Comparing withdrawal- and anxiety-like behaviors following oral and subcutaneous oxycodone administration in C57BL/6 mice

Suzannah S. De Almeida*, Caryssa R. Drinkuth* and Gregory C. Sartor

Excessive prescribing and misuse of prescription opioids, such as oxycodone, significantly contributed to the current opioid crisis. Although oxycodone is typically consumed orally by humans, parenteral routes of administration have primarily been used in preclinical models of oxycodone dependence. To address this issue, more recent studies have used oral self-administration procedures to study oxycodone seeking and withdrawal in rodents. Behavioral differences, however, following oral oxycodone intake versus parenteral oxycodone administration remain unclear. Thus, the goal of the current studies was to compare anxiety- and withdrawal-like behaviors using established opioid dependence models of either home cage oral intake of oxycodone (0.5 mg/ml) or repeated subcutaneous (s.c.) injections of oxycodone (10 mg/kg) in male and female mice. Here, mice received 10 days of oral or s.c. oxycodone administration, and following 72 h of forced abstinence, anxiety- and withdrawal-like behaviors were measured using elevated zero maze, open field, and naloxone-induced precipitated withdrawal procedures. Global withdrawal scores were increased to a similar degree following oral and s.c. oxycodone use, while both routes of oxycodone administration had minimal effects on anxiety-like behaviors. When examining individual withdrawal-like behaviors, mice receiving s.c. oxycodone exhibited more paw tremors and jumps during naloxone-induced precipitated withdrawal compared with oral oxycodone mice. These results indicate that both models of oxycodone administration are sufficient to elevate global withdrawal scores, but, when compared with oral consumption, s.c. oxycodone injections yielded more pronounced effects on some withdrawal-like behaviors.

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Introduction

Opioid use disorder (OUD) remains an immense public health issue in the USA (Centers for Disease Control and Prevention, 2024). High prescribing rates of oxycodone, a semisynthetic mu-opioid receptor agonist, contributed substantially to the current opioid crisis (Jalal *et al*., 2018; Mattson *et al*., 2021). Between 1999 and 2010, prescriptions for oxycodone quadrupled, corresponding with a sharp increase in opioid-related overdose deaths (Strang *et al*., 2020). Even with newer restrictions, oxycodone use continues to be a major risk factor for developing OUD (Scherrer *et al*., 2020). Despite the rise in oxycodone misuse and dependence, most preclinical models have focused on other opioids such as morphine and fentanyl, which have different pharmacokinetics and pharmacodynamics compared with oxycodone (Yoburn *et al*., 1995; Lugo and Kern, 2002, 2004; Lötsch *et al*., 2013). Thus, additional efforts are needed to understand the underlying neurobehavioral mechanisms that drive oxycodone dependence.

Numerous models of oxycodone administration exist, each aimed at examining different aspects of OUD. In rodent studies, oxycodone use and dependence have been primarily modeled using operant intravenous self-administration procedures (Yuferov *et al*., 2018; Zhang *et al*., 2018; Blackwood *et al*., 2020; Kimbrough *et al*., 2020) and experimenter-delivered injections of oxycodone (Enga *et al*., 2016; Wiebelhaus *et al*., 2016; Nasseef *et al*., 2019; Carper *et al*., 2021). To more closely mimic oral misuse of prescription opioids in humans, researchers have recently investigated the behavioral and molecular mechanisms associated with volitional oral oxycodone consumption in rodents (Murphy *et al*., 2021; Iyer *et al*., 2022; Sharp *et al*., 2021; Slivicki *et al*., 2023). While both routes of administration have usefulness in modeling different aspects of OUD, a direct comparison of withdrawal- and anxiety-like behaviors following oral or parenteral administration of oxycodone has yet to be investigated. Given that oxycodone has a high first-pass metabolism in rodents (Chan *et al*., 2008), there is a concern that the behavioral outcomes in oral intake models may be significantly different when compared with repeated oxycodone injection models. Attempting to compare behavioral results from previously published oral and parenterally

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administered oxycodone studies remains a challenge due to variations in methods and data reporting across laboratories. A direct comparison of OUD-related behaviors following oral or parenteral administered oxycodone would help reveal potential disparities between models.

In the present study, we compared changes in anxiety- and withdrawal-like behaviors following oral consumption or subcutaneous (s.c.) administration of oxycodone in male and female mice. Our results indicate that global withdrawal scores (GWS), but not anxiety-like behaviors, are increased similarly following oral and s.c. oxycodone use. Differences in specific withdrawal-like behaviors, however, were observed when comparing s.c. oxycodone administration to oral oxycodone use.

Methods

Subjects

Male and female C57BL/6 mice (10–12 weeks old; Charles River Laboratories; Wilmington, Massachusetts, USA) were single-housed under a reverse 12 h/12 h light/dark cycle (lights off at 9 a.m.) and given *ad libitum* access to food and water. Mice were housed in a temperature-controlled (71.5 °F, 50% humidity) Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at the University of Connecticut and allowed to acclimate to the facility for at least 1 week before experimentation. For all experiments, an equal number of male and female mice were included in each group, and all experiments were conducted during the dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Connecticut and performed in accordance with guidelines established by the US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Oxycodone administration

For oral intake experiments, mice received drinking water with oxycodone (0.5 mg/ml) or drinking water without oxycodone in their home cage bottle for 10 consecutive days ($n = 12$ per group, six males/six females). The concentration of oxycodone used in the drinking water was based on previous studies (Phillips *et al*., 2020; McKendrick *et al*., 2022; Slivicki *et al*., 2023). No taste adulterants were added to the water or oxycodone solutions. The water bottle and mouse weight were recorded each day at 9 a.m. A water bottle in an empty cage was weighed daily as an estimation of leakage ($n = 6$). To account for the mouse's weight, daily intake was also calculated as ml/g and mg/kg (Fig. 1c–g). At the end of 10 days, oxycodone water was replaced with regular drinking water for 72 h. For parenteral administration in different mice, oxycodone or saline was injected twice a day (9 a.m. and 3 p.m.; 10 mg/kg, s.c.) for 10 days ($n = 16$, eight males/eight females per group), similar to previous studies (Azizi *et al*., 2012; Severino *et al*., 2020). Behavioral testing (described below) was performed 72 h after the last oxycodone treatment. This time point was selected based on our pilot data and on previous studies that have demonstrated significant changes in naloxone-precipitated withdrawal effects and anxiety-like behavior in opioid-dependent subjects within this timeframe (Bravo *et al*., 2020; Hassan *et al*., 2020). All behavioral testing was conducted on the same day with at least 2 h between tests, starting with elevated zero maze (EZM), followed by an open field, and ending with naloxone-induced precipitated withdrawal.

