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Heroin vaccine: Using titer, affinity, and antinociception as metrics when examining sex and strain differences

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ABSTRACT

Anti-drug vaccines have potential as new interventions against substance use disorder (SUD). However, given the challenges seen with inter-individual variability in SUD vaccine trials to date, new interventions should ensure a robust immune response and safety profile among a diverse population. This requires accounting for sex and heritable genetic differences in response to both abused substances as well as the vaccination itself. To test response variability to our heroin-tetanus toxoid (Her-TT) immunoconjugate vaccine, we vaccinated male and female mice from several mouse strains including Swiss Webster (SW), BALB/c, and Jackson diversity mice (J:DO). Previous studies with vaccinated male SW mice demonstrated a rare hypersensitivity resulting in mice rapidly expiring with exposure to a low dose of heroin. Our results indicate that this response is limited to only male SW mice, and not to any other strain or female SW mice. Our data suggest that this hypersensitivity is not the result of an overactive cytokine or IgE response. Vaccination was similarly effective among the sexes for each strain and against repeated heroin challenge. Inbred BALB/c and J:DO mice were found to have the best vaccine response against heroin in antinociception behavioral assay. These results highlight the importance of incorporating both male and female subjects, along with different strains to mimic diverse human populations, as new SUD vaccines are being tested.

1. Introduction

Experimental drug conjugate vaccines designed to treat substance use disorder (SUD) are new chemical immunology interventions, which have shown preclinical efficacy in animals but have not yet achieved clinical approval [1–4]. In 2018, the Centers for Disease Control (CDC) released its annual surveillance report in 'Drug-related risks and outcomes' and found that there was a record high in the number of overdose deaths in 2016, primarily attributed to the rise in prescription opioid abuse and heroin; opioid-associated overdose is now the leading cause of accidental death in the United States [5]. Heroin is a schedule I drug, a highly addictive illicit opioid, and a primary player in the U.S. opioid epidemic. Current interventions such as methadone and naltrexone have not been sufficient to address the scope of the problem. Generally, 40–60% of patients who are treated for substance use disorder relapse [5,6]. Thus, new alternatives to combat the opioid crisis are urgently needed.

A major limitation to the advancement of SUD vaccines to market is that therapeutic antibody concentrations are achieved in only a fraction of immunized patients [7,8]. Nevertheless, this shortcoming is rarely addressed in pre-clinical studies. For example, reports of advances with heroin vaccines only have representative samples from one sex and one strain per study (Table S1). Sex is an important consideration in designing a vaccine study for the overall population, especially in regard to the general safety and efficacy of these vaccines [9].

When interpreting data on disparities in nociception, it is important to recognize that there are sex differences in a number of physiological systems that may directly or indirectly affect measures of analgesia [9]. Immune responses also vary between sexes, and adverse effects such as respiratory depression and nausea may

Abbreviations: NHS, N-hydroxysuccinimide; PBS, phosphate buffered saline; BSA, bovine serum albumin; TT, tetanus toxoid; CpG ODN, CpG oligodeoxynucleotides; s.c., subcutaneous; i.p., intraperitoneal; SPR, surface plasmon resonance; 6-AM, 6-acetylmorphine; J:DO, Jackson Diversity:Outbred; SUD, substance use disorder; SW, Swiss Webster.

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occur distinctively by sex [9]. Furthermore, men and women generally abuse drugs for distinct reasons, and substance use disorders may manifest differently in women and men [10]. In 2016, self-reported prevalence of prescription pain relivers was found to be 4.8% for males and 3.8% for females [5]. In murine models, relative sex differences have been previously reported in terms of their differential response to analgesic potency to opioids, including morphine [11].

In addition to understanding the sex-related impact on active vaccination against drugs of abuse, we were also interested in examining vaccine efficacy with respect to inbred and outbred mouse strains. In terms of opioid behavioral responses, several studies have shown that brain opioid binding sites and sensitivities to morphine vary by strain [12]. This underscores the possibility of inter-experimental differences resulting from use of different background strains or pedigrees. Diversity outbred mice are hypothesized to be better predictors of population-wide human responses to chemical exposures due to increased genetic variability [13]. This is also consistent with observed responses to vaccine efficacy in the clinic [7,8]. Although male and female mice have been studied independently, no SUD vaccine study to date has incorporated the use of both sexes across multiple strains within the same design to minimize inter-experimental differences and identify whether sex and background strain are likely to have substantial impacts on SUD vaccine generalizability (Table S1).

In our research group, Taconic Swiss Webster (SW) mice are our traditional mouse models of choice due to their genetic diversity as outbred mice, their rapid availability, and affordability. While it is convenient and useful to benchmark behavioral data for new vaccine development using the same strain and sex, these narrow studies may create an unintended bias based on sex or strain in the experimental results. In order to directly assess this concern, we have now vaccinated males and females of three different strains: outbred Swiss Webster (SW), inbred BALB/c, and outbred Jackson Diversity Outbred (J:DO) mice (Table 1). We were also interested in whether heroin exposure produced any notable differences upon vaccination outcomes in mice, as patients receiving the vaccine are highly unlikely to be drug naïve. Thus, an additional group of SW mice were used to address this question for each sex (Table 1). Within this study, the overall goals were to observe what effect (1) sex, (2) strain, and (3) prior exposure to heroin had on vaccine alteration of phenotypic response to heroin-induced analgesia.

