Combining a candidate vaccine for opioid use disorders with extended-release naltrexone increases protection against oxycodone-induced behavioral effects and toxicity.

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Abstract

Opioid use disorders (OUD) and opioid-related fatal overdoses are a significant public health concern in the United States and worldwide. To offer more effective medical interventions to treat or prevent OUD, anti-opioid vaccines are in development that reduce the distribution of the targeted opioids to brain and subsequently reduce the associated behavioral and toxic effects. Of critical importance is that anti-opioid vaccines should not interfere with medications to treat OUD. Hence, this study tested the pre-clinical proof of concept of combining a candidate oxycodone vaccine (OXY-KLH) with an FDA-approved extended-release naltrexone depot formulation (XR-NTX) in rats. The effects of XR-NTX on oxycodone-induced motor activity and antinociception were first assessed in non-vaccinated naïve rats to establish a baseline for subsequent studies. Next, OXY-KLH and XR-NTX were co-administered to determine whether the combination would affect the efficacy of each individual treatment and found that the combination of OXY-KLH and XR-NTX offered greater efficacy in reducing oxycodone-induced motor activity, thigmotaxis, antinociception, and respiratory depression over a range of repeated or escalating oxycodone doses in rats. These data support the feasibility of combining antibody-based therapies with opioid receptor antagonists to provide greater or prolonged protection against opioid-related toxicity or overdose. Combining anti-opioid vaccines with XR-NTX may provide prophylactic measures to subjects at risk of relapse, and accidental or deliberate exposure. Combination therapy may extend to other biologics (e.g., monoclonal antibodies) and medications against substance use disorders.

Significance statement. Opioid use disorders (OUD) remain a major problem worldwide and new therapies are needed. This study reports on the combination of an oxycodone vaccine (OXY-KLH) with a currently approved OUD therapy, extended release naltrexone (XR-NTX). Results demonstrated that XR-NTX did not interfere with OXY-KLH efficacy and combination of
low doses of XR-NTX with vaccine was more effective than each individual treatment alone to reduce behavioral and toxic effects of oxycodone, suggesting that combining OXY-KLH with XR-NTX may improve OUD outcomes.
Introduction

Opioid use disorders (OUD) are a worldwide public health concern, and the World Health Organization (WHO) estimated that there are 27 million people that suffer from an OUD (WHO, 2018). In the United States, 2.6 million people are diagnosed with an OUD and at least 50,000 opioid-related fatal overdoses are reported annually (Jones et al., 2018). Clinical and epidemiological data highlight that current treatment strategies for OUD are still insufficient to curb the occurrence of opioid-related fatal overdoses (Volkow and Collins, 2017).

Approved medications for OUD largely consist of small molecules targeting opioid receptors. Methadone, an opioid agonist, and buprenorphine, a partial opioid agonist, are effective treatments for OUD but regulations, abuse liability, and diversion complicate their clinical use (Dodrill et al., 2011; Johanson et al., 2012). Naltrexone, an opioid antagonist used to treat OUD, may be less appealing because it lacks agonist replacement effects and requires an abstinence period before treatment can begin. If patients stop taking naltrexone and resume opioid use, there is also an increased risk of overdose due to loss of opioid tolerance (Miotto et al., 1997). The approval of extended release naltrexone formulations (XR-NTX) reduced overall mortality compared to oral naltrexone therapy (Kelty and Hulse, 2012). An XR-NTX intramuscular depot formulation (Vivitrol®, Alkermes) improved antagonist therapy compliance and retention in treatment (Krupitsky et al., 2011; Coviello et al., 2012; DeFulio et al., 2012; Krupitsky et al., 2013). This extended release formulation consisting of NTX embedded in microspheres provides stable plasma NTX concentrations in both animal and human subjects for up to 28 days (Bartus et al., 2003; Dean et al., 2008; Bigelow et al., 2012). XR-NTX decreased hydromorphone effects in human subjects (Bigelow et al., 2012) and blocked fentanyl and hydrocodone effects in rats (Dean et al., 2008). Although XR-NTX improved clinical efficacy of NTX (Krupitsky et al., 2011; Coviello et al., 2012; DeFulio et al., 2012; Kelty and Hulse, 2012),
its use and compliance may still be limited by the need for monthly injections and increased likelihood of overdose during relapse or discontinuation of therapy (Kjome and Moeller, 2011). This suggests the need for developing new alternative treatments for OUD and to test their efficacy in combination with current FDA-approved medications.

Vaccines may provide a safe, long-lasting complementary option to treat OUD. Active immunization with opioid-based conjugate vaccines elicits antibodies that selectively bind the target opioid in serum and extracellular fluids and effectively reduce opioid distribution to brain, ultimately blunting opioid-induced behavior and toxicity (Bonese et al., 1974; Anton and Leff, 2006; Stowe et al., 2011; Pravetoni et al., 2012b). Conjugation of an oxycodone-based hapten (OXY) to native or GMP-grade subunit keyhole limpet hemocyanin (KLH) carrier protein generated a vaccine (OXY-KLH) that reduced the distribution of oxycodone and hydrocodone to brain and their subsequent behavioral effects in mice and rats, including acquisition of oxycodone self-administration and oxycodone-induced gene expression in the midbrain (Pravetoni et al., 2013; Pravetoni et al., 2014a; Raleigh et al., 2017; Robinson et al., 2019). Similarly, an analogous oxycodone vaccine reduced maintenance of oxycodone intravenous self-administration (Nguyen et al., 2018). While anti-drug vaccines have been highly effective in rodents and primates, clinical trials for nicotine and cocaine vaccines showed proof of efficacy in a subset of high responders (Cornuz et al., 2008; Martell et al., 2009; Hatsukami et al., 2011). To increase efficacy against OUD, combining vaccination and XR-NTX could circumvent the shortcomings of both antibody- and small molecule-based interventions.

