TITLE: Development and validation of a novel oral oxycodone self-administration protocol for female and male rats

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RUNNING TITLE: A novel rat oral oxycodone self-administration protocol

ACKNOWLEDGEMENTS: We thank Ashlee A. Dougher, Peter Lenchur, Patrese Robinson-Drummer, and Steve J. Simmons with technical assistance during data acquisition, and Ganesh S. Moorthy, Christina M. Vedar, and Athena F. Zuppa of CHOP's Bioanalytical Core Center for Clinical Pharmacology for oxycodone analysis in blood.

ABSTRACT

The increased abuse of opioids - such as oxycodone - poses major challenges for health and socioeconomic systems. Here we present a novel oral oxycodone self-administration paradigm in rats that recapitulates key aspects of prescription opioid abuse: voluntary drug intake, long-term opioid exposure, measurable levels of dependence, and increased motivation to take drug. Adult female and male Long-Evans rats were given unlimited, home cage access to two bottles containing water (Control rats) or one bottle of water and one bottle of Oxycodone dissolved in water (Experimental rats). Virtually all Experimental rats voluntarily drank oxycodone (~10 mg/kg/day) and escalated their intake over 22 weeks. Females self-administered twice as much oxycodone as males, leading to greater levels of oxycodone in blood, and engaged in more gnawing behavior. Precipitated withdrawal revealed high levels of dependence in both sexes in the total withdrawal score and in individual withdrawal behaviors. Reflecting motivation to drink oxycodone, citric acid at ascendant concentrations suppressed the intake of oxycodone (Experimental rats) and the intake of water (Control rats); however Experimental rats returned to pre-citric acid preference levels whereas Controls rats did not. Finally, long-term exposure to oxycodone reduced the alpha diversity of the gut microbiome compared to water Controls but had varying effects on the relative abundance of select bacterial Phyla. Thus, our model reproduces in female and male rats many of the features of human oxycodone abuse, and provides a paradigm for both understanding mechanisms that mediate long-term voluntary drug use and for exploring potential treatment options.

INTRODUCTION

Opioid misuse and abuse have reached epidemic status worldwide, posing major challenges for health and socioeconomic systems [1–3]. Opioids such as oxycodone are mainstays of pain management [4,5], but the relatively easy access to these medications and their common diversion has led to higher incidence of addiction in the exposed population [3,6,7]. This increase has deadly consequences; in 2014, oxycodone ranked third behind heroin and cocaine in causing overdose deaths [8]. As initial use of opioids can result in dependence that leads to concomitant or replacement use of more accessible, less expensive but more lethal opioids, i.e. heroin or fentanyl, it is critical to understand the factors underlying prescription opioid abuse liability [6].

To understand these factors, preclinical researchers need an animal model of prescription opioid abuse liability that recapitulates key aspects of long-term use - dependence, tolerance, and reinforcement [9–11]. Prescribed oxycodone is typically taken orally, and animal models of oral opioid self-administration have been in use for many years [12–19]; but these models have caveats. In oral models often animals are forced to “self-administer” (drink) an opioid solution by provision of only drugged water, or are provided a drug solution adulterated with an alternative reinforcer, such as sucrose [20]. The high blood levels of drug achieved in oral administration models require concentrated drug solutions that may be aversive to drink. If diluted to increase palatability, the dosing of the opioid is limited [20]. At present, there are no preclinical oral models that offer an animal continuous choice (24 hours/day [h/d], 7 d/week [wk]) between natural rewards (food/water) and oral, unadulterated oxycodone. Such a model would provide a translationally-relevant tool to test interventions and to interrogate variables such as sex differences [21–24] and the gut microbiome [25] that are challenging to study in existing opioid self-administration models. Improved oral opioid abuse models would also

complement IV self-administration models by enabling the study of transition between stages of addiction, thereby facilitating intervention testing.

Here we describe a novel rat model, termed PoVOCC (*Per os* Volitional Oxycodone Continuous Choice), that provides rats with prolonged, unlimited access to oxycodone and leads to escalation, dependence, and marked motivation to ingest the drug. We show PoVoCC recapitulates many features of human oxycodone abuse in female and male rats. We believe PoVOCC will be useful for probing neural and physiological mechanisms that mediate long-term voluntary drug use and exploration of potential treatment options.