2. Materials and methods

2.1. Animals

All studies were performed in compliance with the Scripps Institutional Animal Care and Use Committee (IACUC) and all protocols adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male and female SW and BALB/c mice (Taconic Farms, Germantown, NY; 6–8 weeks old) and Jackson Diversity Outbred mice (J:DO, Jackson Labs, Bar Harbor, ME; 5–6 weeks old) were immunized subcutaneously (s.c.) with 40 μg of heroin-tetanus toxoid (TT) conjugate vaccines on days 0, 14, and 28. The overall vaccination schedule and behavioral experiments are detailed in Figs. 1 and S4. Mice were bled on day 21 and 35 using *retro*-orbital puncture while anesthetized under isoflurane in order to collect approximately 100–150 μL of whole blood. Groups were composed of 16 mice per sex/per strain, and 4 mice for controls per sex/per strain. Mice were group-housed in an AALAC-accredited vivarium and kept on a reverse light cycle (lights on: 9PM-9AM). Mouse weights were measured every week (female cohorts) or every other week (male cohorts) and injection site reactions were measured on the day of antinociceptive testing (**Fig. S5**). In the follow-up study using male SW from Charles River (Wilmington, MA, 6–8 weeks old), these animals were required to undergo quarantine for eight weeks to comply with TSRI IACUC guidelines. Under quarantine, mice were vaccinated, remarked and reweighed every week.

2.2. Vaccine preparation, formulation, schedule

Heroin was obtained from NIDA Drug Supply Program. A second generation heroin hapten was synthesized according to literature procedure and utilized throughout the study (Fig. 1, panel) [14,15]. After preparation of the free carboxylic acid of the hapten, the acid was activated with NHS and then mixed with bovine serum albumin (BSA) or TT (1 mg/mL) in a 1:1 w/w ratio of hapten to protein. Immunoconjugates were allowed to react at room temperature for 20 h using gentle end-over-end mixing. Following conjugation, the solutions were dialyzed against PBS buffer (pH 7.4) using a 10 K molecular weight cut off dialysis cassettes (Thermo Fisher Scientific). Vaccines were cryoprotected with glycerol (50% v/v) and stored at −80 °C. On the day of vaccination, immunoconjugates were defrosted and formulated with 40 ug CpG oligodeoxynucleotide (ODN) 1826 (Eurofins), 1 mg alum (Alhydrogel), and allowed to mix at room temperature for an hour.

2.3. Hapten copy number by MALDI-ToF

The heroin hapten density for immunoconjugates prepared in this study was quantified using MALDI-ToF and ESI-ToF MS analysis and compared to BSA according to literature procedure [14,15]. The immunoconjugates were run through a PD MiniTrap G-10 desalting column (GE Healthcare) and then analyzed by MALDI-ToF. Spectra can be found in the supplementary information (Figs. S1-3).

Table 1
Effects of strain and sex on lethality from a low dose of heroin (2 mg/kg) after exposure to heroin vaccine.

		Male			Female		
Treatment	Strain	Mice Expired	N	% Lethality from Drug	Mice Expired	N	% Lethality from Drug
Vaccinated	Taconic Swiss Webster	6	64	9.4%	0	16	0.0%
	Taconic BALB/c	2[a]	16	0.0%	0	16	0.0%
	Jackson Laboratory J:DO	1[b]	16	0.0%	0	16	0.0%
	Charles River Swiss Webster[c]	1	16	6.3%	n.a.		
Nonvaccinated	Taconic Swiss Webster	0	4	0.0%	0	4	0.0%
	Taconic BALB/c	0	4	0.0%	0	4	0.0%
	Jackson Laboratory J:DO	0	4	0.0%	0	4	0.0%
	Charles River Swiss Webster[c]	0	4	0.0%	0	4	0.0%

[a] One mouse expired from fighting and one from heightened sensitivity to isofluorane during eye bleeds.
[b] Mouse expired from fighting.
[c] Charles River requires an 8-week quarantine, only males were run to test for vendor effect.

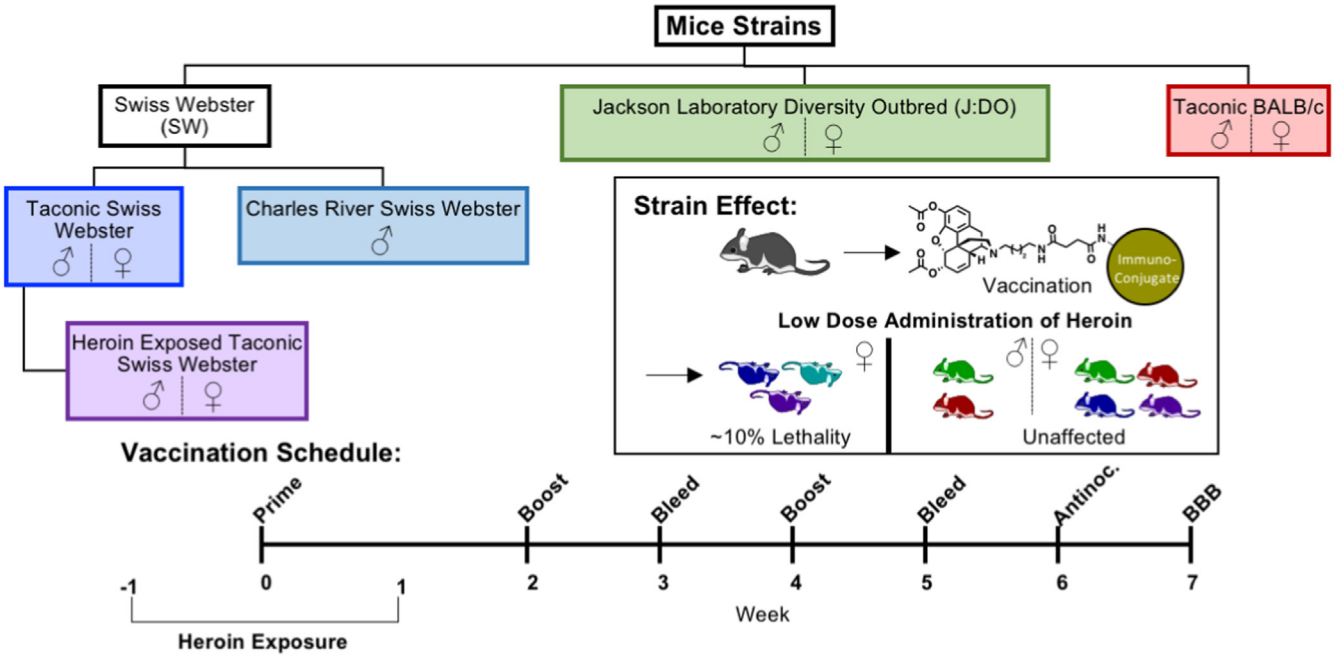

Fig. 1. Overview of mice strains and sexes used in this study with experimental schedule.