Elevated zero maze

Anxiety-like behavior was measured using an EZM apparatus (catalog #341012-M; TSE Systems, Chesterfield, Missouri, USA). Mice were allowed to acclimate in the behavior room with red lights for 30 min before testing. During the test, a mouse was placed on the open arm and allowed to freely explore the apparatus for 5 min. EthoVision tracking software (Wageningen, Netherlands) was used to quantify the time spent in the open arms, as well as the distance traveled. Time spent in open arms was considered a measure of anxiety-like behavior, while distance traveled was measured to quantify locomotor activity (Kulkarni & Sharma, 1991; Singh *et al*., 2007). Between each trial, the apparatus was thoroughly cleaned with 70% ethanol.

Open field

The open field apparatus consists of a 46 cm × 46 cm chamber with opaque gray walls (40 cm high). Each mouse was placed in the middle of the chamber and allowed to freely explore the environment for 30 min, as previously described (Singh *et al*., 2022). EthoVision tracking software was used to quantify time spent in the center, as well as distance traveled. The center zone was defined as a 23 × 23 cm square in the middle of the chamber. Time spent in the center was used as a measurement of anxiety-like behavior, while distance traveled was measured to quantify locomotor activity (Kraeuter *et al*., 2019). Between each trial, the apparatus was thoroughly cleaned with 70% ethanol.

Naloxone-induced precipitated withdrawal

To precipitate withdrawal, mice were injected with naloxone (10 mg/kg, intraperitoneally), similar to previous studies (Wiebelhaus *et al*., 2016; Enga *et al*., 2016). Immediately following naloxone injection, mice were placed individually in a clear plexiglass box, and their behavior was analyzed for 30 min. Mice were scored individually for somatic symptoms of withdrawal, including total number of paw tremors, rearing, grooming, jumps, writhing, and hind limb scratching using methods previously described (Mori *et al*., 2013; Towers *et al*., 2019; Iyer *et al*., 2022). A paw tremor was operationally defined as an isolated event in which the subject's front paws visibly

shook up and down. An up-and-down stroke of the subject's paws was scored as a single paw tremor. A rearing behavior was defined as a single event of the subject standing on its hind legs. Free-standing rearing in the middle of the box as well as rearing while bracing front paws on the wall of the box were both scored as rearing behaviors. Grooming was operationally defined as any self-grooming behavior. Grooming bouts lasting up to 20 s were counted as a single behavior. A jump was defined as an isolated leap into the air with no paws touching the floor of the box. A writhing behavior was defined by an isolated event of stretching behavior in which the subject's back visibly curved down and legs stretched outward. The sum of the somatic signs of withdrawal was used to calculate the GWS, similar to previous studies (Enga *et al*., 2016; Luster *et al*., 2020; Hassan *et al*., 2020). Individual GWS were also transformed into *Z*-scores to account for varying frequencies between individual withdrawal-like behaviors (Bravo *et al*., 2020; Drinkuth *et al*., 2023). *Z*-scores were produced by subtracting a subject's observed score from the sample mean for each behavior and dividing that total by the SD for that behavior. Global withdrawal *Z*-scores for each subject were produced by summing *Z*-scores for each individual withdrawal-like behavior. All behavioral scoring was performed by a trained experimenter blinded to treatment conditions.

Drugs

For oral intake, oxycodone hydrochloride (MilliporeSigma, Burlington, Massachusetts, USA) was dissolved in regular drinking water at a concentration of 0.5 mg/ml. This concentration has been previously used in volitional oral oxycodone models (Phillips *et al*., 2020; McKendrick *et al*., 2022; Slivicki *et al*., 2023). For s.c. injections, oxycodone hydrochloride (MilliporeSigma) was dissolved in 0.9% sterile saline and administered s.c. (10 mg/kg) at a volume of 0.1 ml. Naloxone hydrochloride (R&D Systems, Minneapolis, Minnesota, USA) was dissolved in 0.9% sterile saline and injected intraperitoneally (10 mg/kg) at a volume of 0.1 ml.

