Prophylactic vaccination protects against the development of oxycodone addiction

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ABSTRACT

Abuse of prescription opioids is a growing public health crisis in the United States, with drug overdose deaths increasing dramatically over the past 15 years. Few preclinical studies exist on the reinforcing effects of oxycodone or on the development of therapies for oxycodone abuse. This study was conducted to determine if immunopharmacotherapy directed against oxycodone would be capable of altering oxycodone-induced antinociception and intravenous self-administration. Male Wistar rats were administered a small-molecule immunoconjugate vaccine (Oxy-TT) or the control carrier protein, tetanus toxoid (TT), and trained to intravenously self-administer oxycodone (0.06 or 0.15 mg/kg/infusion). Brain oxycodone concentrations were 50% lower in Oxy-TT rats compared to TT rats 30 minutes after injection (1 mg/kg, s.c.) whereas plasma oxycodone was 15-fold higher from drug sequestration by circulating antibodies. Oxy-TT rats were also less sensitive to 1-2 mg/kg, s.c. oxycodone on a hot water nociception assay. Half of the Oxy-TT rats failed to acquire IVSA under the 0.06 mg/kg/infusion training dose. Oxycodone self-administration of Oxy-TT rats trained on 0.15 mg/kg/infusion was higher than controls; however under progressive ratio (PR) conditions the Oxy-TT rats decreased their oxycodone intake, unlike TT controls. These data demonstrate that active vaccination provides protection against the antinociceptive and reinforcing effects of oxycodone. Anti-drug vaccines may entirely prevent repeated use in some individuals who otherwise would become addicted. Vaccination may also reduce dependence in those who become addicted and therefore facilitate the effects of other therapeutic interventions which increase the difficulty of drug use or incentivize other behaviors.

Keywords: Oxycodone; vaccine; self-administration

INTRODUCTION

Drug overdose deaths in the US have increased dramatically over the past 15 years with most of these attributable to opioids, including prescription drugs. Despite an increase in nonmedical prescription opioid abuse, with the use of heroin and other illicit drugs most often presaged by prescription opioids in new users (Dertadian and Maher, 2014; Mars *et al*, 2014), preclinical research into the addictive properties of oxycodone has been infrequent in comparison with studies on heroin or morphine. Limited studies in mice (Enga *et al*, 2016; Mayer-Blackwell *et al*, 2014; Zhang *et al*, 2015; Zhang *et al*, 2009) and rats (Leri and Burns, 2005; Mavrikaki *et al*, 2017; Secci *et al*, 2016; Wade *et al*, 2015) affirm that oxycodone will reinforce operant responding in intravenous self-administration (IVSA) models, thus, traditional pre-clinical measures of opioid addiction are useful to study the effects of potential therapeutics.

Anti-drug vaccination alters the effects of many drugs of abuse (see (Bremer and Janda, 2017; Lockner and Janda, 2013; Ohia-Nwoko *et al*, 2016) for reviews); vaccines against cocaine (Haney *et al*, 2010; Kosten *et al*, 2002; Martell *et al*, 2009) and nicotine (Cornuz *et al*, 2008; Hatsukami *et al*, 2011) have advanced to clinical trials. Initial studies by Pravetoni and colleagues showed that an oxycodone-specific vaccine could induce antibodies capable of sequestering oxycodone in the blood and lowering brain penetration of oxycodone in rats (Pravetoni *et al*, 2012; Pravetoni *et al*, 2014). Vaccination decreased the antinociceptive effects of a single dose of oxycodone and reduced oxycodone intake during the acquisition of IVSA. This success encourages more comprehensive research on anti-oxycodone vaccines, to further general understanding of how anti-drug vaccinations function in addition to supporting an eventual clinical vaccine for oxycodone abuse.

Janda and colleagues created a vaccine incorporating an oxycodone-hapten conjugated to tetanus toxoid (Oxy-TT) that has shown efficacy in attenuating antinociceptive effects and oxycodone overdose in mice (Kimishima *et al*, 2017). Therefore, it is of interest to determine if the Oxy-TT can reduce voluntary drug-seeking in a rat model. Prior evaluation of anti-drug vaccination efficacy in drug self-administration has typically focused on the effects of vaccination after the acquisition of drug taking behavior, e.g., for vaccines directed against cocaine (Carrera *et al*, 2000; Evans *et al*, 2016; Kantak *et al*, 2000), nicotine (Lindblom *et al*, 2002; Moreno *et al*, 2010) and morphine or heroin (Anton and Leff, 2006; Schlosburg *et al*, 2013). Although there have been a few studies of the effects of prophylactic vaccination prior to self-administration of cocaine (Wee *et al*, 2012), nicotine (LeSage *et al*, 2006a), oxycodone (Pravetoni *et al*, 2014), heroin (Stowe *et al*, 2011) and methamphetamine (Duryee *et al*, 2009; Miller *et al*, 2015), the effects of vaccination in the early stages of addiction are not well understood. Prior studies of

IVSA of oxycodone (Pravetoni *et al*, 2014) and methamphetamine (Duryee *et al*, 2009) suggest that increasing the workload (i.e., the number of lever responses required for each infusion) may decrease intake in the vaccinated groups relative to controls. Another finding with an anti-methamphetamine vaccine suggests, however, that initial higher drug intake may be gradually extinguished over the first several IVSA sessions with no change in workload (Miller *et al*, 2015). Cocaine IVSA is lower in vaccinated animals than in controls under a Progressive Ratio (PR) schedule of reinforcement and increased workload (Wee *et al*, 2012), whereas nicotine IVSA *increases* in the vaccinated rats (LeSage *et al*, 2006a). The present study was conducted to determine if the novel Oxy-TT vaccine directed against oxycodone would be capable of altering the IVSA of oxycodone.