2.4. ELISA protocol

Anti-heroin midpoint titer for each vaccination group and non-vaccinated control mice were determined according to literature procedure as written, except for employing a final incubation time of 8 min with TMB and H_2O_2 before quenching with 2.0 M H_2SO_4 [14,15].

2.5. Antibody affinity determination by SPR

The binding IC_{50} for mouse serum IgGs and free 6-acetylmorphine (6-AM) was determined by competitive binding assay via surface plasmon resonance (SPR) according to literature methods [14,15]. Diluted mouse serum from day 21 and 35 were incubated with serial dilutions of heroin, 6-AM and morphine. IC_{50} values were determined from a 12 point 6-AM dilution curve and derived from a nonlinear fit of the binding curves in PRISM 6 (GraphPad Software, Inc).

2.6. Antinociception

Mice were tested for cumulative heroin response, administered intraperitoneally (i.p.), in suprapinal (hot plate) and spinal (tail flick) behavioral tests on week 6 as previously described [14–16]. In an attempt to prevent or minimize tissue damage, mouse paws and tails were gently placed in cool water after testing at each dose. Data are reported as ED_{50}, which is the concentration of drug where 50% of animals within a group experience the antinociceptive effects of heroin. The ED_{50} values and 95% confidence intervals were determined for each antinociception test and individual treatment groups to determine ED_{50} values.

2.7. Repeat antinociception assay

After performing the full range of antinociception for both hot plate and tail immersion tests (heroin dosage 2 mg/kg–18 mg/kg, i.p.) for both vaccinated and nonvaccinated controls, half of the vaccinated mice and all of the control mice (male and female SW, heroin-exposed SW, BALB/c, and J:DO) were run again twenty-four hours later. After determination of the ED_{50} value (from the previous day), 4 mg/kg heroin (i.p.) was administered to the mice after baseline measurements were taken before administration of drug in the absence of heroin. Upon administration of drug, the analgesia effect was measured.

2.8. Blood brain distribution experiments

Blood brain biodistribution was determined according to literature procedure with minor modifications [17,18]. A calibration curve for using standard solutions of heroin, 6-acetylmorphine, and morphine was constructed (**Fig. S10**). On Week 7 (3 weeks from last boost), mice were injected i.p. (4 mg/kg) with heroin. At five, fifteen, or thirty minutes following injection the animals were fully anesthetized with isoflurane and then rapidly decapitated using a sharp guillotine. The brain and trunk blood were collected. The trunk blood was collected in a 1:1 ratio with acetate buffer (0.1 M sodium acetate/0.1 M acetic acid/50 g/L NaF, pH 6.0), placed on ice for several hours, centrifuged at 10,000 rpm for ten minutes.

Brain tissue was immediately homogenized using a Bullet Blender with zirconium oxide beads (0.5 mm diameter, Thomas Scientific) with acetate buffer and stored on dry ice. Afterwards the homogenate was centrifuged at 2500 rpm for ten minutes. A 100 μL aliquot of the homogenate or plasma was added to 100 μL of spiked heroin, 6-AM, and morphine concentrations (for standard curve, made up in 85:15 ACN:MeOH) or 100 μL of 85:15 ACN:MeOH (for samples), 100 μL of d_9-heroin, d_3-6-acetylmorphine, and d_3-morphine (1 μg/mL in ACN) and 300 μL of ice-cold acetonitrile/methanol (85:15). The mixture was vortexed for 30 s and stored in the −20 ℃ freezer for twenty minutes, followed by centrifugation at 2,500 rpm for ten minutes. A 450 μL aliquot was transferred to another test tube and the samples was evaporated using GENEVAC. The dried sample was taken up in acetonitrile, centrifuged at 10,000 rpm for five minutes and then transferred to vials for LC/MS analysis [17,18]. On average, in processed brain tissue samples, heroin, 6-AM, and morphine eluted at 3.64, 3.24,

and 1.40 min, respectively. For blood samples, heroin, 6-AM, and morphine eluted at 3.75, 3.46, and 1.56 min, respectively.

2.9. Cytokine expression panel of blood and brain samples

A mouse cytokine multiplexed ELISA array was performed by Quansys Biosciences' (Logan, UT) to measure and quantify the following rodent cytokines:IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFNγ, TNFα, MIP-1a, GMCSF, and RANTES. Using male SW mice (Taconic), a separate set of 32 mice were vaccinated with Her-TT and adjuvanted with 40 μg CpG ODN and 1 mg alum/dose. After a full vaccination schedule, mice were injected with 2.0 mg/kg heroin. Samples were tested in triplicate and with internal standards. Plasma and brain homogenates were submitted for analysis.

3. Results and discussion

3.1. Investigation of heroin hypersensitivity

3.1.1. Prior observations in male SW mice

Previously, we observed an unexpected side effect in eight separate anti-heroin vaccine studies, incorporating a total of 147 male SW mice (Taconic), using a combination of three different carrier proteins, five adjuvants, and several formulation parameters, including either glycerol or trehalose as the cryoprotectant (Table S2). The only commonality among the different experimental parameters was the hapten itself and the sex, strain, and vendor of the animal model. This side effect can be characterized as an apparent hypersensitivity to heroin, inducing rapid lethality upon exposure to a small dose of heroin (i.p.). This outcome was found to occur between 6 and 25% of all vaccinated mice, depending on vaccine formulation parameters and sample size. A contingency analysis was used to determine the relative risk for each independent adjuvant and condition (Table S2 and Fig. 2).