Data analysis

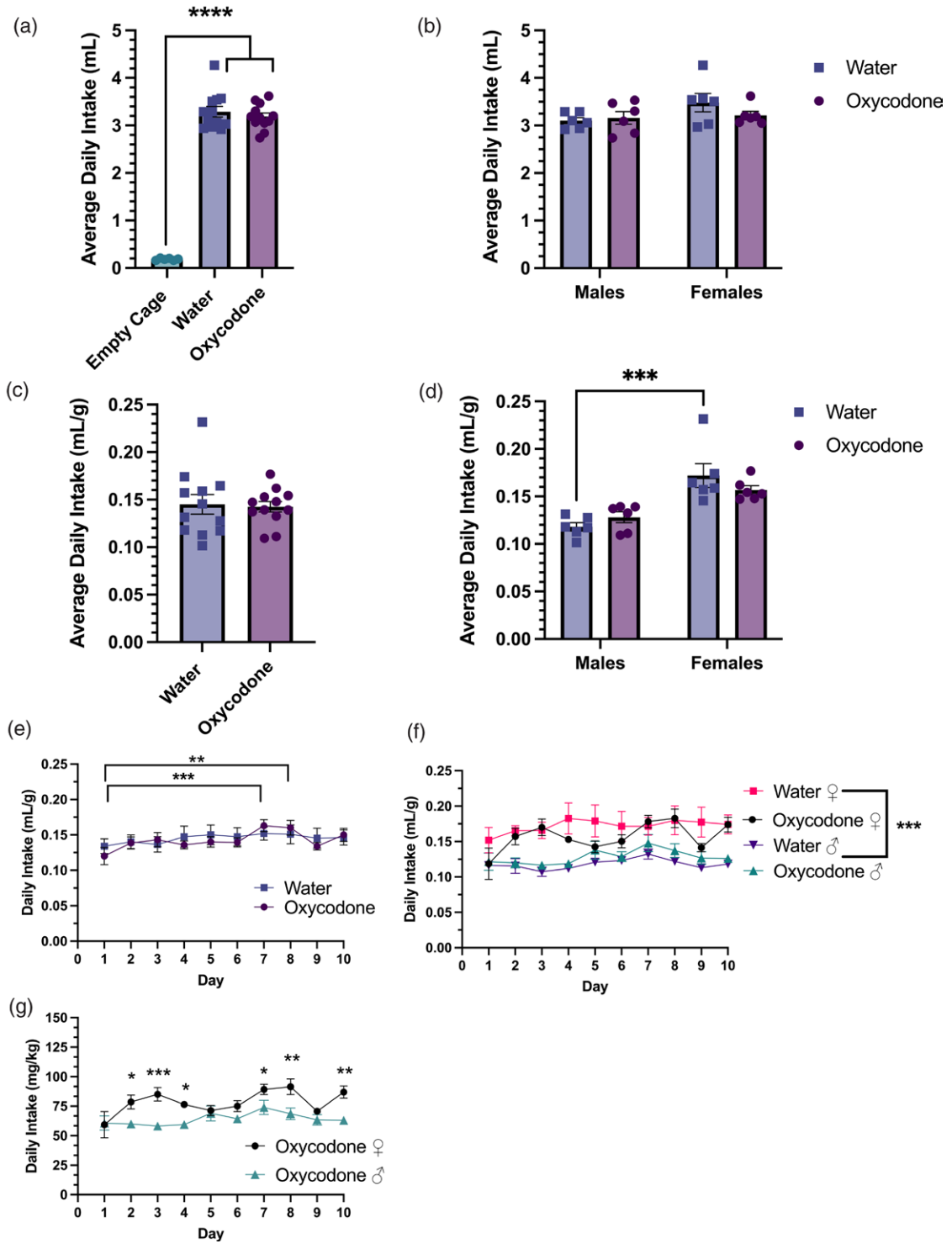
Statistical analyses were performed with GraphPad software (La Jolla, California) Prism 7.0 software (La Jolla, California, USA) or JASP software (Version 0.18. 3; University of Amsterdam, Amsterdam, The Netherlands). The mean values from oral consumption experiments were compared between groups using a Student's *t*-test (nondirectional), a one-way analysis of variance (ANOVA), or a two-way ANOVA. For measuring oral intake across days, a repeated-measure two-way ANOVA was used to assess differences in day × group or day × sex, and a repeated-measure three-way ANOVA was used to investigate day × sex × group. EZM, open field, and naloxone-precipitated withdrawal data were analyzed via two-way ANOVA with treatment (oxycodone vs. vehicle) and route of administration (oral vs. s.c.) as factors. Three-way ANOVA was used to assess sex differences in EZM, open field, and GWS following naloxone-precipitated withdrawal with treatment (oxycodone vs. vehicle), route of administration (oral vs. s.c.), and sex (male vs. female) as factors. The Bonferroni correction was used as a post-hoc comparison for all ANOVAs. All data are expressed as means ± SEM and the level of significance was set to *P* value less than 0.05.

Results

Oral oxycodone consumption in male and female mice

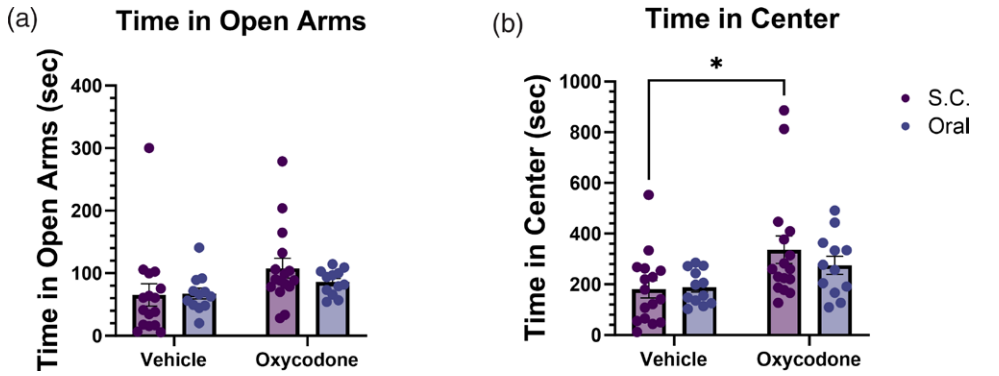
Male and female mice received regular water or oxycodone water (0.5 mg/ml) for 10 days in their home cage, and body weight and intake were measured at the same time each day. A one-way ANOVA revealed a significant group effect when comparing the average daily volume change (ml) of the empty cage group compared with mice in the water and oxycodone groups [$F_{(2, 27)} = 249.3$; $P < 0.0001$] (Fig. 1a). The Bonferroni post-hoc analysis, however, revealed no significant difference in average intake (ml) between the water group and the oxycodone group ($P > 0.05$) (Fig. 1a). When groups were separated by sex, a two-way ANOVA showed no significant difference in average intake (ml) by sex [$F_{(1, 20)} = 2.862$; $P = 0.1062$], group [$F_{(1, 20)} = 0.6356$; $P = 0.4347$], or interaction [$F_{(1, 20)} = 1.630$; $P = 0.2163$] (Fig. 1b). When volume consumed was adjusted for mouse weight (ml/g), no significant difference was found between mice in the water and oxycodone groups [$t_{22} = 0.2180$; $P = 0.8294$] (Fig. 1c). When groups were separated by sex, a two-way ANOVA revealed a sex-dependent effect on consumption (ml/g) [$F_{(1, 20)} = 29.7$; $P < 0.0001$], but no effect between water and oxycodone groups [$F_{(1, 20)} = 0.1$; $P = 0.7398$] or an interaction effect [$F_{(1, 20)} = 2.8$; $P = 0.1125$] (Fig. 1d). The Bonferroni post-hoc analysis revealed that females consumed more water (ml/g) than males ($P < 0.001$), but no difference in oxycodone consumption was observed between sexes ($P > 0.05$) (Fig. 1d). When observing daily intake (ml/g), a repeated-measure two-way ANOVA revealed significant differences across days [$F_{(9, 198)} = 3.2$; $P = 0.0013$] but no group [$F_{(1, 22)} = 0.05$; $P = 0.8294$] or interaction effect [$F_{(9, 198)} = 0.95$; $P = 0.4828$] (Fig. 1e). The Bonferroni post-hoc analysis revealed a significant increase in oxycodone consumption on day 7 ($P = 0.0008$) and day 8 ($P = 0.0028$) when compared with day 1. When comparing daily intake (ml/g) by sex and condition, a repeated-measure three-way ANOVA revealed a significant difference across days [$F_{(9, 180)} = 3.246$; $P = 0.001$] and sex [$F_{(1, 20)} = 29.724$; $P < 0.001$] but no significant differences between groups [$F_{(1, 20)} = 0.113$; $P = 0.740$], group × sex [$F_{(1, 20)} = 2.756$; $P = 0.113$], days × group [$F_{(9, 180)} = 0.973$; $P = 0.464$], days × sex [$F_{(9, 180)} = 1.633$; $P = 0.109$], and days × group × sex [$F_{(9, 180)} = 0.898$; $P = 0.528$] (Fig. 1f). The Bonferroni post-hoc analysis revealed that females consumed more water compared with males ($P < 0.001$), but daily oxycodone intake (ml/g) was not significantly