The goal of this study was to test whether opioid receptor antagonist therapy could be combined with vaccines against opioids to provide better protection against OUD and possibly overdose. To this end, OXY-KLH and XR-NTX were administered alone or in combination to evaluate their
effect on oxycodone-induced antinociception, motor activity, respiratory depression and bradycardia. Results demonstrated that XR-NTX did not impair oxycodone-specific antibody responses to OXY-KLH and did not interfere with the effects of vaccination on preventing oxycodone-induced motor activity. The combination therapy offered better protection than either treatment alone against opioid-induced locomotor activity, respiratory depression, and bradycardia following repeated exposure to high doses of oxycodone. Although it is acknowledged that combining vaccines with antagonists may limit analgesic options for patients, these data suggest that combination of an anti-opioid vaccine with XR-NTX may enhance efficacy of either treatment and may provide extra protection during noncompliance or in treatment-resistant patients.
Materials and methods

Animals
Male Holtzman rats (300-324 grams at arrival) were purchased from Envigo (Indianapolis, IN) housed in pairs on a 12/12 hr light/dark schedule and food restricted to 18 grams per day of standard rat chow. Housing and feeding conditions replicated previous pre-clinical studies of the OXY-KLH vaccine (Pravetoni et al., 2012a; Pravetoni et al., 2013). This study was 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 Hennepin Healthcare Research Institute Animal Care and Use Committee.

Drugs
Oxycodone was obtained through Sigma (St. Louis, MO). Oxycodone doses and concentrations were expressed as the weight of the base. Extended-release naltrexone (Vivitrol®, Alkermes, Waltham, MA) was obtained through the Hennepin County Medical Center pharmacy and its dose was expressed as the weight of the microsphere as provided by the manufacturer.

Vaccine
An oxycodone-based hapten containing a tetracyanine linker at the C6 position (OXY) was first synthesized and then conjugated to chicken ovalbumin (OVA) or the keyhole limpet hemocyanin (KLH) carrier proteins (Pravetoni et al., 2012a). Rats were immunized with either a decamer native KLH or a GMP-grade subunit KLH (dimer KLH, Stellar Biotechnologies). OXY-KLH was mixed with Freund’s adjuvant and administered i.p. as described (Pravetoni et al., 2012a; Pravetoni et al., 2012b). In the final experiment, OXY-KLH was instead adsorbed onto Alhydrogel “85” 2% aluminum hydroxide (Sergeant Chem, Clifton, NJ) and administered i.m. as described (Raleigh et al., 2017; Raleigh et al., 2018) to determine if XR-NTX affected
immunogenicity of a clinically relevant OXY-KLH formulation. To measure oxycodone-specific serum IgG antibodies post-immunization, ELISA 96-well plates were coated with OXY-OVA or unconjugated OVA control in carbonate buffer and blocked with 1% gelatin. Primary antibodies were incubated with goat anti-rat IgG antibodies to measure oxycodone-specific serum IgG antibody titers, as described previously (Pravetoni et al., 2012a).

**Opioid level analysis**

Oxycodone concentrations were measured by gas chromatography/mass spectrometry as previously described (Raleigh et al., 2017; Raleigh et al., 2018) and represented as total drug levels. Briefly, trunk blood was centrifuged at 3100g for 3 min at 4°C and serum collected. Brains were rinsed with distilled water and patted dry. Serum and brains were stored at -20°C until analysis.

**Opioid-induced motor activity and thigmotaxis**

Motor activity studies were conducted during the light cycle. Rats were first given 1 hr to habituate after transport to the behavioral testing room, and then injected with saline or oxycodone, according to the experimental design, and placed in an open field (ENV-510S, Med Associates, St. Albans, VT). The entire apparatus was enclosed in a sound attenuating cubicle. Locomotor activity was monitored via infrared beam breaks and recorded by Activity Monitor 5.0 software (Med Associates). The thigmotaxis index was calculated as the ratio of the distance travelled in the external area to the one travelled in the center, which could be set in the software as a virtual divide (Pravetoni and Wickman, 2008).

**Opioid-induced respiratory depression and bradycardia**
After 1 hr of habituation to the behavioral room, baseline % SaO₂ (arterial oxygen saturation) and heart rate were assessed using a MouseOx (STARR Life Sciences Corp., Oakmont, PA) monitor collar placed around the neck of awake, freely moving rats (n=8 each group). Rats further received oxycodone every 17 minutes s.c. so that their cumulative oxycodone dose at successive intervals was 2.25, 4.5 and 9.0 mg/kg. Fifteen minutes after each oxycodone injection, 10-second average % SaO₂ and heart rate were again measured, taking 2 minutes for each animal and resulting in the 17 minutes oxycodone dosing interval. When the last dose was administrated, data were recorded twice, 15 minutes apart.

**Experiments**

**Experiment 1: Effect of oxycodone on motor activity and thigmotaxis index in non-immunized rats.**

Effect of oxycodone (0, 0.25, 0.5, 0.75 and 1.0 mg/kg, s.c.) on motor activity was established in non-vaccinated naïve rats (n=7 per dose) to determine oxycodone doses to be used in subsequent studies. Rats were first given saline and habituated to the testing environment and activity chambers on three consecutive daily sessions (test days 1, 2, and 3). Oxycodone-induced motor activity was then assessed on alternate sessions (test days 4, 7, 9, and 11). All testing sessions started immediately after injection and activity was recorded for 90 minutes. See Figure 1 for an overview of all experiments.

**Experiment 2: Effect of XR-NTX on oxycodone-induced motor activity, thigmotaxis index, anti-nociception, and oxycodone distribution in non-vaccinated naïve rats.**

The effect of XR-NTX (Alkermes, Waltham, MA) on oxycodone-induced motor activity, thigmotaxis index, and oxycodone antinociception was evaluated in non-vaccinated naïve rats. Rats (n=8-16 per group) received either saline or a range of XR-NTX doses (0, 1, 2.5, 5, 25 and 50 mg/kg, i.m.) on days 0 and 36 to ensure consistent naltrexone serum concentrations
throughout the entire study, because it has been shown that XR-NTX’s behavioral effects and serum naltrexone are no longer detectable by day 36 (Bartus et al., 2003). This formulation has been previously administered to rats at a dose of 50 mg/kg i.m., which has been shown to elicit serum naltrexone concentrations of 4-15 ng/ml for 28 days in rats shortly after administration (Bartus et al., 2003; Dean et al., 2008), which are similar to the therapeutic range observed in humans treated with the same formulation (Bigelow et al., 2012). Rats received saline on days 7, 8, and 9. Rats received 0.5 mg/kg oxycodone s.c. on days 10, 13, 15, 17, and 20. The oxycodone dose was chosen because it elicited a substantial increase in motor activity in a pilot study (Supplemental Figure 1). The thigmotaxis index was calculated for each dose of XR-NTX. Two rats had very high thigmotaxis indices (46.5 and 168.5) in the 5 mg/kg group that were unexplained (Supplemental Figure 2). These were determined to be outliers as detected using ROUT (Q=0.1%) and removed from analysis. On day 43, the effect of XR-NTX (0, 1, 2.5, 5, 25 and 50 mg/kg, i.m.) was tested on oxycodone antinociception using a hot plate set at 54°C as previously described (Pravetoni et al., 2013). Nociception was measured at 30 minutes after a dose of 2.25 mg/kg oxycodone s.c., which was previously shown to elicit a near maximal antinociceptive response in this test (Pravetoni et al., 2012a; Pravetoni et al., 2013). After reaching the nociceptive behavioral end point or the 60-second cut-off, serum and brain were collected for measurement of oxycodone as described previously (Pravetoni et al., 2013). Percent (%) of maximum possible effect (MPE) was measured as post-test latency to respond minus baseline latency to respond divided by 60 seconds (max cut-off) minus baseline latency to respond times 100.