METHODS

Animals and husbandry. Animal procedures were in accordance with federal guidelines [26] and the Institutional Animal Care and Use Committee (IACUC) at the Children's Hospital of Philadelphia Research Institute (CHOP), and were performed in AAALAC-approved facilities at CHOP (AAALAC Unit # 000427, PHS Animal Welfare Assurance #D16-00280 [A3442-01]). Efforts were made to minimize both animal distress and the number of animals used. Female (n=12) and male (n=12) Long-Evans Hooded rats (Envigo, 60-65 day-old) were individually-housed in a vivarium (temperature, 20-23°C; humidity 30-70%: lights on/off: 6:15AM/6:15PM). Food and water were provided *ad libitum* throughout the experiment. (Additional details in **Supplementary Methods.**)

Baseline behavioral assessment. After at least two weeks of individual housing, rats were tested in the open field and for marble burying behavior (**Fig. 1**). Open field was performed as previously described [27,28] with minor modifications. One day later, rats were then assessed

for marble burying, as previously described [29–33] with minor modifications. (Additional details in **Supplementary Methods.**)

Oxycodone self-administration. After behavioral assessment (**Fig. 1**), lixits were removed from all cages and rats were given water via a bottle (Hydropac® or Zyfone bottle) for the duration of the experiment. During Baseline (Weeks -1 and 0, **Fig. 1**), rats were provided two water Hydropacs (in front and back position) in a hopper on the right side of the cage with food placed in between. Starting on Week 1, Control ("Water/Water") rats continued to receive water from both bottles, while Experimental ("Water/Oxycodone") rats received water in one bottle and oxycodone dissolved in water in the other. Oxycodone concentration range delivered was 0.06-0.12 mg/mL, with a goal of individual rat intake of 10 mg/kg/day [34] if they exclusively drank from the oxycodone bottle. From Weeks 3 to 11, the oxycodone bottle was placed in the front hopper position, and from Weeks 12 to 32 it was switched to back hopper position. (Additional details in **Supplementary Methods.**)

Home cage behaviors. Home cage behaviors were observed twice daily, at 7AM and 4PM. A single rat was tracked by one observer during 1-min observation periods for the presence or absence of specific behaviors (**Supplementary Table 1**) indicative of normal activity (i.e. grooming, walking) as well as spontaneous withdrawal-like behavior (i.e. wet dog shakes) as adapted from previous studies [35–39].

Serum assessment of oxycodone levels. Blood was taken via tail vein under isoflurane anesthesia and assessed for oxycodone levels according to prior protocols [40,41]. (Additional details in **Supplementary Methods.**)

Gnawing home cage behavior. At Week 12, each rat cage received 1 nylabone and 1 wood gnawing block (Bio-Serv K3512 medium size). The weight of both the nylabone and block were recorded daily at the same time. The change in weight of each item was calculated to derive a gnawing index, defined as the total amount in grams of nylabone and wood block chewed over time.

Citric acid aversion test. The citric acid (CA) aversion test was guided by prior work [42–45] with significant modifications. Between Week 27 and 29 all rats received ascending concentrations of citric acid (CA, Sigma Aldrich, cat# PHR1071) at 1, 3, and 5mM in the back bottle. The 5mM CA was repeated (5 and 5'). In between each CA exposure, rats received their original bottle (water or oxycodone) for 2 days to allow for return to baseline. Liquid intake, body weight, preference, and deviation from baseline were scored daily.