Fig. 1



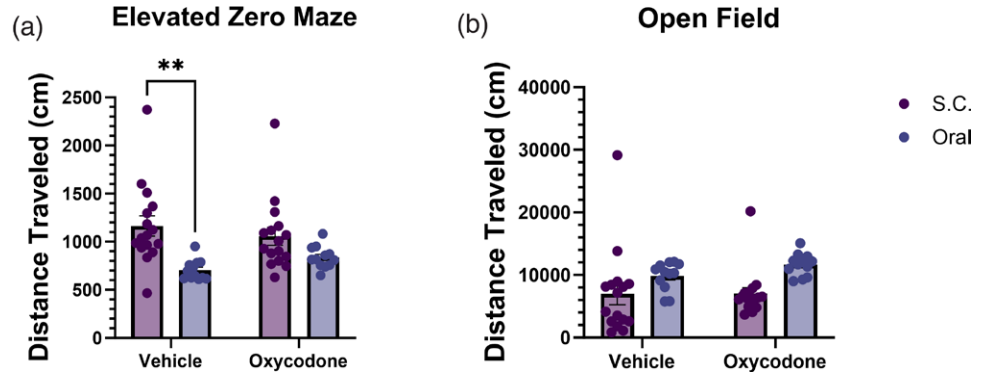
Oral consumption of oxycodone in male and female mice. (a) Mice were assigned regular water or oxycodone water for 10 days ($n = 12$ per group). Average daily volume change (ml) was significantly greater in the water and oxycodone groups compared with an empty cage ($n = 6$). (b) No sex difference in average consumption (ml) was observed when comparing water and oxycodone groups. (c) When accounting for weight (ml/g), no difference in average consumption was observed when comparing water and oxycodone groups. (d) Sex differences in average water intake but not in average oxycodone intake (ml/g) were observed. (e) Comparison of daily water and oxycodone intake (ml/g) revealed a significant increase in oxycodone intake on days 7 and 8 compared with day 1. (f) When comparing daily intake (ml/g) by sex ($n = 6$ per group), females consumed more water compared with males but no differences in daily oxycodone intake were observed between sexes. (g) Daily oxycodone intake (mg/kg) by sex revealed significant differences in oxycodone consumption across multiple days. Data are mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$ indicate a significant difference via Bonferroni's post-hoc test.

Fig. 2



Anxiety-like behavior following oral or subcutaneous (s.c.) oxycodone administration. (a) Compared with vehicle groups, time (s) spent in the open arms during the elevated zero maze test did not differ in mice that received oral or s.c. oxycodone. (b) Compared with vehicle groups, time spent in the center of the open field test differed in mice that received s.c. oxycodone ($n = 12$ for the oral group; $n = 16$ for the s.c. group). Data are mean ± SEM. Data was analyzed via a two-way analysis of variance followed by Bonferroni's post-hoc test. $^{*}P < 0.05$.

