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An oxycodone conjugate vaccine elicits oxycodone-specific antibodies that reduce oxycodone distribution to brain and hot-plate analgesia

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Characterization of a novel oxycodone conjugate vaccine

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ABSTRACT

Opioid conjugate vaccines have shown promise in attenuating the behavioral effects of heroin or morphine in animals. The goal of this study was to extend this approach to oxycodone (OXY), a commonly abused prescription opioid. Haptens were generated by adding tetraglycine $(Gly)_4$ or hemisuccinate (HS) linkers at the 6 position of OXY. Immunization of rats with OXY$(Gly)_4$- conjugated to the carrier proteins BSA or KLH produced high-titer antibodies to OXY and its metabolite oxymorphone with substantially lower IC_{50} values for other structurally-related opioid agonists and antagonists. There was no measurable binding of antibody by the $(Gly)_4$ linker alone or off-target opioids methadone and buprenorphine. OXY(HS) conjugates were less immunogenic despite achieving protein haptenation ratios comparable to OXY$(Gly)_4$-BSA. In rats given a single intravenous dose of OXY, immunization with OXY$(Gly)_4$-KLH increased OXY protein binding and retention in serum while decreasing its unbound (free) concentration in plasma and distribution to brain. Vaccine efficacy correlated with serum antibody titers and it was greatest in rats given the lowest OXY dose (0.05 mg/kg) but was significant even after a larger OXY dose (0.5 mg/kg) equivalent to the high end of the therapeutic range in humans. These effects of OXY$(Gly)_4$-KLH on drug disposition were comparable to those of nicotine or cocaine vaccines that are in clinical trials as addiction treatments. Immunization with OXY$(Gly)_4$-KLH also reduced OXY analgesia in a thermal nociception test. These data support further study of vaccination with the OXY$(Gly)_4$-KLH immunogen as a potential treatment option for OXY abuse or addiction.

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INTRODUCTION

There are an estimated 15 million users of opioids worldwide and 1.2 million in the U.S. Until recently heroin use predominated in the U.S., but over the past 10 years the abuse of prescription opioids has increased dramatically and is now more common than heroin abuse. The rise in prescription opioid abuse has been accompanied by a substantial increase in the incidence of emergency department visits and fatal opioid overdose. Oxycodone (OXY) is the most commonly abused prescription opioid (Compton and Volkow, 2006; Lopez et al., 2009). Treatment options have been developed for heroin addiction, but fewer options have been studied for abuse of OXY or other prescription opioids. Agonist therapies for heroin addiction such as methadone and buprenorphine can be very effective, but their own abuse potential and risk of side effects obligate careful and frequent monitoring, and their therapeutic use is legally restricted to those regularly using substantial quantities of opioid over a sustained period of time (Fareed et al., 2011). Many prescription opioid abusers do not fit this profile because their opioid use is oral rather than i.v. and may be sporadic, yet they still run the risk of overdose, social disruption, and transition to i.v. drug use and addiction. Additional treatment options for prescription opioid abuse are needed (Stotts et al., 2009; Dodrill et al., 2011; Maxwell, 2011).

Vaccines are being studied as a potential adjunct to drug abuse or addiction treatment. They are of interest because they target the drug rather than the brain and therefore lack central nervous system side effects. Addictive drugs are too small to stimulate an immune response but can be rendered immunogenic by conjugation to a foreign carrier protein through a linker arm (Chi, 2011). Such conjugate vaccines stimulate the production of drug-specific antibodies that can bind their target drug in serum and

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extracellular fluid and reduce or slow its distribution to brain. Efficacy in blocking a wide range of addiction-like behaviors has been shown in animals for vaccines directed against nicotine, cocaine, methamphetamine and heroin (Chi, 2011). Nicotine and cocaine conjugate vaccines have entered clinical trials with some early evidence of efficacy and no important side effects (Martell et al., 2009; Hatsukami et al., 2011). A number of morphine vaccines have been developed which produce antibodies that cross react with heroin and its active metabolites, and which block or attenuate the behavioral effects of heroin or morphine in rodents. A desirable feature for such vaccines is that they not bind or block the actions of certain off-target opioids such as methadone or buprenorphine so that these can still be used therapeutically for treating opioid addiction or for analgesia (Wainer et al., 1973; Bonese et al., 1974; Anton and Leff, 2006; Anton et al., 2009; Stowe et al., 2011). Although the immunological and behavioral effects of heroin/morphine vaccines have been studied in animals their effects on opioid pharmacokinetics, which mediate their behavioral actions, have not been reported.

The goal of the current study was to synthesize and evaluate the immunologic and pharmacokinetic effects of candidate OXY conjugate vaccines in rats. Several linkers and carrier proteins were used to assess their immunogenicity and the influence of the degree of protein haptenation on vaccine efficacy. Effects of the lead candidate OXY$(Gly)_4$-KLH vaccine on OXY protein binding in serum, OXY distribution to brain and OXY-induced analgesia were evaluated to provide mechanistic information and to anticipate whether additional study of this vaccine is warranted. Effects of OXY$(Gly)_4$-KLH on OXY-induced hot plate analgesia were studied to measure the ability of this vaccine to block a centrally mediated opioid effect. The large observed effects of OXY$(Gly)_4$-KLH on OXY pharmacokinetics, and its ability to block OXY analgesia, support its further development as a potential adjunct to addiction treatment.

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METHODS

Overview of hapten synthesis (Scheme 1). The 6 position of OXY was selected for linker attachment because it has been used previously to generate effective antibodies to heroin and its active metabolites (Bonese et al., 1974; Anton and Leff, 2006). Two linkers were chosen for evaluation; hemisuccinate because it has been used successfully with heroin conjugate vaccines, and $(Gly)_4$ because preliminary studies using oligoglycine as a convenient means of studying homologous linkers of different lengths suggested that $(Gly)_4$ was the most promising of this series (data not shown).

Compound **1** was obtained as previously reported (Lamont JV, 2003) by the condensation of OXY with *O*-carboxymethoxylamine hemihydrochloride (2-(aminooxy)acetic acid) in refluxing methanol using pyridine as a base. This intermediate was coupled to tetraglycine tertbutyl ester (Gly_4tBu) (Bieniarz C, 1989), using N,N'-Dicyclohexylcarbodiimide (DCC)/ Hydroxybenzotriazole (HOBt) procedure followed by acid hydrolysis to afford $OXY(Gly)_4$ in moderate yield (52% overall). Compound **2** was prepared stereoselectively using a catalytic reduction of the imine formed from the oxycodone and benzylamine, followed by debenzylation (Sayre LM, 1980). Condensation of the intermediate **2** in a manner similar to that previously described for morphine 3-succinyl (Wainer et al., 1972) with succinic anhydride in refluxing pyridine gave OXY(HS) with good yield (82%). Compounds were characterized by ^1H NMR and Mass Spectrometry after purification. Purity of target haptens was determined by elemental analysis.