In an independent experiment, the effect of XR-NTX on the time course distribution and area under the curve (AUC) of serum oxycodone was measured in a separate cohort of non-vaccinated naïve rats injected i.m. with either 50 mg/kg XR-NTX or saline (n=10 or 11, respectively) on day 0. On day 2 rats were anesthetized with ketamine/dexmedetomidine and
implanted with a right jugular vein catheter, which was flushed daily with 200 µl of heparinized saline and locked with 25% glycerol to maintain patency. On day 9 rats were fasted for 12 hr, dosed with 2.25 mg/kg s.c. oxycodone, and serum samples were collected at 15, 30, 60 and 180 minutes. Catheters were flushed with 200 µl heparinized saline between samples and locked with 50 µl of 25% glycerol after the 60-minute time point. Estimates for pharmacokinetic parameters including half-life, maximum concentration ($c_{\text{max}}$), time to reach $c_{\text{max}}$ ($t_{\text{max}}$), area under the curve (AUC), mean residence time (MRT), volume of distribution (Vd/F, volume over the fraction absorbed), and clearance (Cl/F, clearance over the fraction absorbed) were obtained from serum concentrations using noncompartmental methods (Pravetoni et al., 2012a; Pravetoni et al., 2013). Some rats were removed from analyses because fewer data points were collected leading to final group sizes of n=9 for XR-NTX and n=8 for saline.

**Experiment 3: Effect of high doses of XR-NTX on oxycodone-induced motor activity in rats immunized with OXY-KLH.**

The effect of mid and high doses of XR-NTX (5 or 50 mg/kg) on OXY-KLH vaccine behavioral efficacy was tested in rats to assess whether combining antagonist with vaccination would show increased efficacy compared to each individual treatment. Groups (n=8/group) were as follows: 1) KLH + saline; 2) KLH + XR-NTX; 3) OXY-KLH + saline; and 4) OXY-KLH + XR-NTX. Rats received either saline or 50 mg/kg XR-NTX i.m. at 0 and 27 days. Concurrently, rats were immunized with either 100 µg of the OXY-KLH vaccine or the unconjugated KLH administered on days 0, 21, and 42 in Freund’s adjuvant as previously described (28). On day 48, rats were tailbled for titers. On day 55, rats were tested for antinociception as described in **Experiment 2.** On days 60, 61, 62, 63, 66, 68, and 70 rats were tested in motor activity chambers with saline for the first 3 sessions and then challenged with oxycodone (0.5 mg/kg, s.c.) for the next 4 sessions. Because 50 mg/kg XR-NTX completely blunted oxycodone-induced motor activity, rats were held for one month before retesting at a lower XR-NTX dose. Rats were vaccinated on
days 83 and 104. Rats received a 5 mg/kg i.m. dose of XR-NTX on study day 98. On days 116, 117, 118, 119, 122, 124, and 126 rats were tested in motor activity chambers with saline for the first 3 sessions and then challenged with oxycodone (0.5 mg/kg, s.c.) for the next 4 sessions.

**Experiment 4: Effect of low doses of XR-NTX on oxycodone-induced motor activity and thigmotaxis in rats immunized with OXY-KLH.**

The effect of a low dose of XR-NTX (2.5 mg/kg) on OXY-KLH vaccine behavioral efficacy was tested in rats to assess whether combining antagonist with vaccination would show increased efficacy compared to each individual treatment. To this end, a dose of XR-NTX (2.5 mg/kg, i.m.) was used because it produced a sub-optimal reduction of oxycodone-induced motor activity. Rats received either saline or XR-NTX i.m. on days 0, 42 and 70. Concurrently, these rats were immunized with either 100 μg of the OXY-KLH vaccine or the unconjugated KLH on days 0, 21, 42 and 72 in Freund’s adjuvant as previously described (Pravetoni et al., 2012a). Groups (n=8) were designated as follows: 1) KLH + saline; 2) KLH + XR-NTX; 3) OXY-KLH + saline; and 4) OXY-KLH + XR-NTX. On day 49, rats were tailbled for titers. Rats were first tested for baseline motor activity following s.c. saline injections of 1 ml/kg on days 53, 54, and 55 followed by oxycodone-induced motor activity (as described above in “Opioid-induced motor activity and thigmotaxis”) after s.c. injections of 0.5 mg/kg oxycodone on days 56, 59, 61, 63, and 66. To demonstrate increased efficacy of a combination with XR-NTX and vaccination, rats were subsequently challenged with 1.0 mg/kg oxycodone s.c. on testing days 73, 75, and 77, and 2.0 mg/kg on day 80. All sessions recorded motor activity and thigmotaxis.

**Experiment 5: Effect of vaccination with OXY-KLH, alone or in combination with XR-NTX, on oxycodone-induced motor activity, respiratory depression, and bradycardia.**

Rats received 2.25 mg/kg XR-NTX i.m. at 0 and 36 days. Concurrently, rats were immunized i.m. with either 60 μg of the OXY-KLH vaccine or the unconjugated KLH administered at days 0, 21,
and 42. Aluminum hydroxide (90 μg) was used as the adjuvant. Groups (n=8) were as follows: 1) KLH + saline; 2) OXY-KLH + saline; and 3) OXY-KLH + X-R-NTX. On day 49, rats were tailbled for titers. One week later rats were given saline on three consecutive habituation sessions (days 53, 54, and 55). On testing days 56, 59, 61, 63, 66, 68, and 70 rats were given escalating doses of oxycodone s.c. (0.5, 1, 1.5, 2, 2.5, 3, and 3.5 mg/kg, respectively) to track a dose-dependent variation in motor activity. On day 73, rats were tested for oxycodone-induced respiratory depression and bradycardia (as described above in “Opioid-induced respiratory depression and bradycardia”) 15 minutes after each dose of oxycodone that represented cumulative doses of 2.25, 4.5, and 9 mg/kg. Oxygen saturation and bradycardia were also measured 30 min after administration of 9 mg/kg oxycodone.