Fig. 3



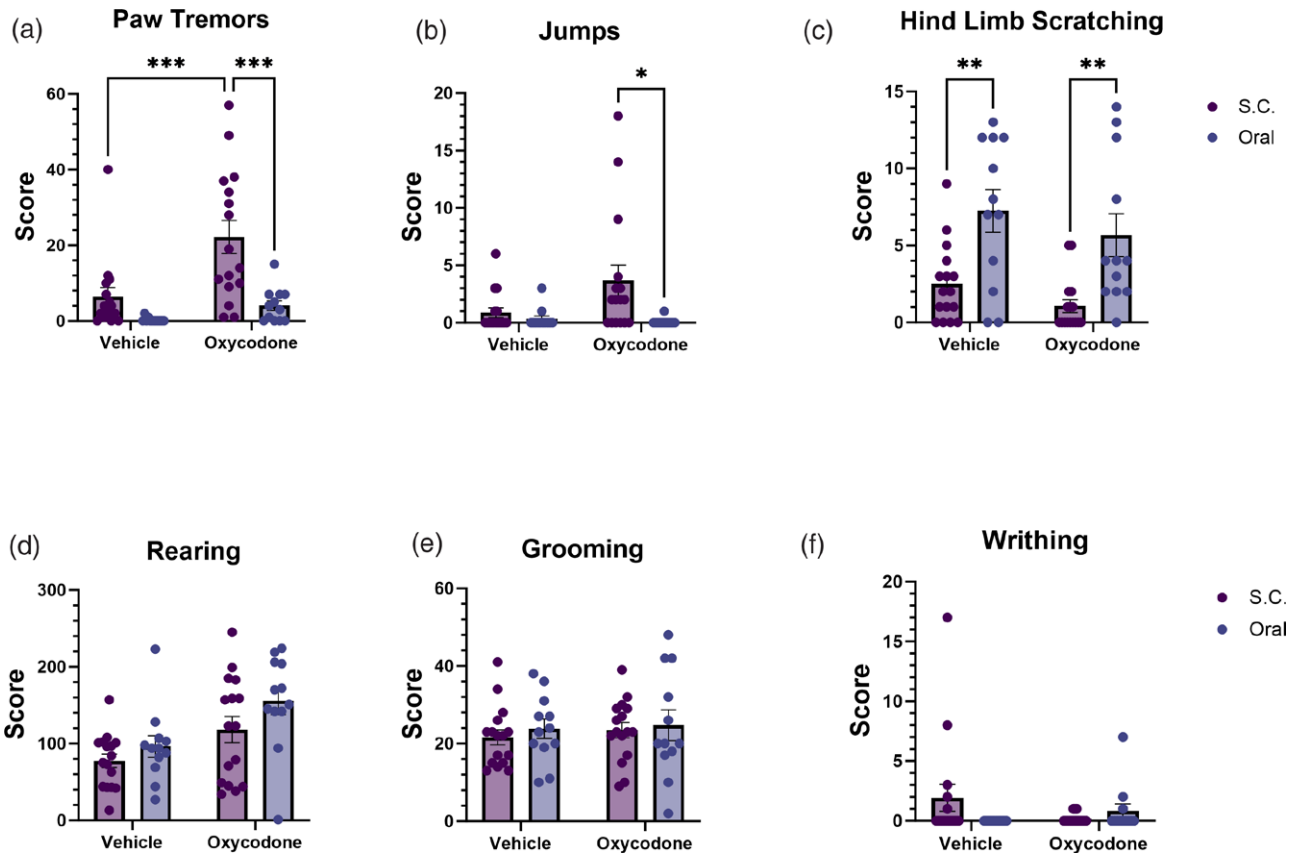
Locomotor activity following oral or subcutaneous (s.c.) oxycodone administration. (a) In the elevated zero maze, distance traveled was increased in mice receiving water compared with s.c. saline but no differences in mice receiving oral or s.c. oxycodone were observed. (b) In the open field, distance traveled did not differ across groups ($n = 12$ for the oral group; $n = 16$ for the s.c. group). Data are mean ± SEM. Data was analyzed via a two-way analysis of variance followed by Bonferroni's post-hoc test. $^{**}P < 0.01$.

different between sexes ($P = 0.086$). When comparing daily oxycodone intake (mg/kg) in males and females, a repeated-measure two-way ANOVA showed a significant difference in sex [$F_{(1,10)} = 15.33$; $P = 0.0029$], days [$F_{(9,90)} = 3.570$; $P = 0.0008$], and days × sex [$F_{(9,\ 90)} = 1.987$; $P = 0.0498$] (Fig. 1g). The Bonferroni post-hoc analysis indicated that females consumed more oxycodone compared with males on day 2 ($P = 0.0124$), day 3 ($P = 0.004$), day 4 ($P = 0.0220$), day 7 ($P = 0.0396$), day 8 ($P = 0.0023$), and day 10 ($P = 0.005$).

Effects of oxycodone on elevated zero maze and open field behaviors

Using the EZM and open field, we examined the effects of oral or s.c. oxycodone administration on anxiety-like behavior (Fig. 2). In time spent in the open arms during the EZM test, a two-way ANOVA revealed a significant main effect of treatment but not route of administration [treatment: $F_{(1,\ 52)} = 4.405$, $P = 0.0407$; route of administration: $F_{(1,\ 52)} = 0.4819$, $P = 0.4906$; interaction: $F_{(1,\ 52)} = 0.7178$, $P = 0.4007$], and Bonferroni's *post hoc* did not reveal significant differences between groups ($P > 0.05$) (Fig. 2a). When measuring time spent in the inner zone in the open field test, a two-way ANOVA revealed a significant main effect of treatment, but not route of administration or an interaction [treatment: $F_{(1,\ 52)} = 8.543$, $P = 0.0051$; route of administration: $F_{(1,\ 52)} = 0.4241$, $P = 0.5178$; interaction: $F_{(1,\ 52)} = 0.6924$, $P = 0.4091$]. The Bonferroni post-hoc comparison revealed that time spent in the inner zone of the open field was significantly increased in mice receiving s.c. oxycodone compared with mice receiving s.c. saline ($P = 0.0357$). Using a three-way ANOVA, no main

Fig. 4



Naloxone-induced withdrawal behaviors following oral or subcutaneous (s.c.) oxycodone administration. In mice administered oxycodone or vehicle, paw tremors (a), jumps (b), hind limb scratching (c), rearing (d), grooming (e), and writhing (f) were measured during naloxone-induced withdrawal ($n = 12$ for the oral group; $n = 16$ for s.c. group). Data are mean ± SEM. Data was analyzed via two-way analysis of variance followed by Bonferroni's post-hoc test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

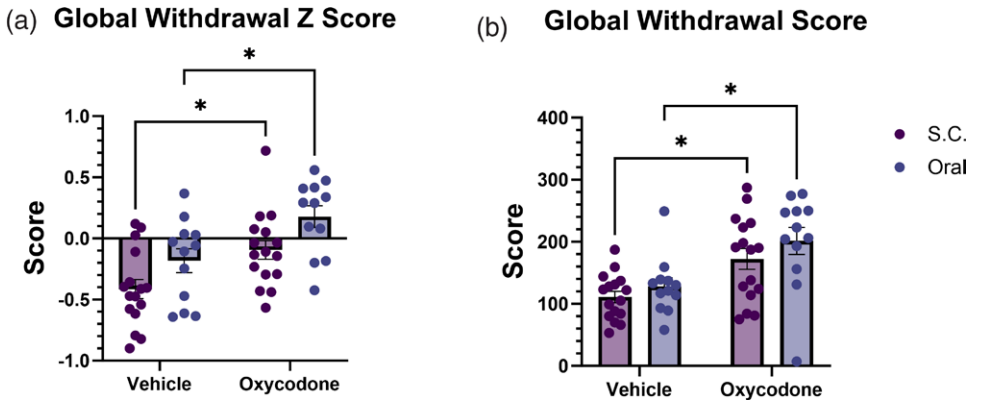
effect of sex was observed for anxiety-like behavior in the EZM [$F_{(1, 48)} = 2.855$, $P = 0.0976$] or open field [$F_{(1, 48)} = 1.322$, $P = 0.2559$] (data not shown).