Precipitated withdrawal. On Week 31 all rats were tested for signs of withdrawal **(Supplementary Table)** based on prior work [38,39,46–50]. (Additional details in **Supplementary Methods.**)

Fecal bolus collection and gut microbiome analysis. Fecal boluses were collected from the home cage at 4PM prior to any treatment (Week -1) and again at Week 28 at 12PM and maintained at - 20°C until processed for library generation**.** After conversion of Illumina MiSew sequencing output to FASTQ files, files were processed using the Divisive Amplicon Denoising Algorithm (DADA2)[51] in R 3.3.2 (https://www.r-project.org/) to resolve fine-scale variation and create a unique amplicon sequence variant table for downstream analysis [52]. Once processed, amplicon sequence variants were used to compare gut microbiome community composition, species richness, and beta-diversity among samples. Community

composition was visualized by the packages Phyloseq [53] and ggplot2 [54] using the plot_bar function for each fecal sample. (Additional details in **Supplementary Methods.**)

Statistical analysis. Most data analysis in **Figs. 1-4** were performed using GraphPad Prism® (version 8; Macintosh) or PAST3 version 3.2 (Macintosh). For gut microbiome data, analyses were performed using R-packages [55]. (Additional details in **Supplementary Methods.**)

RESULTS

Pre-screening for a predictive endophenotype: assessment prior to experimental assignment revealed no overt group differences.

Pre-screening behavioral assessment. Prior to any manipulation, all rats were pre-screened for open field (OF1) behavior, as an indicator of both anxiety and locomotor activity [56,57] and marble burying, as repetitive behavior [58] often associated with anxiety-like phenotypes [59]. For OF1 the total distance travelled (**Fig. 1B**) and number of entries into the center zone (**Fig. 1C**) were similar within groups irrespective of Sex or the to-be-assigned Treatment (Control or Experimental). There was a significant main effect of Sex of duration in the Intermediate zone (**Fig. 1D**), with females spending ~3x more time in Intermediate zone than males. We found no difference in number of marbles buried driven by Sex or to-be-assigned Treatment group (**Fig. 1E**). Thereby at pre-screening the behavior parameters were similar, with the exception that females showed higher levels of novelty-seeking (longer time spent in the Intermediate zone in the OF1 relative to males).

Baseline Water/Water intake. At baseline (average of Week -1 and Week 0, **Fig. 1A**), Control and Experimental rats received two bottles of water in their home cage (**Fig. 1F**). There was

no effect of Treatment or Sex in the average amount of liquid (water) ingested during Baseline (**Fig. 1G**). There was no effect of Treatment and Sex in the preference for the front or the back bottle (**Fig. 1H**). Within Sex, rats had similar body weights (**Fig. 1I**), with females as expected weighing less than males. Thus, the random assignment to either Control or Experimental group was unbiased.

Experimental rats increased their oxycodone preference and drank more oxycodone over time, with females ingesting more than males and having higher oxycodone blood levels.

During the 2-bottle-choice period (see **Fig. 1A**), female Experimental rats increased their total liquid intake from Week 19 compared to Week 1, whereas male Experimental rats did not (**Fig. 2A**). Both females and males increased their preference for oxycodone by Week 9 relative to Week 1. When the bottle position was switched, both females and males maintained their preference for oxycodone, but male rats increased their preference for the oxycodone by Week 17 compared to the week prior to bottle switch (Week 11; **Fig. 2B**). Furthermore, female and male rats self-administered a higher dose of oxycodone over time, with females self-administering a higher dose by Week 9, whereas males self-administered a higher dose by Week 14 (**Fig. 2C**). Females self-administered higher doses than males on Weeks 9 to 12, 17, 18 and 20 to 22. The greater oxycodone intake in females was reflected in higher oxycodone blood levels compared to males (**Fig. 2D**). When dose of oxycodone was correlated with blood levels, the relationship was significant and positive for males, but not significant for females **(Fig. 2E)**.

Experimental rats show higher levels of dependency in precipitated withdrawal and increased gnawing behavior.

Withdrawal in home cage. Daily assessment of home cage spontaneous withdrawal symptoms showed no significant difference between groups when analyzed as individual behavior or when the total sum of these behaviors was collapsed over 22 weeks (**Fig. 3A**). Overall, Experimental rats chewed more nylabone and wooden block in the home cage between Weeks 12 and 22 of oxycodone exposure than did Control rats, whereas Experimental females gnawed more than Control females, Control males, and Experimental males, Experimental males did not differ from Control males (**Fig. 3B**).