**Statistical analyses and calculations**

One- or two-way ANOVA paired with Dunnett’s or Bonferroni post hoc tests (respectively) were used to compare oxycodone antinociception, serum and brain distribution, and oximetry data across groups. Kruskal-Wallis test with Dunn’s multiple comparisons post hoc test was used to compare antinociceptive effects between groups in Experiment 3. Pharmacokinetic parameters were compared between saline and XR-NTX treated groups using unpaired t tests with Welch’s correction was used to compare groups. All statistics were performed with GraphPad Prism (version 8.0, La Jolla, CA).
Results

Experiment 1: Oxycodone-induced motor activity and thigmotaxis index in non-immunized rats.

The effect of varying doses of oxycodone on total traveled distance in motor activity chambers was characterized in the absence of other treatments to determine baseline values (Supplemental Figure 1A). Two-way (treatment x time) repeated measures ANOVA showed a significant effect of time (F(6,162)=32.92; p<0.0001) and interaction (F(24,162)=3.56; p<0.0001), but not treatment (F(4,162)=2.16). The 0.25 and 0.50 mg/kg oxycodone doses did not significantly increase motor activity compared to the saline control group. The 0.75 mg/kg oxycodone dose increased activity on test day 7 (t(4)=3.42; p<0.05), 9 and 11 (respectively t(4)=4.61 and 4.48; p<0.001). The 1.0 mg/kg oxycodone dose increased motor activity on test day 9 and 11 (t(4)=3.53 and 3.49; p<0.05) compared to saline controls, however this dose did not further increase motor activity compared to 0.75 mg/kg. Based on this preliminary study, subsequent testing of the efficacy of XR-NTX, vaccination, or their combination in blocking oxycodone-induced motor activity started from a mid-range dose of 0.5 mg/kg oxycodone. Thigmotaxis index increased as a function of the oxycodone dose (Supplemental Figure 1B), with one-way ANOVA showing a significant effect of treatment (F(4, 27)=3.291; p<0.001) and a significant increase following 0.75 mg/kg oxycodone dose compared to control (q=4.94; p<0.05).

Experiment 2: Effect of XR-NTX on oxycodone-induced motor activity, thigmotaxis index, anti-nociception, and oxycodone distribution in non-vaccinated naïve rats.

Administration of XR-NTX dose-dependently reduced oxycodone-induced motor activity (Figure 2A). Two-way (treatment x time) repeated measures ANOVA showed a significant effect of time (F(7,406)=54.98; p<0.0001), treatment (F(5,58)=9.45; p<0.0001), and interaction (F(35,406)=6.24; p<0.001) on oxycodone-induced activity. XR-NTX significantly decreased oxycodone-induced
motor activity at all doses at and above 5 mg/kg (t=4.01-6.97; p<0.01). One-way ANOVA showed a significant effect of treatment (F[5,23.69]=14.44; p<0.01) on thigmotaxis index. All doses of XR-NTX, except 1.0 mg/kg, were associated with a significant reduction (respectively 2.5-50 mg/kg, t=4.84-7.23; p<0.001) of the thigmotaxis index compared to saline control (Figure 2B). XR-NTX administration reduced oxycodone antinociception (Figure 2C). One-way ANOVA showed a significant effect of treatment (F[5,58]=22.43; p<0.0001) on nociception in the hot plate test. All doses of XR-NTX, except 1.0 mg/kg, significantly reduced oxycodone antinociception (2.5 mg/kg, q=2.7; p<0.05; and 5-50 mg/kg, q=6.96-7.5; p<0.0001, respectively).

One-way ANOVA showed a significant effect of treatment on serum oxycodone distribution (F[3,28]=7.51; p<0.001, Figure 2D) and brain (F[3,28]=5.37; p<0.01, Figure 2E). The two highest doses of XR-NTX (25 and 50 mg/kg) significantly reduced oxycodone in serum (q= 3.83 and 4.29; p<0.01 and p<0.001, respectively) and brain (q=2.74 and 3.61; p<0.05 and p<0.01, respectively) 30 min after s.c. injection of 2.25 mg/kg oxycodone.

In rats, pre-treatment with 50 mg/kg XR-NTX significantly reduced c_{max}, AUC, and Cl/F following a 2.25 mg/kg, s.c. oxycodone challenge compared to saline pre-treated rats (Table 1). Two-way ANOVA showed an effect of time (F[5,63]=24.16; p<0.0001), but no effect on treatment or interaction (Figure 2F). No differences between groups were detected at any single time point.

**Experiment 3: Effect of high doses of XR-NTX on oxycodone-induced motor activity in rats vaccinated with OXY-KLH.**

Oxycodone-specific antibody titers in OXY-KLH and OXY-KLH + XR-NTX (50 mg/kg) treated rats were 41 ± 16 x 10^3 and 84 ± 52 x 10^3, respectively (mean ± SD). There were no differences in titers between groups (Supplemental Figure 3). Only XR-NTX and OXY-KLH + XR-NTX showed reduction in oxycodone-induced antinociception on the hotplate (Supplemental Figure
4). Following s.c. administration of 0.5 mg/kg oxycodone, two-way (treatment x time) repeated measures ANOVA showed a significant effect on time ($F_{(1.954, 54.70)} = 24.08; p<0.0001$), treatment ($F_{(3, 28)} = 20.76; p<0.0001$), and interaction ($F_{(18, 168)} = 8.74; p<0.0001$) (Figure 3A) on motor activity. Vaccination with OXY-KLH alone had no effect on motor activity compared to KLH vaccinated rats. Rats treated with XR-NTX plus OXY-KLH showed a significantly reduction in oxycodone-induced motor activity on every test day ($t_{(43d)} = 3.464$, $t_{(46d)} = 3.454$, $t_{(48d)} = 5.010$, $t_{(50d)} = 3.520; p<0.05$) compared to saline control, as well as following XR-NTX alone ($t_{(43d)} = 4.057$, $t_{(46d)} = 3.891$, $t_{(48d)} = 5.382$, $t_{(50d)} = 4.176; p<0.05$).