Two-way ANOVA was used to assess differences in locomotor activity (distance traveled) in mice receiving oral and s.c. oxycodone (Fig. 3). A significant main effect of route of administration, but not treatment, was observed for locomotor activity in the EZM [treatment: $F_{(1, 52)} = 0.02493$, $P = 0.8751$; route of administration: $F_{(1, 52)} = 16.22$, $P = 0.0002$; interaction: $F_{(1, 52)} = 2.034$; $P = 0.1598$]. The Bonferroni post-hoc comparison revealed that EZM locomotor activity was significantly elevated in mice receiving s.c. saline compared with the mice in the oral water group (Fig. 3a). Similarly, using a two-way ANOVA, a main effect of route of administration, but not treatment, was observed for locomotor activity in the open field [treatment: $F_{(1, 52)} = 0.6144$, $P = 0.4367$; route of administration: $F_{(1, 52)} = 9.713$, $P = 0.0030$; interaction: $F_{(1, 52)} = 0.5344$; $P = 0.4681$] (Fig. 3b), but Bonferroni post-hoc comparison did not reveal significant differences between groups ($P > 0.05$). Using a three-way ANOVA, no main effect of sex was observed for locomotor activity in the EZM [$F_{(1, 48)} = 1.494$, $P = 0.2275$] or open field [$_{(1, 48)} = 2.652$, $P = 0.1100$] (data not shown).

Naloxone-induced withdrawal following oral or subcutaneous oxycodone administration

Next, we examined somatic signs of withdrawal via naloxone-induced precipitated withdrawal. Two-way ANOVA was used to assess differences in individual somatic withdrawal-like symptoms between treatment and route of administration (Fig. 4). When analyzing paw tremors, significant main effects of treatment and route of administration, as well as a significant interaction, were observed [treatment: $F_{(1, 52)} = 11.09$, $P = 0.0016$; route of administration: $F_{(1, 52)} = 17.06$, $P = 0.0001$; interaction: $F_{(1, 52)} = 4.106$, $P = 0.0479$]. The Bonferroni post-hoc test showed that the number of paw tremors were significantly higher in mice receiving oxycodone injections when compared with saline injections ($P = 0.0009$) and when compared with oral oxycodone ($P = 0.0004$) and water groups ($P < 0.0001$)

Fig. 5



Global withdrawal scores following oral or subcutaneous (s.c.) oxycodone administration. Compared with vehicle controls, global withdrawal scores (a) and global withdrawal *Z*-scores (b) were elevated in mice that received oral and s.c. oxycodone administration ($n = 12$ for oral group; $n = 16$ for s.c. group). Data are mean ± SEM. Data was analyzed via a two-way analysis of variance followed by Bonferroni's post-hoc test, $^*P < 0.05$.

(Fig. 4a). No significant effects of treatment or route of administration were observed for jumps [treatment: $F_{(1,\ 52)} = 3.435$, $P = 0.0695$; route of administration: $F_{(1,\ 52)} = 2.405$, $P = 0.1270$; interaction: $F_{(1,\ 52)} = 3.435$, $P = 0.0695$], but Bonferroni post-hoc comparison revealed that the number of jumps was significantly higher in oxycodone-injected mice compared with oral oxycodone mice ($P = 0.0196$) (Fig. 4b). For hind limb scratching, a significant main effect of route of administration, but not treatment or interaction, was observed [treatment: $F_{(1,\ 52)} = 2.511$; $P = 0.1191$; route of administration: $F_{(1,\ 52)} = 24.08$; $P < 0.0001$; interaction: $F_{(1,\ 52)} = 0.005$; $P = 0.9393$]. The Bonferroni post-hoc comparison revealed hind limb scratching was significantly elevated in the oral oxycodone mice compared with subcutaneously injected oxycodone mice ($P = 0.0075$), as well as oral vehicle compared with s.c. vehicle mice ($P = 0.0054$) (Fig. 4c). In rearing, a two-way ANOVA yielded a significant main effect of treatment, but not route of administration [treatment: $F_{(1,\ 52)} = 11.45$, $P = 0.0014$; route of administration: $F_{(1,\ 52)} = 3.624$, $P = 0.0625$; interaction: $F_{(1,\ 52)} = 0.4009$, $P = 0.5294$], and no significant differences were found between groups in post-hoc analysis (Fig. 4d). No significant main effects were observed across treatments for grooming [treatment: $F_{(1,\ 52)} = 0.2891$, $P = 0.5931$; route of administration: $F_{(1,\ 52)} = 0.4437$, $P = 0.5083$; interaction: $F_{(1,\ 52)} = 0.0340$, $P = 0.8543$] or writhing [treatment: $F_{(1,52)} = 0.4219$, $P = 0.5188$; route of administration: $F_{(1,52)} = 0.8378$, $P = 0.3643$; interaction: $F_{(1,\ 52)} = 3.351$, $P = 0.0729$] (Fig. 4e and f). Using a three-way ANOVA, no main effect of sex was observed for paw tremors [$F_{(1,\ 48)} = 0.1480$; $P = 0.2297$], jumps [$F_{(1,\ 48)} = 0.0072$; $P = 0.9324$], hind limb scratching [$F_{(1,\ 48)} = 0.1258$; $P = 0.7244$], rearing [$F_{(1,48)} = 0.1667$; $P = 0.6848$], grooming [$F_{(1,\ 48)} = 3.342$; $P = 0.0738$], or writhing [$F_{(1,\ 48)} = 2.104$; $P = 0.1534$] (data not shown).