Reagents. All commercial reagents and anhydrous solvents were purchased from suppliers and were used without further purification or distillation, unless otherwise

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stated. Analytical thin-layer chromatography (TLC) was performed on plates coated with EM Science silica gel 60 F_{254} (0.25 mm). Compounds were visualized by UV light and/or stained with potassium permanganate solution followed by heating. Flash column chromatography was performed on EM Science silica gel 60 (230 – 400 mesh). NMR (^{1}H) spectra were recorded on a Bruker Avance 400 MHz spectrometer (Bruker, Billerica, MA) and calibrated using an internal reference. Chemical shifts are expressed in ppm and coupling constants (J) are in hertz (Hz). Peak multiplicities are abbreviated: broad, br; singlet, s; doublet, d; triplet, t; and multiplet, m. ESI mode mass spectra were recorded on a BrukerBioTOF II mass spectrometer. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ).

OXY(Gly)$_4$. DCC (1.3 equivalent), carboxylic acid **1** (0.15-0.4 mmol scale, 1 equivalent), and HOBt (1.2 equivalent) were dissolved in 5 mL of anhydrous dimethylformamide (DMF). The solution was cooled to 0°C and placed under a nitrogen atmosphere. After 15 minutes at 0°C, tetraglycine (0.15-0.4 mmol, 1 equivalent) was added. The solution was sealed under a nitrogen atmosphere and was allowed to warm and to stir at room temperature overnight. The reaction mixture was filtered to remove dicyclohexylurea into water (10x initial volume of DMF) and extracted with ethyl acetate. The combined organic layers were dried on magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silicon dioxide (SiO_2) chromatography (DCM/MeOH/NH_4OH : 94/5/1) to afford the desired compound as a pale yellow solid (63% yield). ^{1}H NMR (CD_3OD) δ: 1.39 (m, 1H); 1.49 (s, 9H); 1.62-1.88 (m, 2H); 2.42-2.81 (m, 7H with s, 3H, NCH_3 at 2.68); 3.03-3.19 (m, 2H); 3.37-3.42 (m, 8H); 3.53 (d, 1H, J= 9.4 Hz); 3.61 (m, 1H); 3.84 (s, 3H); 4.67 (s, 2H); 5.07 (s, 1H); 6.83 (d, 1H, $J_{H1\text{-}H2}$= 8.1 Hz); 6.89 (d, 1H, $J_{H2\text{-}H1}$= 8.1 Hz); ESI-TOF MS *m/z*: 673.789 (MH^+).

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To a solution of this ester (0.15-0.4 mmol scale) in DCM (5 mL) was added trifluoroacetic acid (TFA, 20% volume). 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 purified on reverse phase HPLC column (Haisil 100-C18, 5 µm, 10 mm × 250 mm) which was eluted with acetonitrile/H_2O/TFA (70:30:0.1) at a flow rate of 2 mL/min) to provide **OXY(Gly)$_4$** as a white solid (82% yield). The hapten was converted to the TFA salt to facilitate purification and recovery, and TFA was then removed by ion exchange prior to conjugation. ^{1}H NMR (CD_3OD) δ: 1.42 (m, 1H); 1.69-1.91 (m, 2H); 2.48-2.82 (m, 7H with s, 3H, NCH_3 at 2.67); 3.05-3.23 (m, 2H); 3.34-3.38 (m, 8H); 3.48 (d, 1H, J= 9.4 Hz); 3.61 (m, 1H); 3.85 (s, 3H); 4.63 (s, 2H); 5.04 (s, 1H); 6.81 (d, 1H, J_{H1-H2}= 8.1 Hz); 6.88 (d, 1H, J_{H2-H1}= 8.1 Hz); ESI-TOF MS *m/z*: 617.233 (MH^+); analytical calculation for $C_{28}H_{36}N_6O_{10}$: C, 54.54; H, 5.88; N, 13.63. Found: C, 54.25; H, 6.03; N, 12.72.

6α-Amino-14-hydroxydesocodeine (2). A benzene solution containing oxycodone base (2 g, 6.3 mmol), benzylamine (770 mg, 7.15 mmol), and a catalytic amount of *p*-toluenesulfonic acid (5% molar) was refluxed overnight, using a Dean-Stark Trap. The mixture was concentrated, and a solution of $NaBH_4$ (43 mg, 1.1 mmol) in absolute EtOH was added (Sayre LM, 1980). After being stirred under N_2 for 8 h, the resulting solution was diluted with H_20 and concentrated to remove most of the EtOH. Additional diluted NH_4OH was added and the aqueous layer extracted (3xDCM). Organic layers were then dried on magnesium sulfate and concentrated under reduced pressure. This residue was purified by flash chromatography using a DCM/MeOH/NH_4OH mixture (96/3/1) to give the benzylated intermediate as a white solid (78%). ^{1}H NMR ($CDCl_3$): 1.31-1.40 (m, 2H);

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1.52-1.58 (m, 2H); 2.10-2.13 (m, 2H); 2.26-2.29 (m, 2H); 2.49 (s, 3H, NCH_3); 2.54 (m, 1H); 2.91-3.02 (m, 2H); 3.30 (s, 3H, OCH_3); 3.81 (s, 2H); 4.60 (d, 1H, $J_{H5\text{-}H6}$ = 3.7 Hz); 4.73 (m, 1H); 6.42 (d, 1H, $J_{H1\text{-}H2}$= 8.1 Hz); 6.54 (d, 1H, $J_{H2\text{-}H1}$= 8.1 Hz); 7.19-7.36 (m, 5H); ESI-TOF MS *m/z*: 407.538 (MH^+).