After one month, rats were revaccinated and received a lower dose of XR-NTX (5 mg/kg). Following s.c. administration of 0.5 mg/kg oxycodone, two-way (treatment x time) repeated measures ANOVA showed a significant effect on time ($F_{(6, 167)} = 19.10; p<0.0001$), treatment ($F_{(3, 28)} = 3.377; p<0.05$), and interaction ($F_{(18, 167)} = 2.315; p<0.01$) (Figure 3B). Vaccination with OXY-KLH alone had no effect on motor activity compared to KLH vaccinated rats. Rats treated with XR-NTX plus OXY-KLH showed a significantly reduction in oxycodone-induced motor activity on every test day ($t_{(119d)} = 3.632$, $t_{(122d)} = 2.937$, $t_{(124d)} = 2.626$, $t_{(126d)} = 2.727; p<0.05$) compared to saline control, as well as following XR-NTX alone on two days ($t_{(122d)} = 2.640$, $t_{(124d)} = 2.839; p<0.05$)

**Experiment 4: Effect of low doses of XR-NTX on oxycodone-induced activity and thigmotaxis in rats immunized with OXY-KLH.**

Oxycodone-specific antibody titers in OXY-KLH and OXY-KLH + XR-NTX (2.5 mg/kg) were $207 \pm 69 \times 10^3$ and $98 \pm 80 \times 10^3$, respectively (mean ± SD), which were significantly different ($p<0.05$, Supplemental Figure 3). In rats repeatedly challenged with 0.5 mg/kg oxycodone s.c. (Figure 4A), two-way (treatment x time) repeated measures ANOVA showed a significant effect on time ($F_{(7.189)} = 46.54; p<0.0001$), treatment ($F_{(3, 27)} = 15.21; p<0.0001$), and interaction
Vaccination with OXY-KLH alone and in combination with XR-NTX significantly reduced oxycodone-induced motor activity compared to KLH controls (p<0.001).

Subsequently on testing days 73 – 77, rats were challenged with 1.0 mg/kg oxycodone s.c (Figure 4B). Two-way (treatment x time) repeated measures ANOVA showed a significant main effect of treatment (F(3,26)=4.17; p<0.05). Treatment with OXY-KLH combined with XR-NTX reduced oxycodone-induced motor activity compared to KLH controls on testing days 73 and 75 [t(73d)=3.85; p<0.01, t(75d)=3.27; p<0.05, respectively]. No other differences were observed. There was no effect of vaccine or XR-NTX on oxycodone-induced thigmotaxis (Figure 4C). Rats were then challenged with a final dose of 2 mg/kg oxycodone, s.c. but no differences were detected between groups (Figure 4D).

**Experiment 5: Effect of vaccination with OXY-KLH, alone or in combination with XR-NTX, on oxycodone-induced motor activity, respiratory depression, and bradycardia.**

Oxycodone-specific antibody titers in OXY-KLH and OXY-KLH + XR-NTX (2.25 mg/kg) were 203 ± 98 x 10³ and 136 ± 76 x 10³, respectively (mean ± SD). There were no differences in titers between groups (Supplemental Figure 3). Rats (n=8 each group) were first given saline on three consecutive habituation sessions. On alternate sessions afterwards rats were administered 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 mg/kg of oxycodone (s.c.) to track dose-dependent variations in motor activity brought on by oxycodone. Two-way (treatment x time) repeated measures ANOVA showed a significant effect of time (F(7, 147)=12.23), but not of treatment and interaction on oxycodone-induced activity (Figure 5A). Due to the motor depressive effects of oxycodone at 2.5 mg/kg and above, the data was split and examined from 0 – 2 mg/kg oxycodone dosing (Supplemental Figure 5 shows motor activity up to 3.5 mg/kg). Two-way (treatment x time) repeated measures ANOVA showed a significant effect of time (F(4, 84)=24.42), but not of treatment and interaction on oxycodone-induced activity. Combination of XR-NTX and OXY-
KLH vaccine showed a significantly effect on reducing oxytocin-induced motor activity following 1.0 mg/kg oxytocin administration ($t=3.08; p<0.01$) compared to KLH control. No other differences were observed.

Three days following motor activity testing, rats were tested for oxytocin-induced respiratory depression and bradycardia 15 minutes after each dose of oxytocin that represented cumulative doses of 2.25, 4.5, and 9 mg/kg. Two-way (treatment x time) repeated measures ANOVA showed a significant effect of oxytocin dose on $\%$ SaO$_2$ ($F(4, 104)=25.43; p<0.0001$) and heart rate ($F(4, 104)=7.316; p<0.0001$). Two-way (treatment x time) repeated measures ANOVA also showed a significant effect of treatment on $\%$ SaO$_2$ ($F(2, 104)=14.36; p<0.0001$) and heart rate ($F(2, 104)=9.283; p<0.001$). There was no interaction significance for either oxytocin dose or treatment. Combination of XR-NTX and OXY-KLH showed higher $\%$ SaO$_2$ levels after oxytocin administration of 4.5 mg/kg ($t=2.841; p<0.05$), 9$_{15\text{ min}}$ mg/kg ($t=4.229; p<0.001$), and 9$_{30\text{ min}}$ mg/kg ($t=4.091; p<0.001$) compared to KLH control (Figure 5B). Both KLH and OXY-KLH showed reduced $\%$ SaO$_2$ levels at 4.5 mg/kg ($t=3.981, t=4.116, t=5.102; p<0.05$), 9$_{15\text{ min}}$ mg/kg ($t=3.397, t=5.903, t=7.427; p<0.05$), and 9$_{30\text{ min}}$ mg/kg ($t=3.981, t=4.116, t=5.102; p<0.05$) compared to baseline levels, while combination of XR-NTX and OXY-KLH showed reduced $\%$ SaO$_2$ levels only at 9$_{15\text{ min}}$ mg/kg ($t=3.589; p<0.05$).