For GWS, a two-way ANOVA revealed a significant main effect of treatment, but not route of administration [treatment: $F_{(1,\ 52)} = 18.33$, $P < 0.0001$; route of administration: $F_{(1,\ 52)} = 2.185$, $P = 0.1454$; interaction: $F_{(1,\ 52)} = 18.33$, $P < 0.0001$]. The Bonferroni post-hoc analysis revealed a significant increase in both oral and s.c. oxycodone groups compared with their vehicle controls (oral: $P = 0.0197$; s.c.: $P = 0.0263$) (Fig. 5a), but no significant difference in GWS was observed between injected and oral oxycodone groups ($P > 0.05$). To account for varying frequencies of each individual withdrawal behavior, withdrawal scores were transformed into *Z*-scores (Drinkuth *et al.*, 2023; Bravo *et al.*, 2020). Two-way ANOVA revealed a significant main effect of treatment, as well as the route of administration (treatment: $F_{(1,52)} = 15.96$, $P = 0.0002$; route of administration: $F_{(1,\ 52)} = 8.762$, $P = 0.0046$; interaction: $F_{(1,\ 52)} = 0.0549$, $P = 0.8157$). The Bonferroni post-hoc analyses revealed a significant increase in both oral and s.c. oxycodone groups compared with their respective controls (oral: $P = 0.0433$; s.c.: $P = 0.0354$), but global withdrawal *Z*-scores did not significantly differ between mice receiving oral oxycodone and mice receiving s.c. oxycodone ($P > 0.05$) (Fig. 5b). Using a three-way ANOVA, no main effect of sex was observed for GWS [$F_{(1,\ 48)} = 0.2677$; $P = 0.6073$] or GWS *Z*-scores [$F_{(1,\ 48)} = 0.9823$; $P = 0.3266$] (data not shown).

Discussion

With the surge in prescription opioid misuse and dependence, there is an urgent need to develop subject models that accurately recapitulate behavioral features associated with OUD. In previous rodent studies, oxycodone-induced dependence has been demonstrated following repeated experimenter-delivered injections (Enga *et al.*, 2016; Uddin *et al.*, 2021; Carper *et al.*, 2021) and continuous delivery via osmotic mini-pump (Raehal and Bohn, 2011; Mori *et al.*, 2013; Bhalla *et al.*, 2015). To

mimic prescription opioid use and abuse in humans, oral oxycodone consumption models have also recently emerged (Enga *et al*., 2016; Zanni *et al*., 2020), but potential differences in withdrawal- and anxiety-like behaviors following oral and parenterally administered oxycodone have yet to be evaluated. Here, we report somatic symptoms of naloxone-induced withdrawal in male and female mice following 10 days of home cage oral consumption or s.c. injections of oxycodone. We found that both oral and s.c. administration of oxycodone increased GWS to a similar degree, but few changes in anxiety-like behavior were observed across groups. When examining individual withdrawal-like behaviors, mice receiving s.c. oxycodone exhibited a greater number of paw tremors and jumps compared with mice receiving oral oxycodone. In previous studies, increases in paw tremors and jumps were shown to be robust and enduring signs of naloxone-induced withdrawal following chronic oxycodone exposure (Papaleo and Contarino, 2006; Enga *et al*., 2016; Towers *et al*., 2019; Carper *et al*., 2021). Thus, while GWS are increased by both routes of administration, some withdrawal-like behaviors were more pronounced following s.c. injections compared with oral oxycodone. It is important to note, however, that each model may be useful depending on the experimental question. For example, chronic oral opioid use is observed clinically in extended-release opioid prescriptions and methadone maintenance treatments, whereas repeated bolus dosing is more relevant to subjects that inject or snort oxycodone and other opioids.

Despite showing an increase in GWS, the oral oxycodone group did not exhibit an escalation of intake compared with the water group. Similarly, in a volitional two-bottle choice oral oxycodone paradigm, mice did not show a preference for oxycodone over water or escalate their intake across sessions, yet the oxycodone group displayed dose-dependent naloxone-precipitated withdrawal behaviors (Iyer *et al*., 2022). Analogous findings have also been reported in morphine two-bottle choice experiments (Gellert and Holtzman, 1978; Badawy *et al*., 1982). These data indicate that escalation of oral opioid intake is not essential to achieve physical dependence in all oral opioid intake models. The dose and duration of oral oxycodone access are important differences when comparing results between oral oxycodone studies. Using different experimental protocols of oral oxycodone, other studies have reported that mice and rats escalate oral oxycodone intake across sessions (Zanni *et al*., 2020; McKendrick *et al*., 2022; Slivicki *et al*, 2023). For example, Zanni and co-workers (2020) had rats consume oxycodone orally for 22 weeks, whereas in the current study and in the study by Iyer *et al*. (2022), mice consumed oxycodone for 5–10 days (Iyer *et al*., 2022). The context in which oxycodone is consumed (home cage vs. novel environment) may also influence escalation of intake in shorter duration experiments (McKendrick *et al*., 2022), as drugs received in a novel context are potentially more rewarding (Bevins *et al*., 2002). In addition, the availability of food may also influence a subject's preference for natural rewards over illicit drugs (Venniro *et al*., 2020) and could contribute to the lack of escalation in the current experiments. In future studies, limiting access to food during oral oxycodone administration may be considered for translational purposes as subjects with OUD often experience malnourishment (Marabia *et al*., 1989; Nabipour *et al*., 2014).

While sex differences play an important role in prescription opioid abuse propensity (Serdarevic *et al*., 2017), reports of sex differences in rodent models of oral oxycodone use have been mixed. For example, Iyer and co-workers (2022) found that male and female mice do not differ in oxycodone (mg/kg) preference or oral oxycodone consumption in a two-bottle choice drinking paradigm. We found no differences between males and females when oxycodone intake was averaged across the 10 days. When daily oxycodone intake was adjusted for weight (mg/kg), females consumed more oxycodone than males across multiple days. In addition, when comparing water intake by weight (ml/g), females consumed on average more water than males, an effect that has been observed in some mouse strains (Tordoff *et al*., 2008). These results highlight the importance of including a water or control group in oral consumption analysis to ensure that sex-dependent changes are specific to drug intake.