Catalytic hydrogenolysis of the protected intermediate (1g, 2.46 mmol) was conducted at 60 psi in MeOH using 5% molar of $Pd(OH)_2$ for 7 hours. The catalyst was filtered and the filtrate was taken to dryness. This residue was purified by flash chromatography using a DCM/MeOH/NH_4OH mixture (97/2/1) to afford compound **2** as a white solid (93% yield). ^1H NMR ($CDCl_3$): 1.31-1.40 (m, 2H); 1.52-1.58 (m, 2H); 2.10-2.13 (m, 2H); 2.26-2.29 (m, 2H); 2.49 (s, 3H, NCH_3); 2.54 (m, 1H); 2.91-3.02 (m, 2H); 3.30 (s, 3H, OCH_3); 4.60 (d, 1H, $J_{H5\text{-}H6}$ = 3.7 Hz); 4.73 (m, 1H); 6.42 (d, 1H, $J_{H1\text{-}H2}$= 8.1 Hz; 6.54 (d, 1H, $J_{H2\text{-}H1}$= 8.1 Hz); ESI-TOF MS *m/z*: 317.653 (MH^+).

OXY(HS). The amine **2** (80 mg, 0.25 mmol) was dissolved in pyridine and succinic anhydride (30 mg, 0.3 mmol) was added dropwise. The mixture was refluxed for 7 hours. After disappearance of starting material, the solvent was concentrated to dryness and the residue was taken up with toluene (3 times) and concentrated. This residue was purified by flash chromatography using a DCM/MeOH/NH_4OH mixture (88/10/2) to obtain OXY(HS) as a white solid (82% yield) which was then converted to salt for bioconjugation. ^1H NMR ($CDCl_3$): 1.68-1.77 (m, 2H); 1.79-1.91 (m, 2H); 2.17-2.23 (m, 2H); 2.29 (s, 3H, NCH_3); 2.34-2.83 (m, 7H); 3.04 (m, 2H); 3.83 (s, 3H, OMe); 4.09 (m, 1H); 4.63 (d, 1H, *J* = 4.7 Hz); 6.33 (d, 1H, $J_{H1\text{-}H2}$= 8.2 Hz); 6.37 (d, 1H, $J_{H2\text{-}H1}$= 8.2 Hz); 8.03 (d, 1H, *J* = 8.9 Hz, NH); ESI-TOF MS *m/z*: 417.328 (MH^+); analytical calculation for $C_{22}H_{28}N_2O_6$: C, 63.45; H, 6.78; N, 6.73. Found: C, 63.36; H, 6.88; N, 6.72.

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Hapten conjugation. Haptens were first conjugated to bovine serum albumin (BSA) to optimize reaction efficiency. The difference in molecular weight between the conjugated and unconjugated carrier protein was analyzed by MALDI-TOF mass spectrometry and was used to calculate the molecular haptenation ratio (number of moles of hapten attached per mole of carrier protein). Haptens were then conjugated to chicken ovalbumin (OVA) for use as coating antigen for ELISA assay, and to keyhole limpet hemocyanin (KLH) for immunization of rats because this protein is acceptable for administration to humans whereas BSA is not. Hapten 5 mM and N-ethyl-N′-(3 dimethylaminopropyl)carbodiimide hydrochloride (EDAC) cross-linker 50 mM were dissolved in 0.1M 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 4.5. After 10 minutes of stirring BSA, OVA) or KLH (Thermo Fisher Rockford, IL) were added at final concentrations of 2.8, 1.9 or 2.8 mg/ml respectively. These reactant concentrations provided a hapten:protein molar ratio of 120:1 in the reaction mixture for BSA or OVA and produced conjugates with the highest haptenation ratios. Reactions were stirred for 3 hours at room temperature and were terminated by dialysis in 0.05 M PBS for 6 hours at 4^{o} C. The dialyzed conjugates were sterile filtered and stored at 4^{o} C. Determination of the haptenation ratio by mass spectrometry was not possible for KLH conjugates because of its large size (~5-8 million Da).

Vaccination. All protocols were 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. In immunogenicity, distribution and behavioral studies, conjugates were injected intraperitoneally (IP) in a volume of 0.4 ml, at doses of 25-100 µg using complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for 2 subsequent booster injections at 3 and 6 weeks (Pravetoni et al., 2011). A total of 3

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vaccinations were administered respectively at 0, 21 and 42 days. Immunogenicity, distribution and behavioral studies were conducted 7-10 days after the 3rd immunization. Blood was obtained 7-10 days after the last immunization, corresponding to the time of expected peak antibody response, and serum stored at -20° C.

ELISA. ELISA plates were coated with 5 ng/well of OVA conjugate or unconjugated protein control in carbonate buffer at pH 9.6 and blocked with 1% gelatin. $OXY(Gly)_4$-OVA was used as coating antigen. Primary antibodies were incubated with goat anti-IgG antibodies conjugated to horseradish peroxidase. Antibody specificity was characterized by competitive ELISA and IC_{50} values were calculated for those inhibitors which produced distinct plateaus in the maximum percent inhibition. Because plateau values differed, IC_{50} values for each inhibitor were calculated based upon the % inhibition of the optical density of primary antibodies incubated with saline. Finally, in order to establish the relative affinity of anti-OXY antibodies to other structurally-related compounds, cross-reactivity percentage was reported.

Effect of vaccination on OXY distribution. Rats were anesthetized with ketamine/xylazine one week after the final vaccine dose and an indwelling catheter was placed in their right external jugular vein. Blood was withdrawn for ELISA, and OXY was administered as a 10 second infusion. Rats were decapitated 5 minutes later and trunk blood and brain collected.

The effects of immunization with $OXY(Gly)_4$-BSA or $OXY(gly)_4$-KLH doses of 25 and 100 µg were compared using groups of 5 rats and an OXY dose of 0.5 mg/kg administered i.v. Controls were vaccinated with unconjugated protein in place of conjugate. The effects of vaccination on the distribution of OXY doses of 0.05, 0.01 and 0.5 mg/kg were

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compared using groups of 5 rats vaccinated with 100 μg of the OXY$(Gly)_4$-BSA conjugate, 7-10 days after the 3rd immunization. The BSA conjugate was used for this experiment because of the ability to verify the haptenation ratio by mass spectrometry. These OXY doses were chosen as representative of the therapeutic single dose range in humans (Leow et al., 1992; Poyhia et al., 1992).

OXY assay. Drug doses and concentrations are expressed as weight of the base. OXY concentrations were measured by GC-MS and represent the total drug (protein or antibody-bound as well as free) in each sample. Extraction and quantitation of OXY in brain and serum samples was based on a previously described method (Lewis et al., 2005) using d_6-OXY as internal standard (Cerilliant Corp., Round Rock, TX), solid phase extraction, derivatization with BSTFA in 1%TMCS, and analysis on an Agilent 6890 GC with methyl siloxane capillary column and 5973 quadrupole MS (Agilent, Palo Alto, CA). Adaptations to the method included omission of NaF, analysis of 0.5 ml rather than 3 ml samples and an initial 1:4 rather than 1:2 dilution of brain into 0.1 M phosphate buffer pH 6.0, and solid phase extraction of brain homogenate supernatant rather than whole homogenate. The limit of quantitation was 5 ng/ml OXY in serum and 50 ng/ml in brain.