Precipitated withdrawal. In contrast to home cage withdrawal behaviors, Experimental rats presented more precipitated withdrawal symptoms than Control rats at Week 31 for key behaviors analyzed separately: burrow, teeth chatter, high walk, and diarrhea. When total precipitated withdrawal score was analyzed, Experimental females and males presented more withdrawal signs than respective controls (**Fig. 3C**). After precipitated withdrawal, Experimental females lost four-fold more weight than Control females, and Experimental males lost two-fold more than Control males (**Fig. 3D**).

Ascendant concentrations of CA suppressed intake in both Control and Experimental rats, but Experimental rats returned to initial baseline preferences whereas Control rats did not.

When provided ascending concentrations of CA in the back bottle (**Fig. 4A**), all rats suppressed their preference; Experimental males suppressed at 3mM CA whereas the other groups suppressed at 5mM (**Fig. 4B**). When CA was removed during the interspersed

baselines, Experimental rats returned to the initial baseline preference, whereas Control rats maintained their aversion (**Fig. 4C**).

Pre-screening of open field behavior predicted oxycodone intake-related outcomes in both female and male rats.

For an unsupervised picture of the relationship between open field behavior and dependent measures during oxycodone intake (precipitated withdrawal, spontaneous withdrawal, intake, preference, CA, blood, gnawing, etc.), we conducted a factor analysis of all pre-screened open field behaviors. Analysis revealed a single factor from pre-screening open field explained ~71% of the variance each for females (**Supplementary Fig. 1A**) and males (**Supplementary Fig. 1B**). To obtain a single score for each rat, the pre-screening open field behavior measures were multiplied by the factor score of that behavior and summed. Each rat's factor score was then correlated with the dependent measures during the oxycodone intake phase. For females, only gnawing was significantly correlated with the open field measure. For males, significant correlations were seen with precipitated withdrawal weight loss, gnawing, oxycodone dose at Week 22, liquid intake at Week 22, and blood levels (**Supplementary Fig. 1C**).

Graphic representation of the correlation data (**Supplementary Fig. 1D**) revealed a positive relationship between oxycodone intake in female and male rats in Week 22 and pre-screening activity in activity measurements and Center areas of the open field. In both female and males rats we did not observe significant correlations in the post-screening activity.

Chronic oral oxycodone decreases alpha-diversity but has a mild impact on gut microbiome composition.

In both Control and Experimental rats, Bacteroides and Firmicutes dominated the gut microbial composition whereas Actinobacteri, Cyanobacteria, Deferribacteres, Lentisphaerae, Proteobacteria, Tenericutes, Verrucomicrobia were present in low abundance (**Fig. 5A**). Analysis of relative abundance at the Phylum level at Week 28 showed several bacterial Phyla varied between Control and Experimental rats (**Fig. 5B**; remainder of ranks, **Supplementary Tables 2-5**). Alpha diversity at Week -1 vs. Week 28 did not differ in Controls but did in Experimental rats (**Fig. 5C**), with a lower alpha diversity of gut microbial communities seen at Week 28 of Experimental rats. Beta diversity and principal coordinates analysis (PCoA) revealed that rats within treatment groups clustered more closely than those from different treatment groups (**Fig. 5D**).

DISCUSSION

Important aspects of a preclinical model for human prescription opioid abuse liability include prolonged and unlimited drug access, voluntary self-administration when a choice is provided, high levels of intake, drug dependence, and subsequent somatic signs of opioid withdrawal. Here we present a novel oxycodone self-administration paradigm in rats, PoVOCC, that recapitulates these key features of human prescription opioid abuse. In the PoVOCC model, rats given continuous choice between oxycodone and water reliably demonstrated voluntary and escalating intake of oxycodone, measurable levels of dependence, and motivation to take the drug. We believe this preclinical model of prescription opioid abuse - which relies on oxycodone oral voluntary self-administration - is of significant translational relevance for understanding the mechanisms that underlie opioid

addiction and ultimately will contribute to the ongoing research efforts to combat the growing opioid epidemic.

Conceptual and methodological advantages of PoVOCC

The face validity of opioid self-administration models is based on an animal's motivation to seek and to take drug [60] and to do so despite adverse consequences [61]. In the PoVOCC model, rats readily drank oxycodone, and showed a preference for the oxycodone-containing bottle relative to the water bottle. Experimental rats escalated their oral oxycodone intake over time, suggesting it was reinforcing, and rebounded to these high levels of intake following citric acid suppression. Thus, rats in the PoVOCC model show motivation to drink in the face of adverse consequences.