There was no obviously increased risk associated with any single adjuvant or carrier protein component, as some studies precluded an adjuvant or carrier protein completely and the adverse reaction was still observed. In contrast, both nonvaccinated naive mice (n = 25) and mice administered vaccine vehicle or saline (n = 18) did not exhibit hypersensitivity at the 2 mg/kg dose of heroin (i.p.) [14,15,19]. Typically, antinociception assays using heroin were performed with doses starting at 2 mg/kg (i.p.) and ending at 18 mg/kg [14–16,19]. For additional reference, a 160 mg/kg dose of heroin (i.p.) prompted an LD_{50} or lower for vaccinated mice and an LD_{75} for nonvaccinated mice for male SW mice (Taconic) in our hands [15]. We also found that the LD_{50} of heroin for male SW mice (Taconic) through direct intravenous (i.v.) injection was found to be 23.7 mg/kg [20]. This illustrates the magnitude of difference between the 2 mg/kg dose of heroin that induced this hypersensitivity versus the 80-fold (i.p.) and 10-fold (i.v.) larger doses of heroin needed to trigger overdose in these mice.

It should be noted that this hypersensitive lethality is also independent of vaccine efficacy, suggesting that the underlying mechanism behind this phenomenon may be independent of antibody interaction. Moreover, one study determined the isotyping of antibodies from vaccination and found no detectable IgE response from either supersensitive or normal mice, indicating that a maladaptive immune response was not the etiology [19]. Lastly, several operators prepared vaccines, administered vaccinations, and/or administered drug. We selected a vaccine formulation that incorporated elements most likely to generate an adverse response to assess if lethality after exposure to heroin was seen in other mouse strains (Table S2 and Fig. 2). This study included an inbred mouse model, BALB/c, that commonly used in anti-drug vaccine development (Table S1) [21–23].

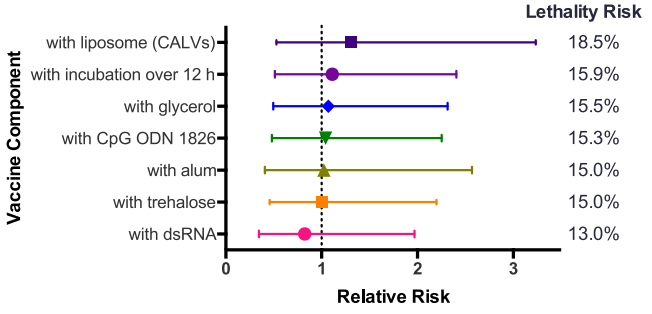

Fig. 2. Relative and lethality risk calculated for each vaccine adjuvant or condition. Relative risk is shown below as mean ± 95% confidence interval. For the Her-TT vaccine used in this study, alum, CpG ODN 1826, glycerol, a prolonged incubation time of 1 h with alum was incorporated into the vaccine formulation. Conjugatable adjuvant lipid vesicles (CALVs) were previously found to be not as effective as the combination of alum with CpG, so were not used in this study as a vaccine component.

3.1.2. Strain and sex-dependent hypersensitivity

In addition, to ensure an adequate sample population, we increased the sample size of our vaccine groups from n = 4–6 mice per group to n = 16 mice per group for each strain and sex combination (Fig. 1 and Table 2). Weight change was monitored for the mice throughout the vaccination schedule as a common indicator of general health (**Fig. S5**). Although the body weight change for male BALB/c was significantly lower than the other mouse strains, the recorded weight according to their weekly age was consistent with the average body weight information for BALB/c (Taconic Biosciences, Inc). After the full vaccination schedule and administration of heroin on the day of the antinociception assay, (Figs. 1 and S4), 6 of the 64 male SW mice (Taconic) expired rapidly after receiving an initial dose of 2 mg/kg (Table 2). Three of the male SW (Taconic) mice were in the heroin-exposed vaccination group, and 3 of the mice were in the heroin-naïve vaccination group. Taken together, the total lethality for this specific strain, sex, and vendor was 9.4%, which was consistent with previous observations.

One male BALB/c mouse expired during the study due to excessive fighting by cage-mates and one from an adverse reaction to isoflurane. A J:DO male mouse also expired due to excessive fighting with cage-mates. It should be noted that general aggressive behavior was noted only for males, regardless of strain; however, fighting was reduced by separating particularly aggressive males from their cage-mates. As expected, no mice in the control groups expired. Surprisingly, there was no hypersensitivity observed for vaccinated SW female mice upon exposure to a low dose of heroin (Table 1).

3.1.3. Effect of heroin exposure on vaccination

Previously it was mentioned that prior heroin exposure is a more likely characteristic of an SUD patient and prior opioid exposure may influence the activity of the vaccine. After exposing both female and male SW to two rounds of 2 mg/kg heroin (i.p.) two weeks apart, midpoint titers, affinity data, antinociception, repeat antinociception, and blood brain distribution experiments were performed (**Figs. S4–S5**). Pre-exposure to heroin did not induce tolerance. For both male and female heroin exposed mice, no hypersensitivity was observed after the first inoculation and exposure to 2 mg/kg of heroin (i.p.). Based on the analyzed data (**Figs. S5–S8**), there was no significant difference found between the two cohorts by any measure.

Table 2
Summary of midpoint titer data and IC_{50} values of polyclonal antibody response to 6-acetylmorphine. Numbers represent means ± SEM for midpoint titer and means for IC_{50} values in nanomolar concentration. Data were tested for statistical outlier evaluation using Grubbs' test and significant outliers were removed. For male mice, no outliers were found, but the number of mice in BALB/c and J:DO were reduced from 16 to 14 and 15, respectively due to mice expiring from fighting. For female mice at week 3, 1 individual was excluded from J:DO, and at week 3, 1 J:DO mouse and 3 heroin exposed SW were excluded.

