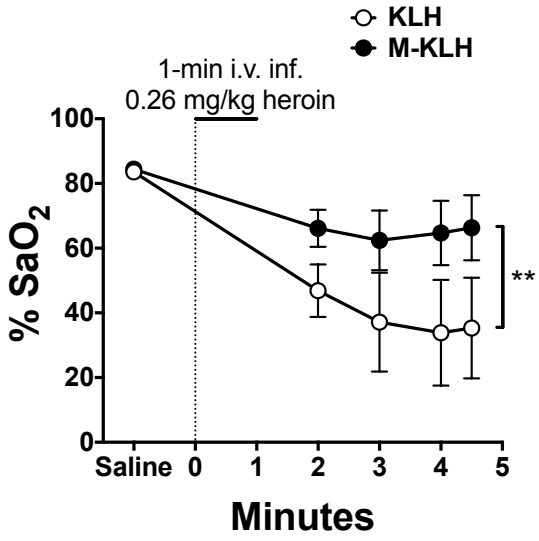

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Figure 4