Protein binding. Equilibrium dialysis was carried out in Sorenson's buffer at pH 7.35 for 4 hours at 37°C using 1 ml Teflon cells (Hieda et al., 1997). The free (unbound) oxycodone concentration was calculated as the product of the % bound and the total serum oxycodone concentration prior to dialysis.

Thermal nociception test. A dose-effect curve was obtained using groups of 6 unvaccinated (naïve) rats in order to select an appropriate OXY dose for further study. Additionally, serum and brain were obtained from the same naïve rats to determine

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oxycodone concentrations 30 min after s.c. dosing. Based upon these data, the effects of immunization were tested in rats before and after receiving OXY 2.25 mg/kg s.c.. Groups of 10 rats were vaccinated with 100 μg of OXY$(Gly)_4$-KLH or unconjugated KLH (controls) and thermal nociception testing was carried out on a hot plate (Columbus Instruments, Columbus, OH) set at 54^{o}C, 7-10 days after the 3rd immunization. Rats were habituated to the testing environment for 1 hr, and then pre-tested on the hot plate to obtain their baseline latency. Two hours later, rats were injected s.c. with OXY and their post-drug latency was obtained 30 min after that. A cut-off value of 60 seconds was used to prevent tissue damage and the maximum possible effect (MPE%) was calculated as: (postdrug latency - baseline latency)/(maximal cutoff - baseline latency) x 100. Licking the hind paw or jumping were considered endpoints for thermal nociception (Lemberg et al., 2006).

Data analysis. Vaccinated groups were compared to unconjugated protein controls using unpaired two-tailed T tests or, for multiple groups, one-way ANOVA followed by Bonferroni's post-test. One subject from the OXY$(Gly)_4$-BSA group and one subject from the KLH group were removed from the analysis of equilibrium dialysis data because the unbound OXY concentrations were <5 ng/ml. The relationship of log titer to brain OXY concentrations was analyzed by linear regression.

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RESULTS

Conjugate vaccine synthesis. Conjugation using a molar hapten:protein ratio of 120:1 in the reaction mixture proved optimal and produced BSA conjugates of OXY$(Gly)_4$ or OXY(HS) with mean haptenation ratios of 16 or 17:1 respectively (Table 1). Hapten was also conjugated to KLH because KLH is suitable for administration to humans whereas BSA is not. Conjugation of KLH could not be quantitated by MALDI-TOF because of its large molecular weight but was confirmed qualitatively by ELISA in which the conjugate was used as the coating antigen. Conjugates of OXY$(Gly)_4$-OVA and OXY(HS)-OVA used as the coating ELISA had haptenation ratios of ≥17:1.

Serum antibody titers and affinity. The OXY$(Gly)_4$-BSA and OXY(HS)-BSA conjugates had nearly identical haptenation ratios yet the conjugate containing the $(Gly)_4$ linker elicited substantially higher titers regardless of the immunogen dose (Table 1). The OXY$(Gly)_4$-OVA conjugate had a similar haptenation ratio but was not further characterized because it was used only as a coating antigen for ELISA. Competitive ELISA using serum from animals immunized with OXY$(Gly)_4$-KLH, with OXY$(Gly)_4$-OVA as the coating antigen (same linker) showed that the resulting antibodies had high affinities (low IC_{50} values) for both the OXY$(Gly)_4$ hapten and OXY without linker (Table 2). The $(Gly)_4$ linker alone showed no measurable competition or binding. Anti-OXY antibodies exhibited 63% cross-reactivity with oxymorphone, a minor but active metabolite of OXY in rat and human (Lalovic et al., 2006; Chan et al., 2008). Affinities for morphine, hydrocodone, hydromorphone, naloxone and naltrexone were substantially lower, and there was no measurable binding of the off-target opioids methadone or buprenorphine.

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OXY distribution. Immunization with OXY$(Gly)_4$-KLH increased retention of OXY in serum measured 5 min after the OXY dose (Fig 1) and reduced distribution of OXY to brain. Immunization with OXY$(Gly)_4$-BSA showed a similar trend toward increased retention of OXY in serum ($p = 0.07$) and, like the KLH conjugate, significantly decreased OXY distribution to brain. There was a trend but no significant difference in efficacy for either immunogen between the 25 and 100 µg doses. However the 100 µg doses produced reductions of over 50% in brain OXY concentrations that differed significantly from controls whereas the 25 µg doses did not, suggesting greater efficacy for the higher immunogen doses (Fig 1). There was a significant correlation between the log serum antibody titers from all vaccinated rats and their respective brain OXY concentration (Fig 1).

Immunization with 100 µg of OXY$(Gly)_4$-BSA altered OXY distribution in a dose-related manner (Fig 2) with substantially larger effects on the lower OXY doses as indicated by the greater percentage change in serum and brain levels at the lower OXY doses. The distribution of OXY to brain was substantially reduced, by 86% in rats receiving 0.1 mg/kg OXY, and the percent reduction could not be calculated at the lowest OXY dose because the brain OXY concentration was below the assay's limit of quantitation.

Protein binding. The protein binding of OXY in the serum of control rats was low (10-12%) but increased markedly to 99% in rats vaccinated with either the BSA or KLH conjugate. The free (unbound) OXY concentration in serum was reduced to 19% (BSA conjugate) or 29% (KLH conjugate) that of controls in vaccinated rats (Table 3).

Thermal nociception test. An OXY dose of 2.25 mg/kg was selected for studying effects of immunogens because it produced a nearly maximal effect on latency in

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unvaccinated naive rats (Fig 3a). In the same naïve rats (n=6) an OXY dose of 2.25 mg/kg, at 30 minutes after s.c. injection, resulted into serum OXY concentrations of 340±50 ng/ml and brain concentrations of 2700±290 ng/ml indicating that concentrations were higher than concentrations obtained with an i.v. dose of 0.5 mg/kg at 5 minutes. Baseline latencies did not differ between groups immunized with OXY$(Gly)_4$-KLH and KLH control (Fig 3b). The MPE% after OXY administration was significantly lower in the group vaccinated with OXY$(Gly)_4$-KLH than in the KLH control group (17±10% vs 69±11%, $p< 0.01$) (Fig 3c).