		Midpoint Titers		IC_{50} (nM)	
Sex	Strain	Week 3	Week 5	Week 3	Week 5
♂	Taconic Swiss Webster	21971 ± 3477	23210 ± 3243	285	90.5
	Taconic BALB/c	20763 ± 7000	22304 ± 5060	441	160
	Jackson Laboratory J:DO	26883 ± 6283	14744 ± 5382	470	44.2
♀	Taconic Swiss Webster	55001 ± 13137	52537 ± 6923	225	170
	Taconic BALB/c	21623 ± 2605	63446 ± 10357	791	180
	Jackson Laboratory J:DO	56424 ± 14679	59854 ± 16799	362	150

3.1.4. Charles River vs Taconic Swiss Webster mice

The hypersensitivity to heroin following Her-TT vaccination in Male SW mice prompted us to explore if this effect was present in all SW mice or just an effect of a random genetic mutation acquired from a vendor over time. To test if this hypersensitivity was related to conditions arising at a specific vendor, we acquired a separate set of 20 male Charles River SW mice, and vaccinated 16 of them. Charles River mice were under quarantine during vaccination, but their housing conditions were similar to mice out of quarantine. Charles River SW mice were vaccinated at the same age (week) as Taconic and the other strains.

After the full vaccination, Charles River SW mice were run in an antinociception assay (Fig. 3). Interestingly, one mouse expired at the low 2 mg/kg dose of heroin (6.3% lethality rate), indicating that hypersensitivity is not solely a vendor effect, but in fact a general trend observed for all SW mice (Table 1). Nonvaccinated mice exhibit an increased tolerance to heroin in supraspinal response (hot plate), which is indicative of higher brain function and different receptor chemistry in the brain. However, for vaccinated mice, the efficacy of the vaccine was generally similar among the two vendor groups (Fig. 3). Based on this comprehensive study, it appears that this hypersensitivity to heroin is limited to only males of the SW strain, is not observed in either sex for the two other strains and does not distinguish between heroin-exposed and heroin-naïve animals.

Mice from Taconic farms were more sensitive to morphine analgesia and toxic effect than Charles River [24]. However, the degree of tolerance observed was similar. In agreement with the literature, nonvaccinated control mice were approximately 2-fold more sensitive to heroin's effects than Charles River, which is attributed to the greater number of brain opioid binding sites. A survey of literature revealed that although Swiss Webster are outbred, mice from Taconic Farms are supersensitive to opioids, showing lethality after administration of 50 or 100 mg/kg of morphine, s.c. [12,24]. The LD_{50} of morphine in these mice is 313 mg/kg, s.c., was well below the dose administered to Taconic mice. Charles Rive mice had an LD_{50} of 745 mg/kg and no lethality was observed at the 50 or 100 mg/kg dose of morphine [12]. This supersensitivity is thought to be caused by the increase number of opioid receptors. Based on the lethality data for morphine, before mice are vaccinated, Taconic mice are 2.2–2.4 times more sensitive to the lethal effect than compared with Charles River mice, which is consistent with the effect observed after vaccination (1.5 times more sensitive).

3.1.5. Cytokine panel

Cytokine production is an important physiological response following T cell activation and proliferation. We hypothesized that the supersensitivity observed in male Swiss Webster mice may have been due vaccine-induced cytokine-release syndrome or also known as a cytokine storm [25]. A cytokine storm is a systemic immune response that involves the potential release of more than 150 inflammatory mediators, including cytokines and chemokines, which precipitates system wide symptoms including shock, cell death, infection, and ultimately organ failure [26]. It is also widely documented that certain vaccines and monoclonal antibodies induce cytokine-release syndrome [27]. Although cytokines are required for several immunoregulatory activities, cytokine storm is characterized by increased levels of TNFα, IFNγ, IL-6, IL-10 (sometimes IL-2 and IL-8) [26].

To test this hypothesis, a separate batch of 32 male Swiss Webster (Taconic) mice were vaccinated with the same heroin vaccine. At week 6, mice were run at baseline and then injected i.p. with 2 mg/kg heroin. Mice exhibiting hypersensitivity were quickly anesthetized and the blood and brain samples were harvested. Samples were then submitted for testing of normal mice and hypersensitive mice. Results are shown in **Figs. S11–S12**. The only significant difference is between IL and 1b in the brain. In both mice and humans, this cytokine induces the cyclooxygenase-2

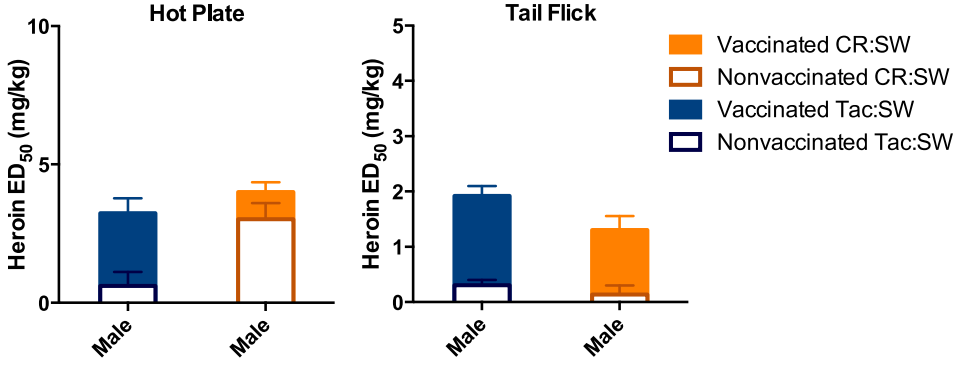

Fig. 3. Effect of animal vendor on heroin vaccine efficacy in antinociception assays. Panel A shows results from hot plate assay. Panel B shows results from tail flick assay. Bars represent means ± SEM, where each vaccinated group is comprised of 16 mice, and control groups are comprised of 4 mice. CR:SW stands for Charles River Swiss Webster; Tac:SW stands for Taconic Swiss Webster. Tac:SW are the same data as shown in Fig. 5 for males. Only males were run in this vendor study.

expression in the brain, which contributes to inflammatory pain hypersensitivity [28,29]. The only other notable trend is that cytokine levels were generally elevated in the blood for control mice and elevated in brain for hypersensitive mice.