METHODS AND MATERIALS

Animals. Adult male Wistar (N=48; Charles River, New York) rats were housed in humidity and temperature-controlled vivaria (23$\pm$1°C) on 12:12 hour light:dark cycles. Cohort 1 (N=24; lower training dose) animals were 10 weeks old (mean weight 288.3 g; SD 12.8) at the start of the study. Cohort 2 (N=24; higher training dose) animals were 13 weeks old (mean weight 408.9 g; SD 30.6) at the start of the study. Animals had *ad libitum* access to food and water in their home cages. All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute and in a manner consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Hapten Synthesis. The oxycodone hapten (Oxy) was designed with an activated linker extending from the bridgehead nitrogen to directly react with the surface lysines of carrier protein tetanus toxoid (TT, **Figure 1**) or BSA. Oxycodone hapten was synthesized according to previously published methods from the Janda laboratory with slight modification in the reductive amination and amide bond formation steps (Kimishima *et al*, 2016). Detailed synthetic methods, characterization data, and immunoconjugation protocols can be found in the Supplemental Information.

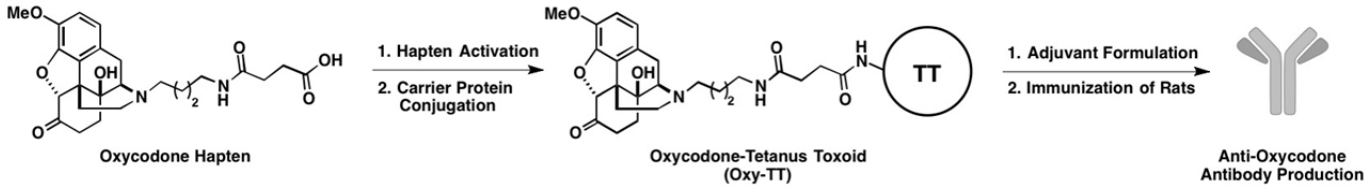

Figure 1. The general vaccine strategy using an oxycodone hapten-immunoconjugate vaccine.

Vaccine Formulation. Vaccines were formulated the day of immunization using 13:1:5 (v/v/v) mixture of Oxy-TT (1.0 mg/ml in PBS) or control TT (1.0 mg/ml in PBS), CpG ODN 1826 (5 mg/ml in PBS), and Alhydrogel® (alum, 10 mg/ml, InvivoGen). Rats were administered the conjugate vaccine (Oxy-TT; N=24) or tetanus toxoid only control (TT; N=24) on Weeks 0, 2, 4, 8 and 18 for Cohort 1 and on Weeks 0, 2, 4, 8 and 20 for Cohort 2. The immunization protocol was adapted from a vaccination protocol previously reported (Nguyen *et al*, 2016a; Nguyen *et al*, 2017). Additional details are provided in the Supplemental Information.

Drugs. Oxycodone hydrochloride used for this study was obtained from Sigma-Aldrich (St. Louis, MO) and 3,6-Diacetylmorphine HCl (Heroin HCl) was obtained from NIDA Drug Supply. Drugs were expressed as the salt and dissolved in physiological saline (0.9%). For tail-withdrawal studies, rats were administered 0.9% saline vehicle, oxycodone (0.5, 1.0, 2.0 mg/kg) or heroin (1.0 mg/kg) via subcutaneous (s.c.) injection.

Intravenous Catheterization and Self-Administration Procedure. Rats were anesthetized with an isoflurane/oxygen vapor mixture (isoflurane 5% induction, 1-3% maintenance) and prepared with chronic intravenous catheters as described (Miller *et al*, 2013; Nguyen *et al*, 2016b) on week 4 of the vaccination protocol. Rats were allowed to self-administer under a Fixed Ratio 1 (FR1) contingency for 18 sessions. Successful acquisition in the first 18 sessions was defined as an average of 7 or more infusions across two sequential days; the day of acquisition was defined as the first day. Median splits were determined by ranking animals on their mean number of infusions obtained across 18 sessions, and are referred to as Upper and Lower. The 19th session was a PR session with the respective training dose for all animals. In the PR paradigm, the required response ratio is increased after each reinforcer delivery within a session (Hodos, 1961; Segal and Mandell, 1974) as determined by the following equation (rounded to the nearest integer): Response Ratio=5e^(injection number**j*)–5 (Richardson and Roberts, 1996). The *j* value is as specified for a given experiment, i.e., PR^{J2} refers to a session with the *j* value set to 0.2. Additional details are provided in the Supplemental Information.

<u>Cohort 1:</u> Sessions 20-21 were FR1/training dose and Session 22 featured saline substitution. Rats responding under the training conditions for Sessions 31-44 with pre-session administration of 0.0, 0.003, 0.01 or 0.03 mg/kg naloxone, i.p. in an order randomized across the groups. There was negligible difference attributable to naloxone pre-treatment. Training conditions were restored for Sessions 28-30 (i.e., FR1) and thereafter the schedule was FR5 for Sessions 31-44 and FR10 for Sessions 45-63. The PR^{J2} schedule was in effect for Sessions

64-70 and FR1 for Sessions 71-73. Mean infusions in Sessions 28-30 were used to re-rank individuals for the median split for this part of the experiment to determine effects of schedule changes on current, rather than acquisition, drug preference phenotype. This resulted in 3 individuals in the original lower half of the TT group (TT Lower) switching with Upper half individuals and one individual in the original Oxy-TT Lower half switching with an Upper half individual.

Cohort 2: Sessions 20-23 were PR^{J2} sessions with doses (0.0, 0.06, 0.15, 0.3 mg/kg/inf) presented in randomized order. There was negligible difference attributable to dose and the subsequent experiments focused on FR/PR transitions at a fixed per-infusion dose. Sessions 24-25 restored the training dose under FR1. Sessions 26-30 were PR^{J2} and sessions 31-35 were PR^{J3} (0.15 mg/kg/inf). Sessions 36-37 were FR1 (0.15 mg/kg/inf). Sessions 38-39 were FR1 (0.06 mg/kg/inf) and session 40 was PR^{J3} (0.06 mg/kg/inf).