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DISCUSSION

The OXY$(Gly)_4$-KLH immunogen elicited high titers of OXY-specific antibodies which were highly selective for OXY and its active metabolite oxymorphone and had substantially lower affinities for a variety of off-target opioids. Vaccination of rats with this immunogen increased OXY binding and retention in serum, decreased the free OXY concentration in serum and decreased OXY distribution to brain following administration of clinically relevant doses of OXY. Vaccination also substantially reduced OXY-induced analgesia in a test of thermal nociception highlighting its ability to attenuate a centrally-mediated behavioral effect of OXY. These data support further study of this immunogen as a potential therapeutic agent for OXY abuse.

Linker composition and length contribute to conjugate vaccine immunogenicity (Kubler-Kielb et al., 2006) but the extent of this contribution is often difficult to interpret because the corresponding haptenation ratios for the conjugates being compared are not reported. Greater conjugate immunogenicity could be due to enhanced recognition by antigen presenting or B cells but could also be due to differences in carrier protein haptenation since higher haptenation ratios are generally associated with greater immunogenicity (Carroll et al., 2011). In the current study haptenation ratios for the OXY$(Gly)_4$-BSA and OXY(HS)-BSA conjugates were nearly identical yet OXY$(Gly)_4$-BSA elicited higher ELISA titers. Greater immunogenicity in this case was therefore due to differences in the linker *per se* rather than haptenation efficiency. Since $(Gly)_4$ and HS differ in both composition and length, it is not clear from the available data which of these factors contributed.

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Competitive ELISA showed that the full hapten OXY$(Gly)_4$- had a somewhat higher affinity for vaccine-generated antibodies than did OXY alone. Similar data with a nicotine conjugate vaccine suggest that linkers may contribute to the epitope (part of an antigen) that is recognized by the immune system (Hieda et al., 1997). This is not surprising given the small size of nicotine or OXY. Oligoglycine was used as a linker in this study because it provided a convenient means of studying and optimizing linker length, and $(Gly)_4$ was shown in preliminary studies to be an effective linker. A potential concern with using a peptide linker is cross-reactivity with native proteins containing $(Gly)_4$ sequences. This is unlikely because the minimum size generally associated with peptide immunogenicity is 8-12 amino acids (Mayrose et al., 2007). In support of this, the $(Gly)_4$ linker alone produced no measurable inhibition in competitive ELISA. Cross reactivity of antibodies with native proteins would therefore not be expected.

Antibodies generated by OXY$(Gly)_4$-KLH cross reacted with oxymorphone, an active but minor metabolite of OXY in rat and human (Poyhia et al., 1991; Lalovic et al., 2006). It is unlikely that the binding of oxymorphone is necessary for an effective OXY vaccine, since it is largely undetected in human plasma, however oxymorphone is marketed as an analgesic and its abuse has now been reported (2011). The OXY$(Gly)_4$-KLH vaccine could therefore be of interest with regard to oxymorphone abuse. Our findings are similar to a previous report of an OXY immunogen which also used the 6 position for linker attachment and elicited antibodies with similar IC_{50} values for OXY and oxymorphone (Findlay et al., 1981).

A desirable feature of an OXY vaccine is the production of antibodies which do not bind other opioids that might be needed for therapeutic use in vaccinated individuals. These

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include methadone, buprenorphine and naltrexone which are used for opioid addiction treatment, and naloxone which is used to reverse excessive opioid effect. Antibodies from the OXY$(Gly)_4$-KLH vaccine had no measurable affinity for methadone or buprenorphine, and IC_{50} values substantially lower for naltrexone and naloxone than for OXY. Their clinical use would not be expected to be impeded by vaccination, although this remains to be demonstrated. Cross-reactivity with other abusable opioids could be a useful feature of an OXY vaccine, but this was found only for oxymorphone. Since OXY and oxymorphone differ only in their 3 position substituent, this position does not appear to contribute importantly to the drug-binding epitope. In contrast, absence of a hydroxyl group at the 14 position (hydrocodone and hydromorphone) was sufficient to greatly reduce the IC_{50} value of these ligands. These findings help to define the structural features of OXY contributing to epitope recognition.

There are very limited data regarding effects of opioid vaccines on opioid pharmacokinetics, and none pertaining to OXY. Berkowitz et al. reported a 7 to 30-fold increase in the serum concentration of ^{3}H-dihydromorphine in mice immunized with a morphine-BSA conjugate vaccine compared to controls, and slower radiolabel elimination (Berkowitz and Spector, 1972). Rats or mice passively infused with immune serum from vaccinated rabbits showed reduced distribution of ^{3}H-dihydromorphine to brain (Berkowitz et al., 1974). Hill et al. found no increase in ^{14}C-morphine concentration in the serum of rabbits immunized with a similar vaccine but reported modestly prolonged radiolabel elimination (Hill et al., 1975). The use of radiolabel rather than specific drug assays is an important limitation of these studies. In the current study, using GC-MS for OXY assay, vaccination with OXY$(Gly)_4$-BSA greatly enhance OXY retention in serum and reduced OXY distribution to brain by up to 84%. This magnitude of effect is similar to that of nicotine and cocaine vaccines that block addiction-related

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behaviors in rats and which are in clinical trials (Fox et al., 1996; Pentel et al., 2000). Single oral OXY doses used in humans commonly range from 0.07 to 0.38 mg/kg of immediate release formulations. Corresponding peak or early serum OXY concentrations are typically 10-100 ng/ml (Leow et al., 1992; Poyhia et al., 1992). In the current study, OXY was administered over a comparable dose range of 0.05 to 0.5 mg/kg, but i.v. instead of orally, and produced serum OXY concentrations equaling or exceeding the peak levels from oral dosing in humans. Serum OXY concentrations after s.c. dosing at 2.25 mg/kg, as used in the hotplate analgesia experiment, were even higher. Vaccination was effective in altering OXY distribution and hot plate analgesia despite this rigorous challenge. Greater effects of vaccination on drug distribution at lower OXY doses was anticipated based on data with other addiction vaccines, since vaccine efficacy is greatest when the antibody to drug ratio is highest (Keyler et al., 2005).

The large effect of immunization on OXY distribution was mirrored by its behavioral efficacy. Immunization with OXY$(Gly)_4$-KLH reduced OXY-induced analgesia in a test of thermal nociception, a centrally-mediated effect of opioids. These observations suggest that further study of vaccination using models of OXY addiction are warranted.