Based on our comprehensive sex and strain studies, the observed hypersensitivity to heroin is centralized to only one sex of one specific strain. Although the mechanism is not clear, we are confident that it is not the result of an IgE response, operator error, or a cytokine storm and occurs in strains from two separate vendors.

3.2. Effect of sex and strain on vaccine efficacy

3.2.1. Midpoint titer and affinity data

Both male and female vaccinated mice exhibited a robust polyclonal antibody response against 6-acetylmorphine, the primary psychoactive metabolite of heroin (Table 2). Generally, midpoint titers increased for both males and females between week 3 and week 5, after receiving a boost at week 4. Only J:DO males' titer decreased substantially by week 5. Another notable observation was that female mice largely had higher titers than their male counterparts. BALB/c mice had similar responses, then diverged at week 5, with females having a higher titer of 63,446 vs. 22,304. Utilizing SPR measurements, the strains can be ranked based on highest to lowest anti-6-AM antibody affinity: J:DO > SW > BALB/c Unexpectedly, although female subjects had higher titers than male subjects, male mice developed antibodies with better affinity for 6-AM than female mice, with J:DO males exhibiting nanomolar IC_{50} values. Regardless, this decrease in titers has been previously observed [30–33].

3.2.2. Antinociception assay

In order to gauge the efficacy of our heroin vaccine, we employed behavioral assays to assess the antinociceptive effect of heroin between vaccinated and unvaccinated animals. In agreement with affinity data, J:DO mice performed the best in the supraspinal response to heat stimuli (hot plate assay, Fig. 4A). In Fig. 4, the filled-in bars represent the ED_{50} of vaccinated mice, and unfilled bars represent nonvaccinated control mice. Response to heroin significantly differed in vaccinated and nonvaccinated J:DO male and female mice in both behavioral models. The response also differed significantly between vaccinated and nonvaccinated BALB/c mice, but only in the spinal response to heat stimuli (tail flick assay, Fig. 4B).

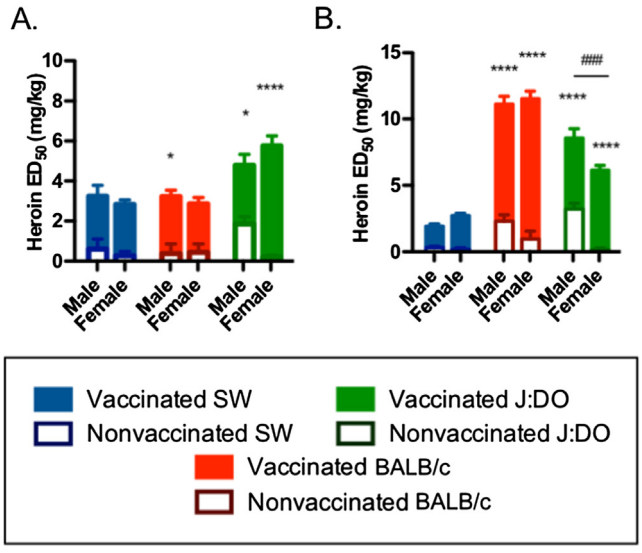

Fig. 4. Effects of strain and sex on heroin vaccine efficacy in antinociception assays. Panel A shows results from hot plate assay. Panel B shows results from tail flick assay. Bars represent means ± SEM. A two-way ANOVA was performed for each antinociception assay, followed by a Tukey's *post hoc* comparison test. * Denotes vaccine effect. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus control nonvaccinated mice. $^{\#\#\#}$ Denotes sex difference, $P < 0.05$ for both male ($P < 0.001$) and female ($P < 0.0001$) in tail flick by a two-way ANOVA.

Generally, males and females of the same strain performed similarly in both antinociception assays, except for J:DO response to the tail flick assay. Based on a two-way ANOVA analysis for hot plate, strain differences were observed between J:DO vs other female strains ($P < 0.0001$). For tail flick, a two-way ANOVA shows that strain differences were observed amongst all strains for both male ($P < 0.001$) and female ($P < 0.0001$). Potency values for each sex and strain are given in **Fig. S9**, where potency ratio is defined by the ED_{50} of vaccinated mice normalized by ED_{50} of nonvaccinated control mice. Based on the potency values, J:DO females performed the best and J:DO males performed the worst. However, these potency ratio values are extremely biased on the response of nonvaccinated control mice, and obscure overall information on vaccine performance by strain. Interestingly, male J:DO mice were the least sensitive to the antinociceptive effects of heroin of all strains and sexes (Fig. 4). Alternatively, female J:DO mice were extremely sensitive to heroin, therefore resulting in an extremely large potency ratio. Largely, female control mice were more sensitive to heroin than their male counterparts. Taken together, it appears that the Her-TT vaccine works the best in the inbred strain BALB/c and the outbred stain J:DO, and is the least effective in SW mice (Taconic).

3.2.3. Repeat antinociception assay

In a realistic scenario of heroin drug abuse, an individual with SUD is likely to engage in more than one challenge of an opioid [34]. Yet, contiguous, cumulative, daily antinociceptive testing can cause tissue damage. To model this repeated administration behavior, we included a repeat antinociception challenge. However, we only acquired two measurements: a baseline measurement and antinociception at a single dose on day two, in order to minimize potentially-confounding tissue damage. In order to determine the appropriate dosage for repeated antinociception rather than an arbitrarily assigned dose, we dosed the animals at the calculated ED_{50} of the vaccine. Only half of the vaccinated cohorts were run in this study, due to logistical limitations (*i.e.*, immunizations, bleeds, assays, and experiments were split over two days due to the large number of animals required to be run in behavioral experiments see **Fig. S4**, day 44).