Combination of XR-NTX and OXY-KLH reduced the extent of bradycardia induced by 9 mg/kg oxytocin ($t=2.917, t=2.881; p<0.05, 15$ and 30 minutes after injection of the drug, respectively) compared to the KLH control and compared to OXY-KLH alone ($t=3.358; p<0.01$) (Figure 5C). Only KLH alone showed reduced $\%$ SaO$_2$ levels at 9$_{15\text{ min}}$ mg/kg ($t=4.281; p<0.05$) and at 9$_{30\text{ min}}$ mg/kg ($t=3.587; p<0.05$) compared to baseline levels.
Discussion

This study provided pre-clinical proof of concept for combination therapy against OUD by coadministration of an FDA-approved extended release antagonist formulation and a candidate vaccine against the prescription opioid oxycodone. The major findings were: 1) XR-NTX blocked oxycodone-induced motor activity and oxycodone antinociception in rats in a dose-dependent manner, 2) XR-NTX reduced distribution of oxycodone to serum and brain as well as the AUC, 3) XR-NTX minimally interfered with the OXY-KLH vaccine ability to induce oxycodone-specific serum antibodies, and 4) the combination of XR-NTX with OXY-KLH showed greater effect than each treatment alone in reducing motor activity and anxiolytic effects, as well as protecting against reduction of % SaO₂ and heart rate induced by repeated doses of oxycodone in rats. Together, these data support the combination of anti-opioid vaccines with XR-NTX for the treatment of OUD and possibly prevention of opioid-induced toxicity associated with fatal overdose (e.g., respiratory depression).

To develop an animal model to test the effect of combining anti-opioid vaccines with XR-NTX and compare their efficacy to each individual treatment, the effects of XR-NTX on oxycodone-induced motor activity and hotplate analgesia were tested in rats. Very low doses of naltrexone (naltrexone:oxycodone dose ratio of 1:10⁵) have been shown to enhance the motor activity induced by oxycodone (Leri and Burns, 2005). Higher naltrexone doses used in the current study (naltrexone:oxycodone dose ratio between 1:1.5 – 1:25) reduced oxycodone-induced locomotor activity in a dose-dependent manner. Yet, differences in experimental design complicate interpretation of pre-clinical data across studies. Abstinence from opioid use following XR-NTX has been demonstrated in humans with doses of 380 mg (5.4 mg/kg in a 70kg human, (Nunes et al., 2015)) suggesting that these higher XR-NTX doses, as those used
in the current study, are more typical of those used clinically. Combining XR-NTX and vaccine concurrently so that lower doses of both could be used and side effects minimized would be the ultimate goal.

XR-NTX dose-dependently decreased oxycodone-induced antinociception in the hot plate test. Although this effect has not previously been demonstrated using oxycodone, similar effects have been shown for XR-NTX against morphine, fentanyl, and hydrocodone (Bartus et al., 2003; Dean et al., 2008). In these previous studies, very large doses of XR-NTX (50 mg/kg) were used to demonstrate that XR-NTX can block antinociception. The current data extend this range of efficacy to much lower XR-NTX doses (down to 2.5 – 5 mg/kg). However, different opioids may require more or less XR-NTX depending on their relative potency at opioid receptors.

Unexpectedly, XR-NTX dose-dependently decreased distribution of oxycodone to serum and brain of in rats. A second experiment showed that XR-NTX reduces the $c_{\text{max}}$, clearance, and AUC following s.c. administration of oxycodone. These findings suggest that XR-NTX alters the pharmacokinetics of oxycodone and the overall exposure of oxycodone in rats. It is not clear why XR-NTX produces these effects. While it is possible that XR-NTX affected oxycodone absorption from the injection site into blood in this study it is unlikely because XR-NTX and oxycodone were administered at different sites and times. Another possibility is that naltrexone interfered with analysis of oxycodone, however, this was not seen in preliminary $\textit{in vitro}$ studies. While XR-NTX could have increased oxycodone clearance, accounting for both serum and brain oxycodone levels being lower in animals receiving naltrexone, we are aware of no reports of such an effect. The reduced oxycodone brain concentration in presence of naltrexone could be due to an effect of naltrexone on opioid transport across the blood brain barrier (BBB). For
instance, it has been shown that oxycodone, a cationic opioid agonist, is actively transported across the BBB (Bostrom et al., 2006), although it is not clear if oxycodone is a substrate for the well-characterized P-glycoprotein transporter (Bostrom et al., 2005) or for another organic cation transporter (Okura et al., 2008; Sadiq et al., 2011). Interactions on transport across the BBB have been shown between oxycodone and other CNS-acting drugs (Nakazawa et al., 2010). However, naltrexone has been demonstrated to not have any interaction with P-glycoprotein transporters, so it’s unlikely that naltrexone affected these transporters to affect oxycodone pharmacokinetics (Metcalf et al., 2014). Despite the unknown specific mechanism of this pharmacokinetic interaction, XR-NTX did not impair the efficacy of vaccination for blocking oxycodone-induced motor activity, which was the main focus of this study.

XR-NTX significantly lowered oxycodone-specific antibody titers only in selected experiments. XR-NTX’s effect on antibody titers was less evident when OXY-KLH was administered i.m. in aluminum adjuvant. Similarly, the immunomodulatory properties of naltrexone (or its XR-NTX formulation) seemed dose-dependent because this effect was significant at a low dose of XR-NTX (2.5 mg/kg), but not following a high dose (50 mg/kg). In a previously published study, XR-NTX did not interfere with the ability of OXY-KLH to induce antibodies in rats (Raleigh et al., 2017). In contrast, naltrexone has been shown to have immunostimulant activity in one study (Sacerdote et al., 1997), whereby administration of 10 mg/kg naltrexone 60 min prior to tissue collection and analysis showed elevated interleukin-2 (IL-2) production, suggesting elevated T cell activation (Liao et al., 2011). Chronic exposure in the current study versus acute exposure in the Sacerdote et al. study may explain these differences. Other studies have shown the use of naltrexone as an adjuvant to increase IgG1 and IgG2a antibody levels for vaccines against various parasites in mice (Tappeh et al., 2013; Mohammadzadeh Hajipirloo et al., 2014; Khorshidvand et al., 2016; Azizi et al., 2018). The authors suggested that naltrexone may
promote a TH₁ and cellular immune response by increasing local pro-inflammatory neuropeptides and may also block opioid receptors on innate immune cells to promote local inflammation. Interference at the B cell receptor could reduce B cell activation and hapten-specific antibody production. Nevertheless, the immunomodulatory effect of XR-NTX in the current study was minimal and all observed oxycodone-specific IgG antibody titers were within the range previously described for OXY-KLH (Pravetoni et al., 2012a; Pravetoni et al., 2012b; Pravetoni et al., 2013; Laudenbach et al., 2015; Raleigh et al., 2018).