Nociception. Tail withdrawal latency was assessed before, and 30 minutes after, injection of oxycodone (0.5, 1.0 or 2.0 mg/kg, s.c.) or heroin (1.0 mg/kg, s.c.) using a 52°C water bath and a 15 s maximum interval (Gold et al., 1994). Studies were conducted across the course of IVSA (pre- and post-acquisition training) to verify *in vivo* efficacy of the vaccine. Tests were performed on Weeks 5, 10 and 27 for Cohort 1 and on Weeks 5, 9 and 11 for Cohort 2.

Determination of Plasma and Brain Oxycodone. In Cohort 1, Oxy-TT (N=11) and TT rats (N=11) blood was collected 30 minutes after 1.0 mg/kg oxycodone, s.c., on Week 14 (6 weeks from last boost). Blood and brain tissue were collected 30 minutes after 1.0 mg/kg oxycodone, s.c., (N=5, Oxy-TT; N=6, TT) or 2.0 mg/kg oxycodone, s.c., (N=5 per group) on Week 29. In Cohort 2, blood was collected from rats (N=12, Oxy-TT; N=10, TT) 30 minutes after 2.0 mg/kg oxycodone, s.c. on Week 26 (6 weeks from last boost). In Week 27, blood was collected 15 and 30 minutes after 1.0 mg/kg oxycodone, s.c., and brains were collected at the 30 minute time point. Oxycodone was quantified using LCMS using spiked d_6-oxycodone (Cerilliant, Round Rock, TX). See Supplemental Information for details.

Data Analysis. Intravenous self-administration data were analyzed with repeated-measure Analysis of Variance (rmANOVA) with Vaccine Group (TT Upper/Lower and Oxy-TT Upper/Lower) as a between-subjects factor and Time (Session) as a within-subjects factor. Tail-withdrawal latencies were analyzed with rmANOVA with Vaccine as a between-subjects factor and with Time (and therefore Drug treatment) as a within-subjects factor. Plasma data

were analyzed with two-way rmANOVA with vaccine Group as a between-subjects factor and Drug condition as the within-subjects factor as repeated measure factors. Brain concentrations were analyzed by unpaired t-tests. Significant effects within group were followed with post hoc analysis using Tukey correction for all multi-level factors and Sidak for two-level factors. Criterion for significant results was set at $p<0.05$, and all analysis used Prism for Windows (v. 6.07 and 7.03; GraphPad Software, Inc, San Diego CA).

RESULTS

Conjugation of Oxy-TT Vaccine. The oxycodone hapten (Oxy) and Oxy-TT immunoconjugate (**Figure 1**) were prepared as described previously (Kimishima *et al*, 2016) with minor modification, described in detail in the Supplementary Information. MALDI-ToF analysis of Oxy-BSA confirmed successful conjugation with approximately 10-16 moles of Oxy hapten per mole of surrogate carrier protein (**Figure S6**; Supplemental Information).

Vaccination Protects Against Self-Administration of Oxycodone. Using a threshold per infusion dose of oxycodone (0.06 mg/kg/inf; Cohort 1) under fixed-ratio (FR) schedule of reinforcement, rats steadily increased intake across the first 18 sessions of training (**Figure 2A**; See details of FR schedule in Materials and Methods). Analysis of the data confirmed a significant effect of Session [$F(17,357)=14.54$; $p<0.0001$] but not of Group or of the interaction of factors. Only 58% of the Oxy-TT group met the acquisition criterion (average of 7 infusions across two sessions), whereas all of the TT animals met criterion (**Figure 2C**). Analysis of the acquisition data by median split (**Figure 2E**) confirmed a significant effect of Group [$F(3,19)=7.83$; $p<0.005$], of Session [$F(17,323)=16.95$; $p<0.0001$] and of the interaction [$F(51,323)=2.84$; $p<0.0001$]. Collapsed across session, significantly more infusions were obtained by the Oxy-TT Upper group compared with each of the Lower half groups. See Supplemental Information **Figure S8A** for the percent meeting acquisition within sub-group. Under a higher per infusion dose (0.15 mg/kg/inf; Cohort 2), rats increased oxycodone intake over the first 18 sessions of acquisition, with the Oxy-TT group eventually obtaining more infusions (**Figure 2B**). The analysis confirmed significant effects of Session [$F(17,357)=19.89$; $p<0.0001$], of vaccine Group [$F(1,21)=6.02$; $p<0.05$] and of the interaction [$F(17,357)=3.22$; $p<0.0001$]. In this Cohort, 83% the TT and Oxy-TT animals met acquisition criterion by averaging 7 infusions across two sessions (**Figure 2D**). Analysis of the acquisition data by median split (**Figure 2F**) confirmed a significant effect of Group [$F(3,19)=11.72$; $p<0.0005$], of Session [$F(17,323)=19.05$; $p<0.0001$] and of the interaction of factors [$F(51,323)=1.62$; $p<0.01$].