Results from the repeated antinociception experiments are shown in Fig. 5. Day 1 values are extrapolated from the standard antinociception assay run at baseline and at 4 mg/kg. It is apparent that mice have experienced minor tissue damage, despite efforts to mitigate damage through strict cutoff exposure times and treatment with cool water, based on the increase in average baseline times for nonvaccinated control mice over time for all mice except male SW. Despite this, it appears that the vaccine is still efficacious after repeated exposure to heroin in both assays, particularly for the BALB/c and J:DO mice. SW mice had modest efficacy over two exposures of heroin (blue bars, Fig. 5).

A Tukey's multiple comparisons test for male mice in the tail flick assay revealed that comparison between Day 1 and 2 at 15 min was not significant for any group except J:DO, but even in this case the vaccinated group performed the same and the control group performed worse, likely due to tissue damage. Both vaccinated BALB/c and J:DO were significantly less affected by heroin than SW mice at both Day 1 ($P < 0.0001$) and Day 2 ($P < 0.0001$ and $P < 0.001$, respectively, Tukey's). Results from Tukey's post hoc analysis for the hot plate assay showed that there was no significant difference between Day 1 and 2 at 15 min for SW mice, but

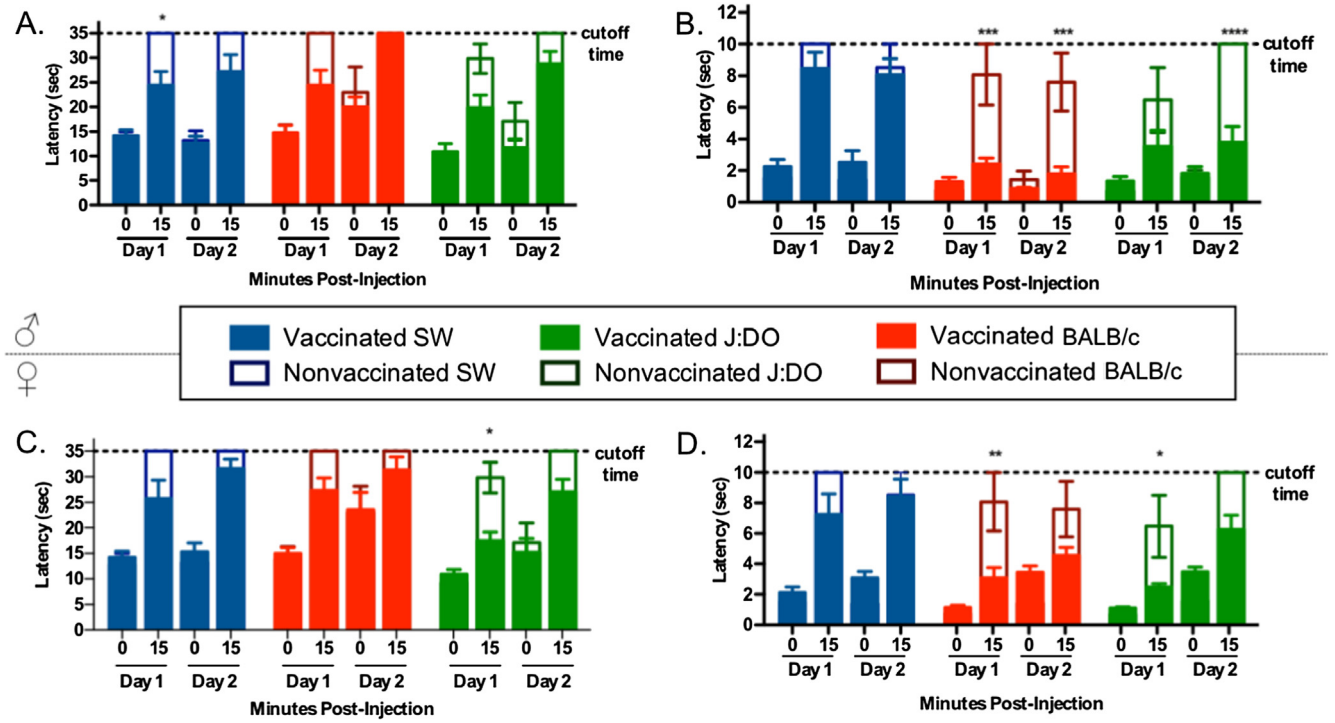

Fig. 5. Repeated antinociception study. Panel A and C show results from hot plate assay. Panel B and D show results from tail flick assay. Solid bars represent means ± SEM for vaccinated mice (n = 8), where SW, BALB/c, and J:DO mice correspond to blue, red, and green solid bars. Clear bars represent means ± SEM for nonvaccinated control mice (n = 4), where SW, BALB/c, and J:DO mice correspond to blue, red, and green unfilled bars. Study shows that vaccines are efficacious after repeated exposure to heroin (4 mg/kg, i.p.) in both assays. A and B are males; C and D are females. * Denotes vaccine effect. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus control nonvaccinated mice by a two-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