Antibodies are too large to cross the blood brain barrier and drug bound to antibody is also excluded from the brain. The primary action of addiction vaccines is presumed to be the binding of drug to antibody in serum, reducing the unbound or free drug concentration and its subsequent distribution to brain (Keyler et al., 2005; Pravetoni et al., 2011). Changes in OXY protein binding and the unbound concentration of OXY in serum after vaccination with OXY$(Gly)_4$-KLH were substantial and strongly support this mechanism of action. In addition to reducing the early distribution of drug to brain,

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nicotine vaccines also slow nicotine distribution to brain and nicotine elimination (Keyler et al., 1999). These additional effects of vaccination on nicotine disposition may contribute to their effects on drug-related behavior and it will be of interest to examine these parameters for OXY vaccines.

The role of addiction vaccines in treatment is as yet unclear since they are in early stages of development. It is likely that they will be used as adjunctive therapies or in combination with other medications rather than as single modalities. Antibodies generated by the OXY$(Gly)_4$-KLH vaccine did not appreciably cross react with methadone, buprenorphine or naltrexone so that the therapeutic use of these medications as a treatment for addicts with sufficiently regular and severe opioid use would still be possible. The combination of vaccine and agonist therapy is of interest because a substantial minority of opioid addicts continue to use their opioid of choice even while on methadone maintenance therapy (D'Aunno and Vaughn, 1992). It is of course possible that someone abusing OXY, the opioid targeted by this vaccine, could switch to abusing a different opioid. Combining a vaccine with other adjunctive therapies would presumably help to minimize this possibility. It may also be feasible to combine multiple vaccines directed at different opioids to provide broader coverage. This strategy is widely used for infectious diseases by combining multiple unrelated vaccines, e.g. measles, mumps and rubella for convenience. Because the immune system has the capacity to respond to multiple simultaneous challenges, the individual vaccine components retain their individual immunogenicity when combined in this manner. Early data with nicotine vaccines suggest that this strategy can be generalized to conjugate vaccines as well (Keyler et al., 2008). Whichever approach is adopted, the development of an effective OXY vaccine represents a first step toward investigating whether this is a viable therapeutic strategy.

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Authorship contributions

Participated in research design: Pravetoni, Pentel, Portoghese

Conducted experiments: Pravetoni, Tucker and Harmon

Contributed new reagents or analytic tools: Harmon and Le Naour

Performed data analysis: Pravetoni and Pentel

Wrote or contributed to the writing of the manuscript: Pravetoni, Tucker and Pentel

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FOOTNOTES

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b) Part of this work was presented at the College on Problems of Drug Dependence 2011 meeting, in Ft. Lauderdale, FL.

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FIGURE LEGENDS

Figure 1. Effects of two vaccine doses on OXY distribution

Rats immunized with either 25 or 100 μg of conjugate (5 per group) received 0.5 mg OXY i.v. and samples were collected 5 min later. Data (mean±SD) represent the total OXY concentration in serum or brain for animals immunized with (A) OXY$(Gly)_4$-BSA or (B) OXY$(Gly)_4$-KLH. Numbers over the data bars are the percent change compared to control. Vaccination with OXY$(Gly)_4$-KLH increased retention of OXY in serum and both immunogens decreased OXY distribution to brain, * $p<0.05$ compared to controls (C) There was a significant correlation between the log serum antibody titer and the brain OXY concentration. Closed symbols are values for all rats receiving either conjugate vaccine shown in panels A and B, and the open symbol is the mean±SD for control rats from those experiments.

Figure 2. Effect of vaccination on several OXY doses

Rats were immunized with 100 μg of OXY$(Gly)_4$-BSA and treated as in Fig 1 but received different OXY doses i.v.. Effects on OXY distribution were greatest at the lower OXY doses as indicated by the percent of control concentrations. Data are represented as mean±SD, * $p<0.05$, ** 0.01 compared to control, # OXY concentrations too low to quantitate.

Figure 3. Effect of vaccination on OXY-induced analgesia

Rats were immunized with 100 μg of OXY$(Gly)_4$-KLH or KLH control and tested for blockage of OXY-induced analgesia on a hot plate thermal nociception test. (A) Dose-response relationship for OXY effect on MPE% at 30 minutes after injection. (B) Rats immunized with OXY$(Gly)_4$-KLH or KLH control exhibited similar baseline latencies. (C)

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Effects of 2.25 mg/kg OXY administered s.c. were blunted in rats vaccinated with OXY$(Gly)_4$-KLH compared to controls immunized with unconjugated KLH. Data are expressed as mean±SEM, ** $p<0.01$ compared to control.

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Table 1 Conjugate haptenation ratios and serum antibody titers

Linker	Carrier	Haptenation Ratio	Immunogen Dose	Serum titer (x 10^3)
$(Gly)_4$	BSA	16	25	80 ± 70
$(Gly)_4$	BSA	16	100	130 ± 90
$(Gly)_4$	KLH	-----	25	170 ± 110
$(Gly)_4$	KLH	-----	100	110 ± 60
(HS)	BSA	17	100	11 ± 11
(HS)	KLH	-----	100	20 ± 14

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Table 2 Competitive binding IC_{50} values and cross-reactivity %*

Inhibitor	IC_{50} (μM)	Cross-reactivity %
$OXY(Gly)_4$	0.003	>100
OXY	0.017	100
Oxymorphone	0.03	63
Naloxone	1	1.2
Hydromorphone	4	0.4
Naltrexone	5	0.4
Hydrocodone	10	0.2
Morphine	20	0.1
$(Gly)_4$	------	
Methadone	------	
Buprenorphine	------	
Nicotine	------	

* $OXY(Gly)_4$-OVA used as ELISA coating antigen

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Table 3 Oxycodone serum protein binding

	Total OXY ng/ml	OXY % Bound	Unbound OXY ng/ml
BSA	160 ± 150	12 ± 2	140 ± 130
OXY$(Gly)_4$-BSA	3000 ± 1100***	99 ± 1***	30 ± 15
KLH	80 ± 20	10 ± 3	70 ± 20
OXY$(Gly)_4$-KLH	3200 ± 1500 **	99 ± 1***	20 ± 15*

Group sizes n=4 or 5. * $p<0.05$, ** 0.01 and ***0.001 compared to controls.