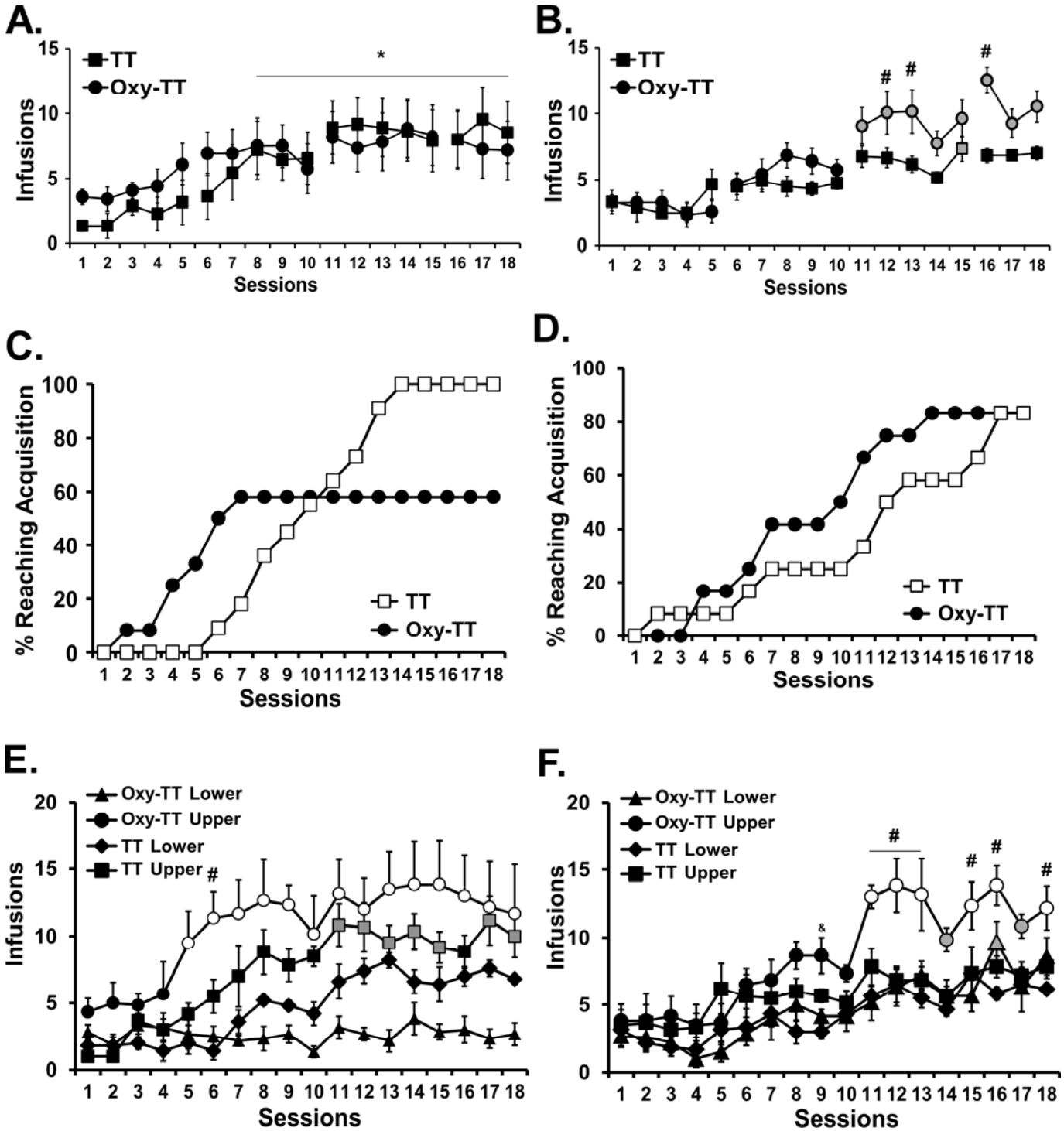

Figure 2. Vaccination attenuates intravenous self-administration of low dose oxycodone. *Mean (±SEM) oxycodone infusions obtained by rats in A) Cohort 1 (TT; N=11 and Oxy-TT; N=12) and B) Cohort 2 (TT; N=12 and Oxy-TT; N=11). Post hoc tests: A significant difference from each of the first three sessions, across Groups is indicated with *. Cumulative percentage of each group reaching acquisition criterion (average of 7 infusions across two sessions) in C) Cohort 1 and in D) Cohort 2. Mean (±SEM) infusions obtained by the Upper and Lower (TT Lower N=5) halves of the vaccine groups in E) Cohort 1 and F) Cohort 2. Broken lines between sessions 5 & 6, 10 & 11 and 15 & 16 represent the 70 h weekend interval. Post hoc tests: In panels E and F, the grey symbols indicate a significant difference from the first three sessions within-group. Open symbols reflect significant differences from the first three sessions and between Upper and Lower halves within each vaccine group. A significant difference between vaccine Groups or between the Upper halves of each vaccine group is indicated with #.*

Oxy-TT Vaccine Decreases Motivation for Oxycodone Self-Administration. By increasing the workload required for reinforcement, the first PR probe (See details of PR schedule and *j* values in Materials and Methods) in Cohort 1 produced a significant reduction in the number of infusions earned for the Oxy-TT rats only (**Figure 3A**); there was a significant effect of schedule condition [$F(1,21)=9.35$; $p<0.01$] but not of vaccine Group. The analysis of the saline substitution (**Figure 3B**) confirmed significant effects of vaccine Group [$F(1,20)=4.46$; $p<0.05$] and of Drug treatment condition [$F(1,20)=28.63$; $p<0.0001$], but not of the interaction of factors. Following further training under increasing FR schedule, the TT group obtained more oxycodone infusions under PR (Sessions 64-70) compared with the final FR10 sessions (Sessions 45-63), whereas the Oxy-TT group only increased intake following transition back to the FR1 (Sessions 71-73) condition (**Figure 3C**). The rmANOVA confirmed significant effects of Session [$F(8,136)=1301$; $p<0.0001$] and of the interaction of vaccine Group and Session factors [$F(8,136)=2.610$;$p<0.05$]. See **Figure S9** for sub-group effects of incrementing the FR, loss of catheter patency prevented subgroup analysis beyond this stage of the experiment.

Oxy-TT rats trained under a higher per infusion dose (Cohort 2) also self-administered less oxycodone under the higher workload conditions of a PR schedule (**Figure 3D**). The analysis confirmed an interaction of Group with reinforcement schedule [$F(1,20)=19.68$; $p<0.0005$]. In the subsequent FR, PR^{J2}, PR^{J3}, FR transitions fewer infusions were obtained in both PR^{J2} and PR^{J3} relative to each of the FR conditions within the Oxy-TT group (**Figure 3E**). The analysis confirmed a main effect of schedule [$F(3,54)=10.45$; $p<0.0001$] and interaction of Group with reinforcement contingency [$F(3,54)=6.23$; $p<0.005$]. Finally, the analysis of the final FR (0.15 mg/kg/inf), FR (0.06 mg/kg/inf), PR^{J3} (0.06 mg/kg/inf) conditions (**Figure 3F**) confirmed a significant impact of the dose/schedule factor [$F(2,36)=62.15$; $p<0.0001$] and an interaction of this factor with Group [$F(2,36)=3.51$; $p<0.05$].