In other studies, female mice and rats orally consumed more oxycodone than males (Kimbrough *et al*., 2020; Zanni *et al*, 2020; Slivicki *et al*, 2023). The differences in oral intake may be attributed to several factors including oxycodone metabolism, as the enzymes that metabolize oxycodone (e.g. CYP3A4 and CYP2D6) are influenced by sex hormones (Arguelles *et al*., 2022). In intravenous self-administration studies, however, no sex differences were detected in plasma or brain oxycodone levels, indicating that sex differences in oxycodone use are not always related to pharmacokinetic factors (Mavrikaki *et al*., 2017). Similar to oxycodone intake, sex differences in rodent withdrawal-like behaviors following opioid use have been mixed, with some studies showing no sex differences (Towers *et al*., 2019), while others reported that female rats exhibit less intense but more protracted withdrawal than males (Bobzean *et al*., 2019). Clearly, additional work is needed to elucidate these discrepancies in sex-dependent opioid use and withdrawal.

Using the elevated plus maze and open field, previous studies have shown decreased anxiety-like behaviors during opioid withdrawal (Hodgson *et al*., 2008; Buckman *et al*., 2009; Bruijnzeel *et al*., 2022), while others have reported the opposite effect (Harris and Gewirtz, 2004; Bravo *et al*., 2020). Similarly, we also obtained mixed results, as mice receiving s.c. oxycodone injections, but not oral oxycodone, spent more time in the center of the open field compared with the vehicle group, while no

changes between groups were observed in EZM. These different outcomes may be attributed to the relatively high baseline anxiety in C57BL/6 mice (Miller *et al*., 2010; Marchette *et al*., 2018), making it difficult to obtain consistent results. The time point in which anxiety-like behaviors are measured following the last opioid exposure may also influence the results, as earlier time points may be more sensitive in detecting changes in anxiety-like behaviors (Becker *et al*., 2017; McKendrick *et al*., 2020). While some studies have previously shown that oral opioid administration increases locomotor activity in an open field test (Iyer *et al*., 2022; Berríos-Cárcamo *et al*., 2022), other studies investigating locomotion during opioid withdrawal have revealed reduced locomotion (Doherty *et al*., 2013) or no differences in locomotor activity (Ma *et al*., 2007), suggesting that locomotor activity during withdrawal may vary depending on the experimental conditions.

Some limitations should be considered when interpreting the current results. First, in these experiments, mice in the oral oxycodone group did not have an alternative drinking option, nor was the frequency or time spent consuming oxycodone over a 24-hour period measured. Recently, Slivicki and co-workers (2023) addressed these issues by using a lickometer device to show that the frequency of oxycodone drinking was significantly elevated compared with water drinking and that the majority of oxycodone drinking occurred within the first few hours of the dark phase. Second, the timing of the anxiety and withdrawal tests following the final oxycodone treatment might influence the behavioral outcomes. In previous studies, increases in naloxone-induced precipitated withdrawal have been observed as early as a few hours and up to several weeks after the last opioid exposure (Papaleo and Contarino., 2006; Bravo *et al*., 2020; Towers *et al*., 2019; Carper *et al*., 2021). Some withdrawal-like behaviors, however, do attenuate as a function of time (Carper *et al*., 2021). Further, although precipitated opioid withdrawal in OUD patients remains an important issue in emergency care settings (Oakley *et al*., 2021; D'Onofrio *et al*., 2023), spontaneous withdrawal is more common in opioid-dependent patients but was not examined in the current experiments. In rodent models, spontaneous withdrawal-like behaviors are much weaker compared with naloxone-induced withdrawal (Carper *et al*., 2021), and identifying potential differences becomes a challenge due to a floor effect. Nevertheless, future studies using precipitated and spontaneous withdrawal are needed to determine time-dependent behavioral differences following oral versus s.c. oxycodone administration. Third, the oral bioavailability of oxycodone in rodents is an important factor that should be taken into consideration in the current and future studies. For instance, a study in rats reported that the bioavailability of parenterally administered oxycodone is approximately 57%, whereas the oral bioavailability of oxycodone is between 1.2 and 5.0% (Huang *et al*., 2005; Chan *et al*., 2008). In humans, oral bioavailability of oxycodone is approximately 69% (Nieminen *et al*., 2009), which is more similar to oxycodone bioavailability in rodents following parenteral administration. It is also important to note that opioids have effects outside of the brain (e.g. gut microbiome) that contribute to the disease state, and the route of opioid administration may also differentially alter these factors to influence behavior (Wang *et al*., 2018; Ren and Lotfipour, 2020; Antoine *et al*., 2022). Finally, molecular mechanisms of oxycodone dependence were not investigated in the current experiments. In a recent study, however, ΔFosB, a transcription factor involved in drug-induced neuroplasticity (Nestler *et al*., 2001), was found to be elevated in the reward-related brain regions following oral oxycodone consumption (Iyer *et al*., 2022), similar to parenteral opioid administration studies (Wang *et al*., 2005; Núñez *et al*., 2010). In addition, an enhancement of excitatory neurotransmission in nucleus accumbens medium spiny neurons was observed in mice following oral oxycodone self-administration (Slivicki *et al*., 2023), a mechanism that has been linked to drug dependence and relapse-like behaviors (Ma *et al*., 2014; Pascoli *et al*., 2014; Madayag *et al*., 2019).

Overall, growing evidence indicates that oral oxycodone subject models are effective at recapitulating some behavioral aspects of prescription opioid dependence. By showing similar GWS following oral and parenteral oxycodone administration, the current results suggest that oral oxycodone self-administration is a reliable procedure to model prescription opioid abuse. Ongoing efforts to optimize the experimental conditions that accurately mimic oxycodone dependence and withdrawal may enhance our understanding of OUD and lead to more effective treatment options.

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Conflicts of interest

There are no conflicts of interest.

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