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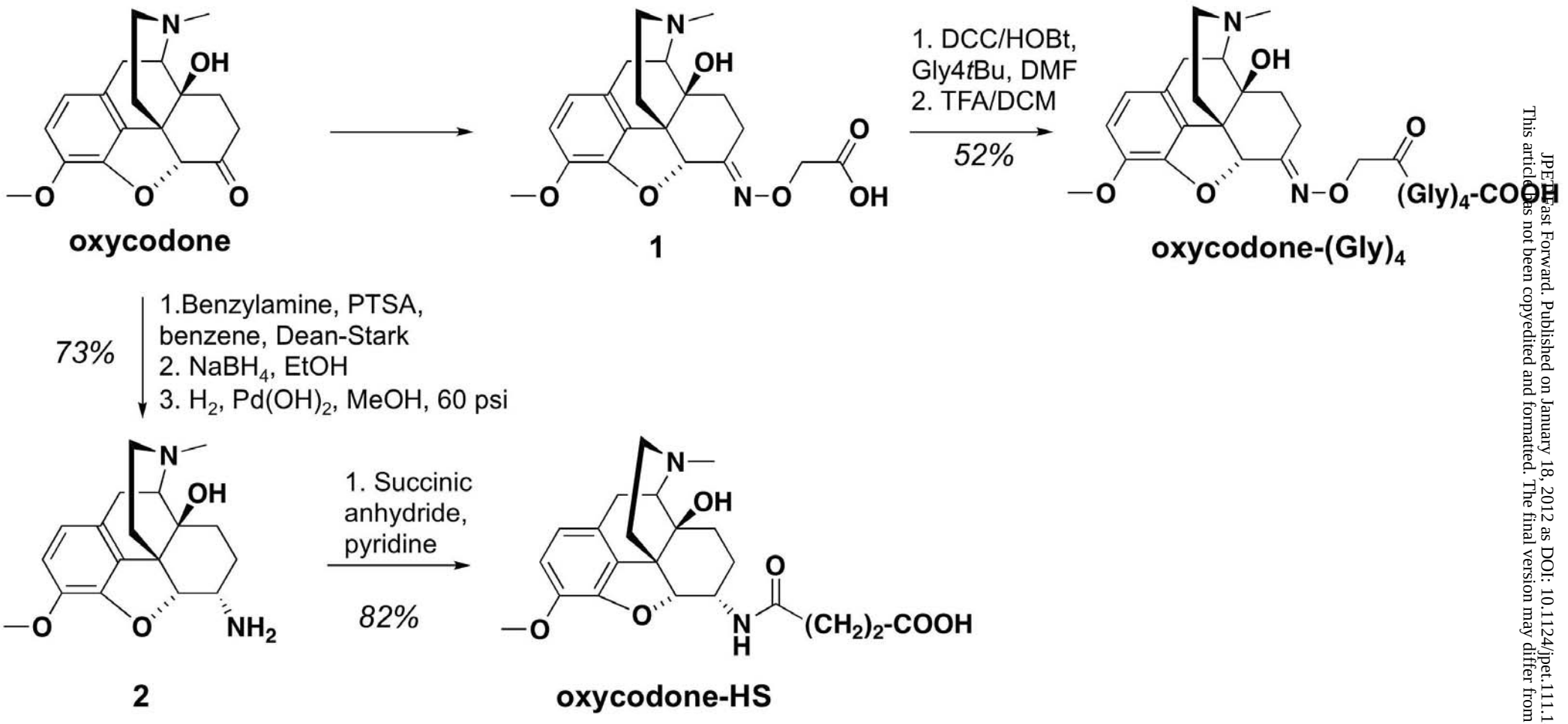

Scheme 1. Oxycodone haptens synthesis; chemical structures and reaction details. Intermediates are numbered in bold type.