Vaccine Attenuates Antinociceptive Effects of Oxycodone. The effects of oxycodone on nociception were first determined prior to self-administration training in Cohort 1 (N=11-12) and Cohort 2 (N=12) rats. For Cohort 1 (**Figure 4A**), the rmANOVA confirmed significant main effects of vaccine Group [$F(1,21)=19.45$; $p<0.001$], of Time [$F(4,84)=116$; $p<0.0001$], and of the interaction [$F(4,84)=8.802$; $p<0.0001$]. For Cohort 2 (**Figure 4B**), the analysis also confirmed a significant effect of vaccine Group [$F(1,22)=6.36$; $p<0.05$], of Time [$F(8,186)=17.1$; $p<0.0001$] and of the interaction [$F(8,176)=5.39$; $p<0.0001$]. The dose-dependent attenuation of the antinociceptive effects of oxycodone in Oxy-TT rats persisted throughout the study (**Figure S11**; Supplemental Information). Ultimately, the attenuation of oxycodone antinociception was observed up to 8-10 weeks (data not shown) after the most recent

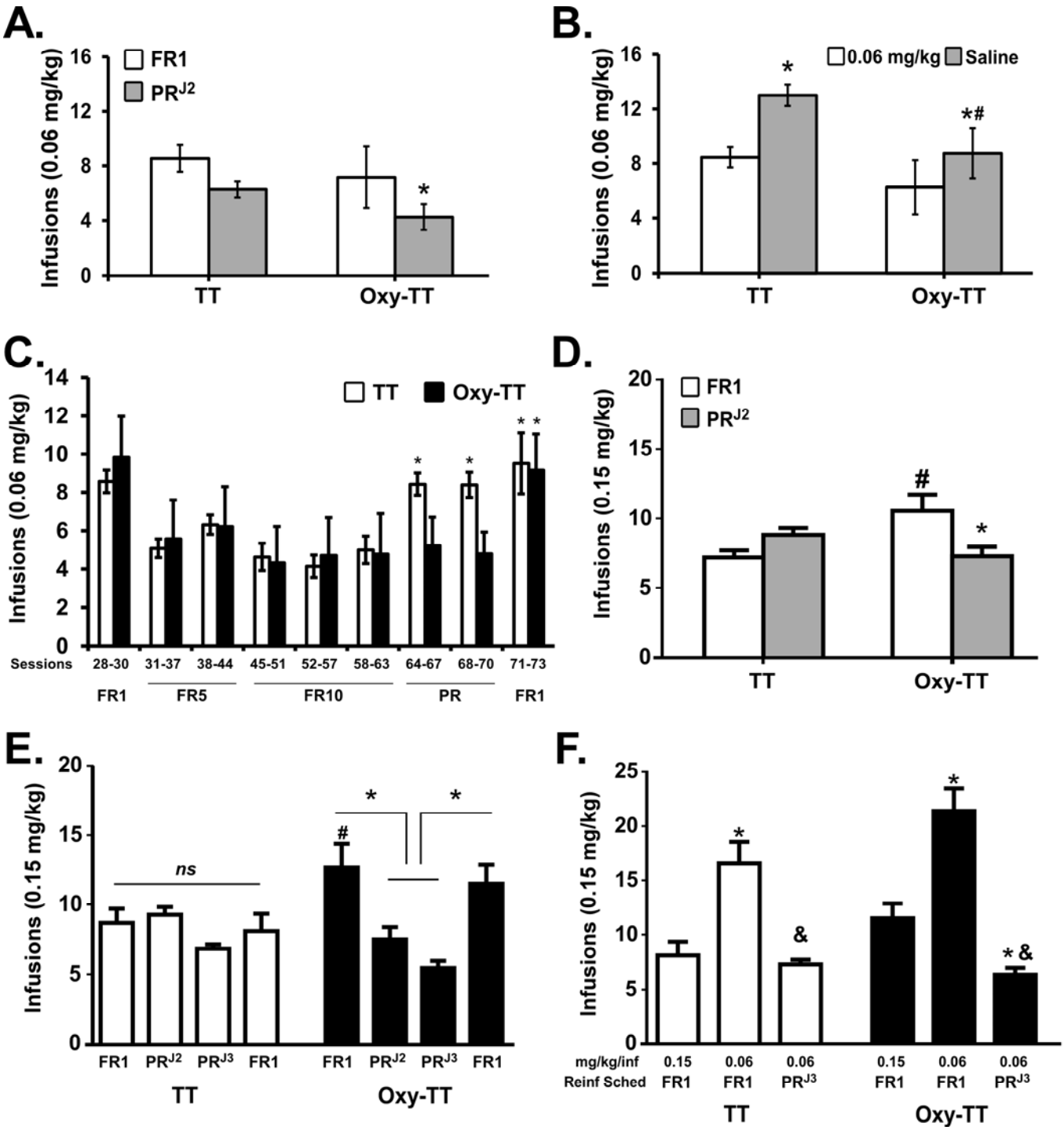

Figure 3. Vaccination decreases motivation for oxycodone self-administration under increased workload. *Mean (+SEM) oxycodone infusions obtained by TT (N=11) and Oxy-TT (N=11-12) vaccinated groups in Cohort 1 under A) PR conditions or B) during a saline challenge. Post hoc tests: A significant difference within-group is indicated with * and between the groups with #. C) Training under FR1 to FR10 followed by PR challenges showed that TT (N=11) rats obtained more infusions compared to Oxy-TT (N=8) rats. Post hoc tests: A significant difference from the FR 10 (Sessions 45-63) condition is indicated with *. Mean (±SEM) infusions of oxycodone for Oxy-TT (N= 9-11) and TT (N= 11) vaccinated rats under D) PR conditions and B-C) subsequent challenges with intermittent changes in workload (PR^{J2}-PR^{J3}). Post hoc tests: A significant difference between vaccine groups for a given schedule of reinforcement condition is indicated by #, a difference from FR (0.15 mg/kg/inf) within group by * and from FR (0.06 mg/kg/inf) within group by &. ns indicates no significant difference was confirmed.*

vaccination boost. Finally, Oxy-TT vaccination did not protect against heroin (1.0 mg/kg, s.c.)-induced antinociception which was assessed in Cohort 2 (**Figure 4C**). The rmANOVA confirmed significant effects of vaccine Group [F(1,22)=6.33; *p*<0.05] and of Time [F(4,88)=28.45; *p*<0.0001].