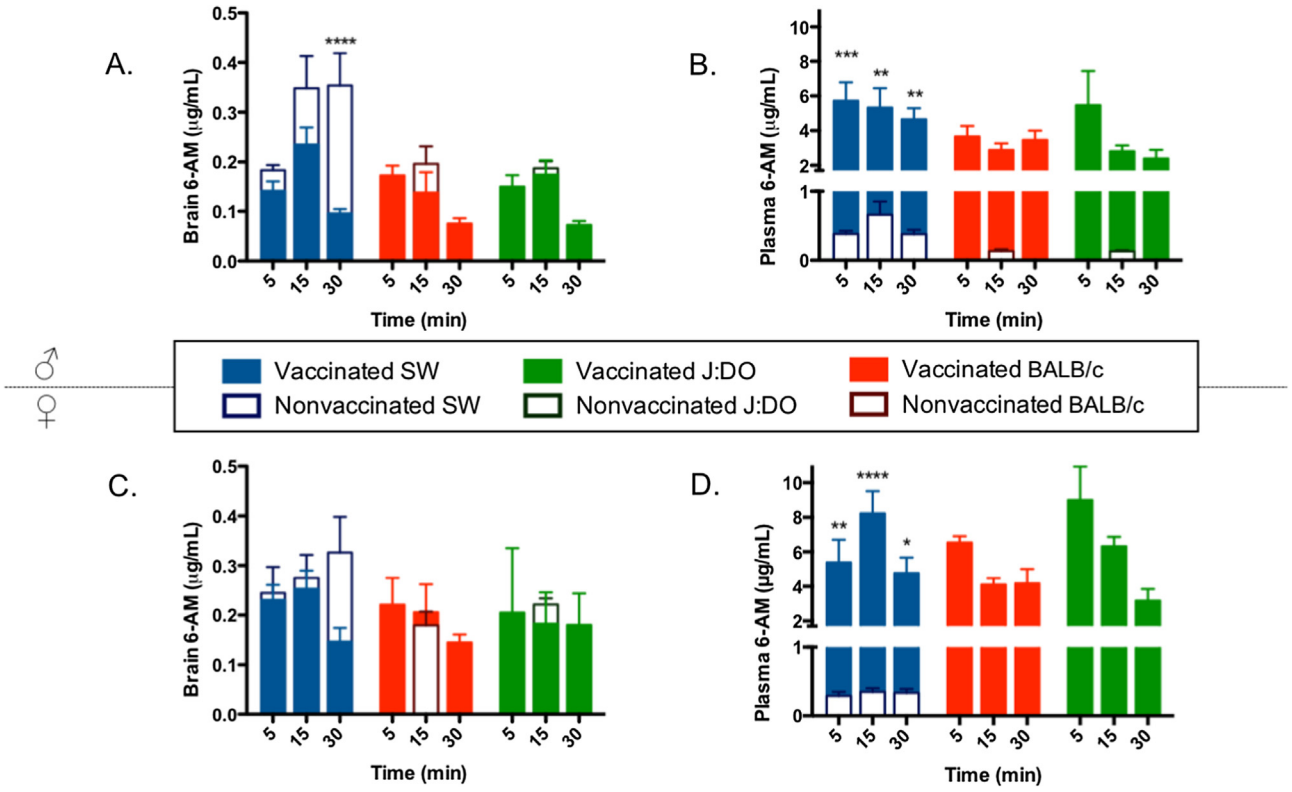

Fig. 6. Blood brain distribution study. Panels A and B show 6-acetylmorphine concentrations in the brain and blood for males, respectively. Panels C and D show 6-AM concentrations in the brain and blood for females, respectively. Bars represent means + SEM, where each group is comprised of 4–6 mice. Samples were collected at five, fifteen, and thirty minutes post intraperitoneal injection of 4 mg/kg heroin. Filled in bars represent vaccinated mice, and clear bars represent nonvaccinated control mice. * Denotes vaccine effect. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus control nonvaccinated mice by a two-way ANOVA. Control values for female BALB/c and J:DO at 15 min were excluded due to limitations on quantitative assessment of 6-AM concentrations from samples collected for these groups (panel D).

there was a significant difference for BALB/c and J:DO strains, indicating either increased susceptibility to tissue damage on the hot plate or decreased vaccine efficacy. Nonvaccinated controls of BALB/c and J:DO also exhibit an increased latency time between Day 1 and 2 but these changes are not significant. Overall, it is generally true that the Her-TT vaccine remained efficacious after repeated exposure to heroin, especially in the tail flick assay (Fig. 5).

3.2.4. Blood brain distribution study

As a terminal experiment, mice were administered 4 mg/kg heroin (i.p.) and brain tissue and blood were harvested at five, fifteen, or thirty minutes post drug administration at week 7 (day 49–50, **Fig. S4**). Results for both males and female mouse strains are shown in Fig. 6. Based on the analysis, all vaccinated mice sequestered 6-AM in the periphery (Fig. 6). Female mice exhibited higher concentrations of 6-AM in both the plasma and brain tissue compared to males (Fig. 6). Generally, the vaccinated strains performed similarly in blocking 6-AM from the brain. It should be noted that one limitation of this study is that drug dosing is based on the overall animal weight, however, the general size of the brain does not necessarily correlate to body size (**Fig. S11**). For example, J:DO male mice are significantly smaller in body mass than male SW mice but are not significantly different in brain size (the same trend is observed for female SW mice compared to male SW mice). Although brain tissue is processed based on its weight, the dosing the animal receives varies based on sex or strain and may artificially inflate or deflate the concentration of 6-AM observed in processed samples.

4. Conclusion

Vaccines against drugs of abuse are new immuno-chemical interventions that may assist in the treatment and prevention of overdoses in the U.S. opioid epidemic. Although preclinical studies with animal models may not reflect true pharmacological properties encountered in humans, a concerted effort to incorporate larger samples sizes, both sexes, and multiple strains is a step in the right direction. We previously observed a rare hypersensitivity to a low dose of heroin. Based on a study incorporating both sexes, and three different strains of mice from three different vendors, we found that this hypersensitivity was localized to only male SW mice at a lethality rate of 6.3–9.4% and was not based on cytokine storm or an allergic IgE reaction. Moreover, our study shows that, males and females generally have similar responses to blockade of heroin-induced analgesia within a strain. In addition, BALB/c and J:DO mice outperformed our standard Taconic SW model in behavioral assays in both measures. Lastly, a repeated antinociception assay indicated that vaccine efficacy was maintained over multiple challenges of heroin.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Author Contributions

C.S.H., C.J.W., K.D.J designed the research strategy and experimental plan. C.S.H. synthesized heroin and fentanyl haptens and prepared the immunoconjugate vaccines. C.S.H. and L.C.S. performed all animal experiments including: ELISAs, vaccination, weighing, antinociception assays, and blood brain distribution studies. B.E. performed animal bleeds. B.Z. ran the SPR experiments. C.S.H., C.J.W., and L.C.S. analyzed the data. C.S.H. wrote the manuscript and supporting information. L.C.S. made all figures and tables in the manuscript and supporting information. C.S.H., L. C.S., C.J.W., and K.D.J. edited the manuscript. All authors have given approval to the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.061.

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