Drug-induced motor activity has been previously used as a means of screening vaccination efficacy against opioids and other drugs of abuse (Carrera et al., 2001; Cornish et al., 2011; Li et al., 2011; Wee et al., 2012; Raleigh et al., 2013; Robinson et al., 2019). Fewer studies have characterized oxycodone-induced motor activity (Leri and Burns, 2005; Niikura et al., 2013), or tested the pre-clinical efficacy of XR-NTX in preventing oxycodone-induced behaviors (Bartus et al., 2003; Dean et al., 2008). In the current study, vaccination with OXY-KLH alone had mixed effects on oxycodone-induced motor activity. At low oxycodone doses (0.5 mg/kg), OXY-KLH reduced oxycodone-induced motor activity if antibody titers were above $200 \times 10^3$, but not if titers were under $50 \times 10^3$. At oxycodone doses at or above 1.0 mg/kg, vaccine alone did not alter motor activity. In contrast, a previous publication demonstrated that OXY-KLH in alum reduced oxycodone-induced motor activity following a much larger oxycodone dose of 5 mg/kg in mice (Robinson et al., 2019). However, rats and mice demonstrate differences in metabolism of oxycodone and may in part explain why oxycodone doses differed so much between studies (Ishida et al., 1982). In fact, doses of oxycodone that exceeded 2.0 mg/kg began showing reduced motor activity compared to lower doses, suggesting that these doses are not appropriate for testing vaccine and XR-NTX effects.
Combination of OXY-KLH and XR-NTX using high doses of XR-NTX (50 mg/kg) had no effect over XR-NTX alone due to the high XR-NTX dose. However, human doses are around 5.4 mg/kg (Bisaga et al., 2018). Thus, high XR-NTX doses may not reflect real-world application of a combination therapy. In the current study, XR-NTX doses that were half of typical human doses (2.25-2.5 mg/kg) were combined with OXY-KLH and demonstrated reduced motor activity compared to controls. Further, combination of XR-NTX and OXY-KLH was more effective than vaccine alone in reducing oxycodone-induced respiratory depression and bradycardia. Only one study has previously measured the effect of XR-NTX (at a dose of 50 mg/kg) on opioid-induced respiratory depressive effects (respiratory rate) and found that pretreatment with XR-NTX prevented fentanyl-induced respiratory depression (Dean et al., 2008). Combined, these data support the use of a combination therapy of OXY-KLH and XR-NTX.

The clinical efficacy of vaccines against prescription opioids, and against opioids in general, may be limited by highly variable and low serum antibody concentrations achieved in immunized subjects or by the occurrence of opioid users switching or transitioning between prescription opioids and other opioids, such as heroin or fentanyl. These limitations can be overcome by combining vaccination with medications targeting opioid receptors. Opioid antagonists may provide additional protection at the beginning of the vaccination regimen prior to development of clinically effective serum antibody titers and may enhance efficacy of vaccination in people who are relatively poor vaccine responders by providing additional blockage against the effects of prescription opioids. Additionally, if vaccination does not interfere with the efficacy of XR-NTX, patients wishing to change their type of treatment may do so without fear of losing efficacy during the transition.
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Authorship contributions:

Participated in research design: Accetturo, Pravetoni

Conducted experiments: Raleigh, Accetturo

Contributed new reagents or analytical tools: Pravetoni

Performed data analysis: Raleigh, Accetturo, Pravetoni

Wrote or contributed to the writing of the manuscript: Raleigh, Accetturo, Pravetoni
References


vector blocks cocaine psychostimulant and reinforcing effects.

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Footnotes

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Figure legends

**Figure 1.** Design overview of Experiments 1 through 5. XR-NTX doses were given i.m. with subscripts denoting mg/kg dose received. Sal refers to saline administration. The gap in the x axis in Experiment 2 depicts a separate cohort of animals. HP + PK refers to antinociceptive testing after s.c. administration of 2.25 mg/kg oxycodone on the hotplate set to 54°C followed by blood and brain collection while PK\textsubscript{2.25} refers to the pharmacokinetic study. For Experiments 3 through 5, vaccinations are shown as Vax # with # denoting order of vaccination and subscripts denoting Freund’s Adjuvant (FA) or aluminum (Al). Blood draws for titer analyses are shown as ‘Titers’. Motor activity sessions are shown from start to finish, with subscripts denoting s.c. oxycodone doses. %SaO\textsubscript{2} + HR refer to oximetry and bradycardia testing in Experiment 5.

**Figure 2.** Effect of XR-NTX on oxycodone-induced motor activity, thigmotaxis index, antinociception and distribution in naïve rats. Rats (n=8 each group) received 0, 1, 2.5, 5, 25, or 50 mg/kg XR-NTX (i.m.). A) Rats then received 0.5 mg/kg oxycodone s.c, and their activity was measured for 90 minutes. B) Thigmotaxis measurements were recorded during motor activity. C) Antinociception was measured using the hot plate (set to 54°C) 30 min following s.c. administration of 2.25 mg/kg oxycodone. D) and E) Serum and brain oxycodone levels were measured following antinociception. F) Separately, non-vaccinated naïve rats (n=8) were administered either 50 mg/kg XR-NTX i.m. or saline and blood collected at 15, 30, 60, 120, and 180 min. Data are expressed as mean ± SEM. Thigmotaxis index was calculated as the ratio of the distance travelled in the external area to the one travelled in the center. Two-way ANOVA with Bonferroni post-test (A and F) or one-way ANOVA using Dunnett’s multiple comparisons test (B, C, D, and E) were used to compare groups to saline. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 from saline control.
Figure 3. Effect of high dose of extended-release naltrexone on oxycodone-induced motor activity in rats immunized with OXY-KLH. A) Rats (n=8/group) received either saline or 50 mg/kg XR-NTX i.m. on 0 and 27 days and immunized with either 100 μg of the OXY-KLH vaccine or the unconjugated KLH administered on days 0, 21, and 42 in Freund’s adjuvant and tested in motor activity chambers. B) Rats were then vaccinated on days 83 and 104. Rats received a lower XR-NTX dose of 5 mg/kg i.m. on day 98. Rats were subsequently tested in motor activity chambers following s.c. doses of saline and 0.5 mg/kg oxycodone. Data are expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-test was performed to compare groups to controls. * p<0.05 and ** p<0.01, from KLH + saline.

Figure 4. Effect of low dose of extended-release naltrexone on oxycodone-induced activity and thigmotaxis in rats immunized with OXY-KLH. Rats received either saline or XR-NTX i.m. on days 0, 42 and 70. Concurrently, rats were immunized with either 100 μg of the OXY-KLH or KLH on days 0, 21, 42, and 72 in Freund’s adjuvant. A) Rats were tested in motor activity chambers following s.c. doses of saline and 0.5 mg/kg oxycodone. Then, rats received 1.0 mg/kg oxycodone s.c. and B) motor activity and C) thigmotaxis were measured. D) Motor activity was measured following a s.c. challenge of 2.0 mg/kg oxycodone. Two-way ANOVA with Bonferroni post-test or one-way ANOVA using Dunnett’s multiple comparisons test were performed to compare to KLH controls. Data are expressed as mean ± SEM. * p<0.05 and *** p<0.001 different from KLH + saline control and from KLH + XR-NTX.