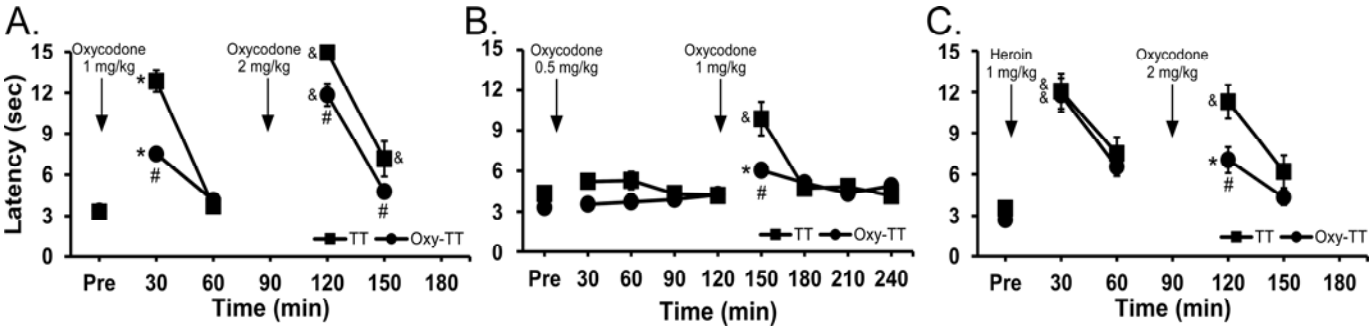

Figure 4. Vaccination ameliorates antinocicpetive effect of oxycodone. *Mean (±SEM) tail-withdrawal latency in Oxy-TT (N=12) vaccinated rats as compared to TT (N=11-12) control rats prior to self-administration training in A) Cohort 1 and B) Cohort 2. C) A post-acquisition test using a non-contingent injection heroin (1 mg/kg, s.c.) showed no difference between vaccine Groups, but persistence of the difference after oxycodone injection (2 mg/kg, s.c.). Post hoc tests: Significant differences between the groups is indicated with #. Significant differences within group compared with Pre are indicated by * and compared with the most prior observation before a drug injection (60 or 120 min) with &.*

Vaccine Protects from Brain Penetration of Oxycodone. The efficacies of the Oxy-TT and control TT vaccines to elicit anti-oxycodone antibodies (Abs) were assessed by an ELISA (**Figure S10;** Supplemental Information). No anti-oxycodone Abs were detected in the control TT group. Plasma oxycodone (ng/mL) was significantly higher in the Cohort 1 Oxy-TT rats compared to the corresponding TT group following injection of oxycodone (1.0 mg/kg, s.c., **Figure 5A**), which was confirmed by an unpaired t-test [t(20)=7.708, *p*<0.0001]. In Cohort 2, plasma oxycodone was also higher in Oxy-TT rats compared to TT rats (**Figure 5B**) as confirmed by significant effects of vaccination Group, [F(1,22)=73.35; *p*<0.0001]), of Drug condition [F(2,44)=24.13; *p*<0.0001], and of the interaction [F(2.44)=14.98; *p*<0.0001]. Plasma oxycodone levels in TT rats 30 minutes after injection of 2 mg/kg oxycodone were approximately doubled compared to plasma levels after injection of 1.0 mg/kg oxycodone (**Figure 5B insert**), whereas plasma oxycodone in Oxy-TT rats were similar, possibly reflecting the binding capacity of the Abs. Analysis of brain tissue from Cohort 2 after injection of 1.0 mg/kg, s.c., showed significantly lower brain concentrations of oxycodone in Oxy-TT rats compared to TT rats (**Figure 5C**; [t(19)=4.569, *p*<0.001]).