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Figure 1

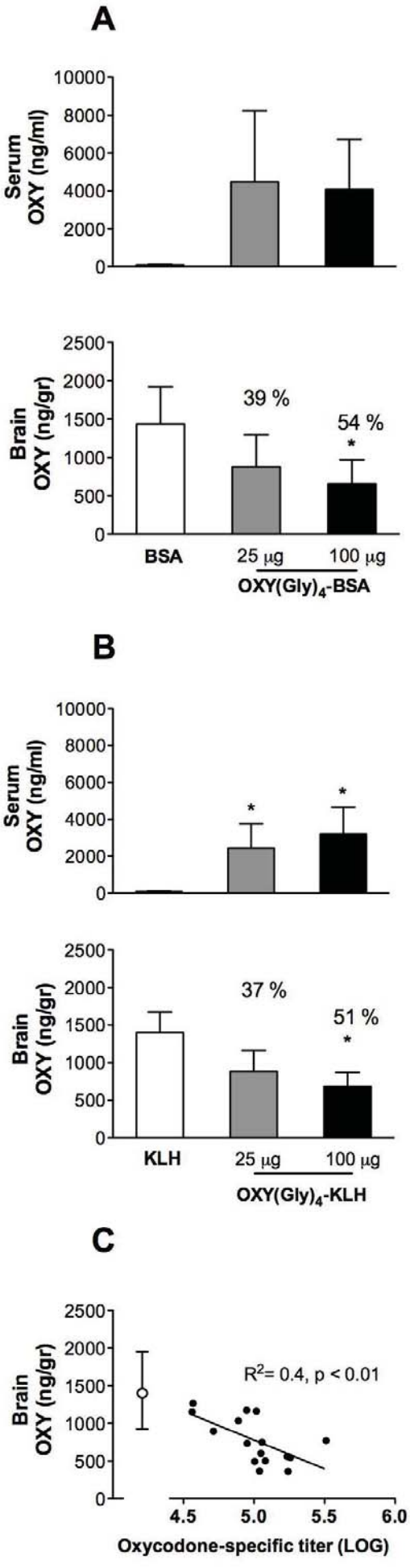

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Figure 2

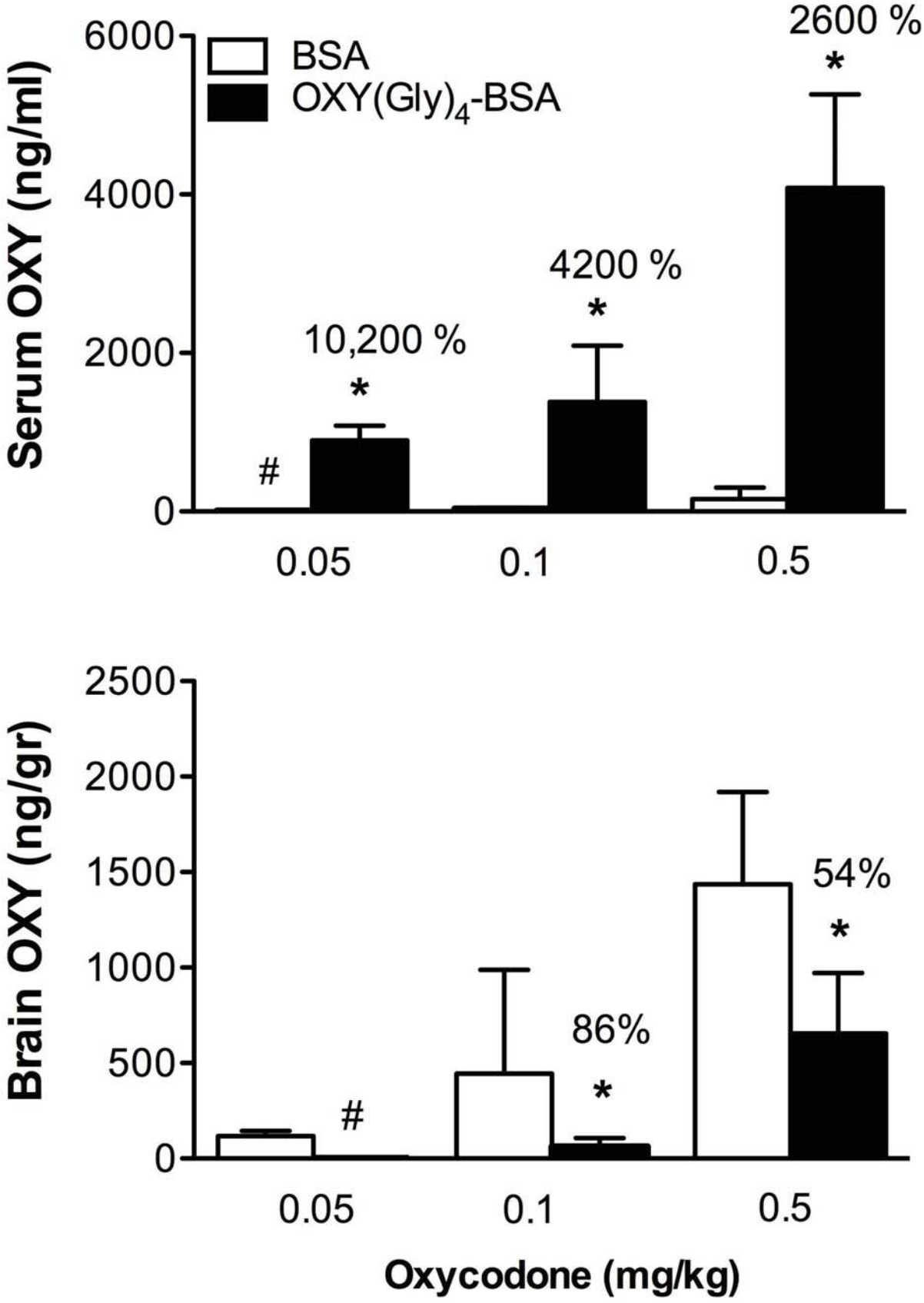

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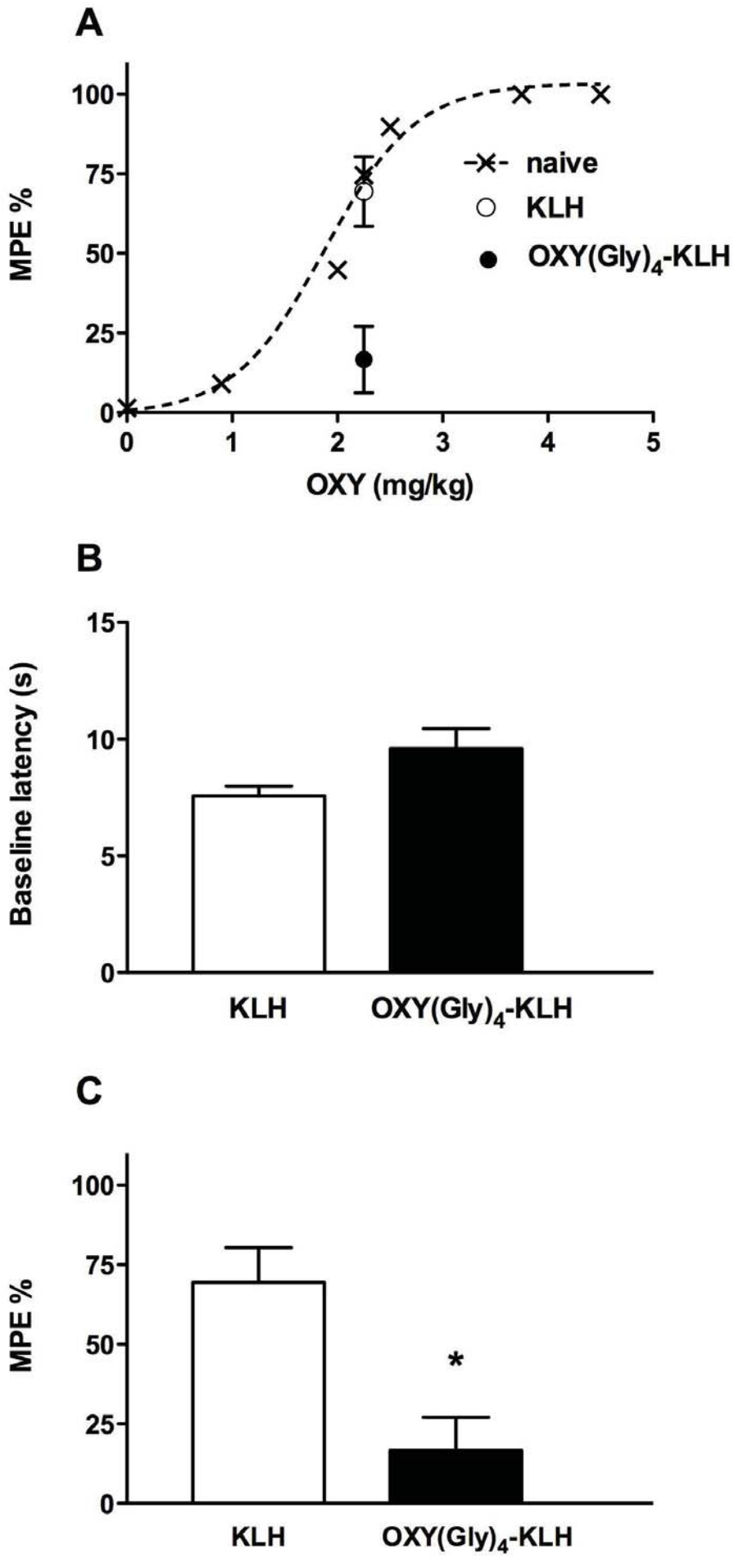
Figure 5



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