Figure 5. Dose-dependent effect of oxycodone on motor activity, oxygen saturation, and heart rate of rats treated with OXY-KLH vaccine, alone or in combination with XR-NTX. Rats (n=8/group) received either saline or XR-NTX i.m. on days 0, 42 and 70. Concurrently, rats were immunized with either 100 μg of the OXY-KLH or KLH on days 0, 21, and 42. Aluminum hydroxide (90 μg) was used as the adjuvant. A) Motor activity was measured following saline or
0.5, 1.0, 1.5, and 2.0 mg/kg of oxycodone s.c. B) % SaO₂ and C) heart rate were measured at baseline and then after administration of cumulative oxycodone doses every 17 minutes s.c. Data are expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-test was performed to compare groups to controls and a separate two-way ANOVA with Bonferroni post-test was performed to compare each group to its baseline. * p<0.05, ** p<0.01, and *** p<0.001 different from saline control; ** p<0.01 different from OXY-KLH; * p<0.05 different from baseline.
Tables

Table 1. Effect of XR-NTX on pharmacokinetic parameters of oxycodone

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>XR-NTX</th>
<th>t value</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Half-life (hr)</td>
<td>1.04±0.48</td>
<td>0.94±0.40</td>
<td>0.4525</td>
<td>0.6574</td>
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<td>t_max (hr)</td>
<td>0.56±0.27</td>
<td>0.34±0.13</td>
<td>2.078</td>
<td>0.0604</td>
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<tr>
<td>c_max (ng/mL)</td>
<td>440.8±112.1</td>
<td>309.1±64.6</td>
<td>3.006</td>
<td>0.0101</td>
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<tr>
<td>AUC_{0-inf} (hr*ng/mL)</td>
<td>799.7±217.0</td>
<td>525.4±158.5</td>
<td>2.998</td>
<td>0.0093</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>1.71±0.72</td>
<td>1.42±0.54</td>
<td>0.9395</td>
<td>0.3627</td>
</tr>
<tr>
<td>V/F (L/kg)</td>
<td>4.33±1.92</td>
<td>5.81±1.47</td>
<td>1.1806</td>
<td>0.0913</td>
</tr>
<tr>
<td>Cl/F (L/hr/kg)</td>
<td>2.99±0.77</td>
<td>4.61±1.27</td>
<td>3.143</td>
<td>0.0091</td>
</tr>
</tbody>
</table>

Effect of XR-NTX on the pharmacokinetic parameters of oxycodone following a s.c. dose of 2.25 mg/kg oxycodone in saline treated (n=9) and XR-NTX treated (n=8) rats. Estimates for pharmacokinetic parameters include half-life, maximum concentration (c_max), time to reach c_max (t_max), area under the curve from zero to infinity (AUC_{0-inf}), mean residence time (MRT), volume of distribution (Vd/F, volume over the fraction absorbed), and clearance (Cl/F, clearance over the fraction absorbed). Data are expressed as mean ± SD. * p<0.05 and ** p<0.01 compared to saline treatment.
Experiment 1

Motor activity

0 - oxycodone

0 - 0.25, 0.5, 0.75, 1.0 oxycodone

Experiment 2

XR-NTX

0, 1, 2.5, 5, 25, 50

Motor activity

0 - 0.5 oxycodone

0 - 1, 2.5, 5, 25, 50

XR-NTX

50

HP + PK

PK

2.25 oxycodone

Experiment 3

XR-NTX

50

Motor activity

0, 0.5 oxycodone

XR-NTX

50

Motor activity

0, 0.5 oxycodone

Vax 1

FA

Vax 2

FA

Vax 3

FA

Titors

HP

Vax 4

FA

Vax 5

FA

Experiment 4

XR-NTX

2.5

Motor activity

0, 0.5 oxycodone

XR-NTX

2.5

Motor activity

0, 1, 1.5, 2, 2.5, 3, 3.5 oxycodone

Vax 1

FA

Vax 2

FA

Vax 3

FA

Titors

Vax 4

FA

Experiment 5

XR-NTX

2.25

Motor activity

0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 oxycodone

Vax 1

AI

Vax 2

AI

Vax 3

AI

Titors

%SaO2 + HR

Figure 1
Oxycodone (0.5 mg/kg, sc) + XR-NTX (0-50 mg/kg, im)

Saline

Oxycodone

Total distance (cm)

Day after start of study

0 1 2 5 25 50

0 mg/kg

1.0 mg/kg

2.5 mg/kg

5 mg/kg

25 mg/kg

50 mg/kg

0 mg/kg

1.0 mg/kg

2.5 mg/kg

5 mg/kg

25 mg/kg

50 mg/kg

Total distance (cm)

Thigmotaxis Index

XR-NTX (mg/kg)

Thigmotaxis Index

XR-NTX (mg/kg)

% MPE

XR-NTX (mg/kg)

Serum oxycodone (ng/ml)

XR-NTX (mg/kg)

Brain oxycodone (ng/gr)

XR-NTX (mg/kg)

Figure 2
Oxycodone (0.5 mg/kg, sc) + XR-NTX (50 mg/kg, im)

Total distance (cm)

Day after start of study

Saline | Oxycodone

Figure 3
**Oxycodone (0.5 mg/kg, sc) + XR-NTX (2.5 mg/kg, im)**

- Saline
- OXY-KLH + saline
- KLH + XR-NTX
- OXY-KLH + XR-NTX

**Oxycodone (1.0 mg/kg, sc) + XR-NTX (2.5 mg/kg, im)**

- Saline
- OXY-KLH + saline
- KLH + XR-NTX
- OXY-KLH + XR-NTX

**Oxycodone (1.0 mg/kg, sc), + XR-NTX (2.25 mg/kg, i.m.)**

**Oxycodone (2.0 mg/kg, sc), + XR-NTX (2.25 mg/kg, i.m.)**

Figure 4
Figure 5

A. Variation in activity from baseline (%) with different doses of oxycodone.

B. % SaO2 levels with different doses of oxycodone.

C. Heart rate (bpm) with different doses of oxycodone.