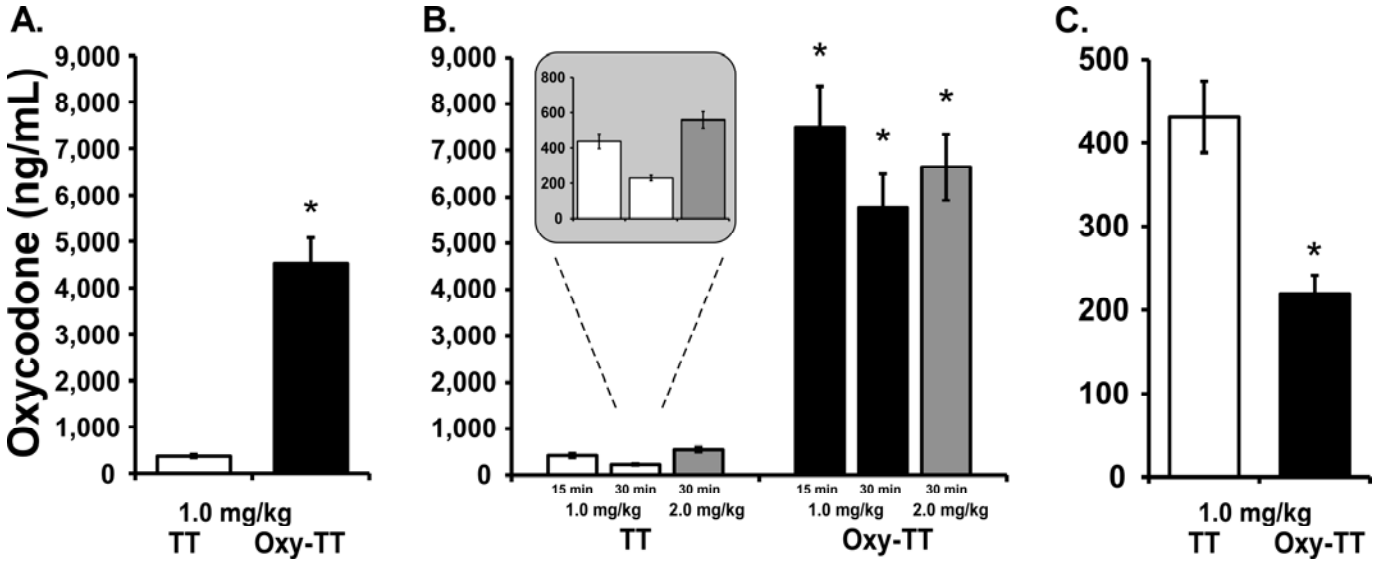

Figure 5. Analysis of oxycodone biodistribution in plasma and brain. *A) Mean (±SEM) oxycodone concentrations in plasma, 30 min after injection of oxycodone (1.0 mg/kg, s.c.,) in TT and Oxy-TT groups (N=11 per group) of Cohort 1. B) Mean (±SEM) oxycodone concentrations in plasma at 15 or 30 min after injection of oxycodone (1.0 or 2.0 mg/kg, s.c.) in TT (N=10) and Oxy=TT (N=11; blood could not be obtained from one animal in this experiment) groups of Cohort 2. The insert shows that TT plasma levels at 30 min were twice as high after 2 mg/kg injected versus 1 mg/kg. C) Mean (±SEM) oxycodone concentrations in whole brain tissue at 30 min after injection of oxycodone (1.0 mg/kg, s.c.) in TT (N=10) and Oxy-TT (N=11) groups of Cohort 2. Post hoc tests: A significant difference between vaccine Groups is indicated with *.*

DISCUSSION

This study shows that active vaccination with Oxy-TT provides functional protection against the reinforcing effects of oxycodone in rats, *in vivo*. First, the study demonstrated efficacy of the Oxy-TT vaccination using antinociception and pharmacokinetic assays under non-contingent drug exposure conditions, thus confirming basic biological efficacy of the vaccination. Second, the Oxy-TT vaccination resulted in about half of animals failing to acquire stable levels of intravenous self-administration using threshold training conditions under which all of the control animals met acquisition criteria. Finally, the Oxy-TT vaccination resulted in greater susceptibility of those animals that had established consistent self-administration (under either lower or higher per-infusion dose training conditions) to reduce their intake under a PR schedule of reinforcement. This latter finding, combined with evidence of reduced brain penetration of oxycodone, suggests anti-drug vaccination may be effective in synergy with other interventions which help to reduce drug use.

The tail-withdrawal test demonstrated that the Oxy-TT groups of each Cohort were less sensitive to the antinociceptive effect of an injection of oxycodone in a dose-dependent manner. This was observed prior to the start of self-administration training and on subsequent time points throughout the course of the self-administration

study. The post-acquisition test in both Cohorts (**Figure S11**; Supplemental Information) indicated some degree of tolerance to the antinociceptive effects of oxycodone (i.e. after the 1.0 mg/kg dose) compared with the pre-acquisition test, most parsimoniously attributed to the oxycodone exposure during many sessions of self-administration. Importantly, the tail-withdrawal test in Cohort 2 (Week 11) found that while Oxy-TT animals were less sensitive to the effects of *oxycodone*, all animals were equally affected by *heroin* (**Figure 4c**). This shows the selectivity of the protection afforded by anti-oxycodone vaccination and also demonstrates that the relatively higher cumulative number of self-administered infusions in the Cohort 2 Oxy-TT group did not convey significant differential cross-tolerance to heroin, and by inference, differential tolerance to oxycodone. This provides further converging evidence that the higher IVSA in the Cohort 2 Oxy-TT group did not result in higher ongoing drug exposure to the central nervous system. Similar dose-relationships were observed in the pre-acquisition tail-withdrawal latencies across Cohorts 1 and 2 despite different group mean antibody titer (**Figure S10**; Supplemental Information). These non-contingent studies therefore highlight the lack of simple correlation of antibody titer with *in vivo* outcome, and confirm antibody hapten binding (as detected via ELISA) does not necessarily equate to functional protection *in vivo*. As a minor caveat, surface plasmon resonance analysis of binding interactions confirmed that the polyclonal response presented tighter binding to free drug in Cohort 1 compared to Cohort 2 (**Figure S12**; Supplemental Information). This increased affinity may have partially compensated for the difference in titer across Cohorts. Nevertheless, there were similar results observed in Cohort 2 Oxy-TT rats from pre-acquisition through to the final assessment, despite the final titer in Cohort 2 having decreased substantially compared with their titer at the time of the pre-acquisition test. This is also consistent with a lack of difference in titer within-Cohort that was associated with the median-split in the distribution of self-administration preference. Similarly, LeSage and colleagues reported that there was no correlation of titer with nicotine self-administration (LeSage *et al*, 2006b).

The pharmacokinetic experiments showed that the Oxy-TT vaccinated rats from each Cohort had approximately 12-15-fold higher plasma oxycodone 30 minutes after 1 mg/kg oxycodone (s.c.) and in Cohort 2 it was shown this coincided with about half the brain levels of oxycodone, compared with TT controls, at the end of the study (**Figure 5**). Interestingly, the plasma oxycodone levels the Cohort 2 TT controls were dose dependent (i.e., about twice as high after 2 mg/kg compared with the 1 mg/kg injection) but no dose-dependency was found in the Oxy-TT rats. This may indicate that the saturation point of protection for those animals was between 1 and 2 mg/kg. Correspondingly antinociceptive effects of oxycodone were observed after 2 mg/kg, s.c., but not a 1 mg/kg,

s.c., in the Oxy-TT group. This functional protection of the central nervous system from a given non-contingent dose of oxycodone demonstrates biological efficacy of the vaccine.

The acquisition of oxycodone IVSA under the lower per-infusion-dose training (Cohort 1) was associated with significant intra-group differences in the Oxy-TT rats. The Lower half of the distribution obtained many fewer infusions than the TT control group's Lower half while the Upper half of the Oxy-TT group self-administered more oxycodone compared with either half of the TT group distribution. Interestingly this intra-group difference in IVSA was not associated with any difference in the blunting of the antinociceptive effects of oxycodone either before or after the acquisition training, nor any significant difference in antibody titer (not shown). This lack of difference in functional efficacy between Oxy-TT groups in Cohorts 1 and 2 is consistent with prior studies that have not typically found strong correlations between individuals' titer and behavioral responses to drug (LeSage *et al*, 2006a); the affinity of antibody to drug may provide a more reliable correlation (Lockner *et al*, 2015). The number of infusions obtained at the end of the initial FR1 acquisition interval in the Cohort 2 study was only 45% higher in the Oxy-TT animals while the post-mortem brain tissue study showed a 49% lower brain penetration of oxycodone. This suggests there may have been a ~26% lower brain oxycodone level in the Oxy-TT group when 0.15 mg/kg/inf was available under FR1 at the end of the acquisition phase and 34% lower brain oxycodone when 0.06 mg/kg/inf was available under FR1 near the end of the study.

Another major outcome of this study was the Oxy-TT vaccinated rats that did acquire stable IVSA exhibited greater sensitivity to reduce intake under an increased workload to obtain oxycodone. This was observed in the Cohort 2 rats despite the fact that a greater number of infusions were obtained by the Oxy-TT group when only one lever response was required per infusion. When the difficulty was increased by introducing a PR schedule, Oxy-TT rats obtained fewer infusions compared with the TT group and significantly fewer infusions than their own intake under FR1. In comparison, the TT group did not alter their intake significantly in response to the increased workload. A similar sensitivity of the Oxy-TT group to increased workload was also observed in Cohort 1. When the effort of obtaining oxycodone was increased by making infusions contingent on a higher, fixed number of responses (FR5, then FR10), both groups self-administered less oxycodone compared with the FR1 condition. However, once a PR schedule was introduced the TT group responded for *more* drug because the cumulative response requirement for the number of infusions obtained was *lower* than in the prior FR10 sessions (the PR^{J2} required only 9 responses for the fourth infusion, and fewer for the prior three infusions), but the Oxy-TT group did not similarly increase their intake. Upon restoration of the FR1 schedule, the Oxy-TT animals increased to match the TT group which did not further elevate their intake compared to the intake under PR. These results are both consistent with

the Oxy-TT animals being much more sensitive to increasing the demand of obtaining drug infusions. A similar reduction in self-administration under a PR contingency with a cocaine vaccine has been previously reported (Wee *et al*, 2012). This result is behavioral confirmation that the Oxy-TT group experienced less brain exposure during the course of the IVSA training, resulting in a less-dependent state despite the greater number of infusions self-administered. Again, assuming the Oxy-TT rats' brain oxycodone levels were ~51% of the TT rats' concentrations, functional exposure under PR was 42% (first PR), 41% (PR^{J2}; 0.15 mg/kg/inf and PR^{J3}; 0.15 mg/kg/inf) and 44% (PR^{J3}; 0.06 mg/kg/inf) lower in the Oxy-TT group compared with the controls. Overall this suggests that any behavioral compensation for the reduced brain penetration of drug was incomplete and insufficient to produce brain oxycodone levels as high as those achieved by the TT control group.

The behavioral results of this study have important implications for the interpretation of the efficacy of anti-drug vaccination in pre-clinical models and the application to eventual clinical trials. The results from Cohort 1 illustrate the likely success of prophylactic vaccination prior to any drug experience. Some subset of the vaccinated animals will never acquire high levels of self-administration behavior, particularly given a threshold level of drug exposure. This is consistent with the prior finding of Pravetoni (Pravetoni *et al*, 2014) which used the same 0.06 mg/kg/infusion training dose, although that study also used an increasing Fixed-Ratio across acquisition sessions which complicates interpretation. Delayed acquisition of stable IVSA was also reported in a study of the efficacy of an anti-methamphetamine vaccine (Miller *et al*, 2015). It is also notable that the amount of oxycodone self-administered depended on how much effort was required to obtain each infusion, most pointedly in Cohort 2. Increasing the workload unmasked the fact that the Oxy-TT vaccinated group were less motivated to seek oxycodone even if they obtained more infusions under easy access (FR1) conditions. This makes it more likely that prior results for oxycodone (Pravetoni *et al*, 2014) and methamphetamine (Duryee *et al*, 2009) self-administration in vaccinated rats was due to the workload increasing across training sessions rather than behavioral extinction.

These results suggest a critical reconsideration of the design and interpretation of human clinical trials in at least two particulars. First, these data encourage consideration of trials for prophylactic efficacy since the Lower half of the Cohort 1 Oxy-TT distribution did not establish a consistent drug-taking pattern. Human drug dependence is a minority outcome among those that sample a given drug (Schramm-Sapyta *et al*, 2009) and it is intuitively obvious that preventing the establishment of dependence from the outset is a sure way to reduce drug-related harms. Even though the Cohort 2 Upper half of the Oxy-TT distribution reached stable, higher patterns of self-administration during acquisition, their intake was more easily reduced compared with the TT animals. The increased intake of oxycodone in the Upper halves of each of the Oxy-TT vaccinated groups might naively be

interpreted in a human clinical context as a significant failure, since drug-taking is usually the main outcome measure of clinical trials (Hoogsteder *et al*, 2014; Martell *et al*, 2009). If, as in these preclinical studies, the human users' drug intake fails to fully compensate for the reduced brain levels of drug this could actually be a treatment success that results in lower levels of dependence over time. This might also facilitate the effects of other therapeutic interventions, similar to the effects of increasing the workload under PR conditions in the present results. In summary, this study showed *in vivo* efficacy of a vaccine directed against oxycodone across pharmacokinetic, non-contingent administration and self-administration assays. The manner by which self-administration was affected provides new insights into how anti-drug vaccines might be tested and applied in the human clinical context.

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