In contrast to our PoVOCC model, other opioid self-administration paradigms rely either on oral intermittent access (OIA) or intravenous (IV) self-administration [16,20,35,62–70]. Both OIA and IV self-administration models lead to dependence and tolerance, and IV models demonstrate drug-seeking motivation. OIA models mimic a common route of administration of humans (oral), whereas the rapid onset of IV drug effects enables assessment of contingent cues and behaviors. However, these models have limitations. High first-pass metabolism of drug in OIA opioid models results in a short bioavailability and longer latencies to induce the "high" compared to IV models [40]. IV self-administration models often rely on short-term access, which confounds assessment of drug effects vs. those caused by daily withdrawal [9,20,71]. We believe the PoVOCC model addresses some of these issues. Its advantages include: a) the translational relevance for orally-delivered drugs, b) voluntary intake and choice between natural rewards (food/water) and oxycodone, c) the chronicity of drug available 24h/7d over months, d) the non-invasive methodological approach, and e) the

absence of confounders, such as adulteration of oxycodone solution to encourage oral intake or stress the animal to induce intake.

PoVOCC models sex differences in oxycodone intake and blood levels

Prescription opioid abuse liability is likely sex-dependent [72,73]. There is a more rapid increase in the rate of prescription opioid abuse in women than men [8]. Sex hormones appear to play a role in opioid dependence and reinforcement [48,72,74–79], including in oral self-administration protocols [80]. Sex differences are also apparent in the pharmacokinetic, metabolic, and analgesic effects of oxycodone [40,81,82], and in the liability for opioid abuse [83,84]. In preclinical studies, females self-administer more opioids, are more vulnerable to their reinforcing effects, and become more physically-dependent than males [23,24,36,75,77–79,85–87]. In our PoVOCC model, both sexes readily drank oxycodone but females self-administered two-fold more oxycodone than males, with resultant five-fold higher blood levels. Female rat preference for oxycodone peaked early and remained high, whereas male rat preference peaked later; these differences were independent of oxycodone concentrations. Female individual differences during pre-screening were less predictive of intake, self-administered dose, blood levels, and gnawing behavior than male individual differences. Thus, the PoVOCC model appears suited to interrogate sex differences under a choice oxycodone self-administration paradigm.

Although we did not investigate sex differences in pharmacokinetics, the large difference in dosing we observed could have been driven by sex differences in oxycodone metabolism that are reported in humans [85,88] and in some, but not all, studies in Sprague Dawley rats [40,83]. In our PoVOCC model with Long Evans rats, one reasonable - but as yet untested - explanation for the higher total liquid intake in females is their attempt to keep oxycodone

blood levels constant. This would argue in favor of a faster elimination rate (but see [40]), lower oxycodone sensitivity and/or different first pass metabolism, resulting in greater intake at the same concentration compared to males. Further studies on blood sampling at earlier and multiple time points, with measures of metabolites, are warranted to test this hypothesis.

PoVOCC as a model of dependence and stereotyped gnawing behavior

In contrast to short-term access paradigms [9,20,71], our long-term free-access PoVOCC model allows investigation of if and how rats titrate their oxycodone intake to avoid withdrawal. Indeed, there was negligible spontaneous withdrawal in our long-term access paradigm. There are caveats though; our sampling for spontaneous withdrawal was time- and behavior-limited, and a more thorough analysis of spontaneous withdrawal signs in the PoVOCC model is warranted. In contrast to the lack of spontaneous withdrawal, both female and male Experimental rats showed robust precipitated withdrawal signs [35,46,69,89], with ~4-fold more naloxone-induced signs of physical dependence relative to Control rats. Thus, the PoVOCC model will be useful for assessing opioid dependence and for assessing the efficacy of treatments targeted to relieve withdrawal.

Despite no significant differences in spontaneous withdrawal, in the PoVOCC model Experimental - but not Control - rats engaged in stereotyped gnawing behavior. Stress can induce stereotyped behaviors, including gnawing, which are mediated by endogenous opioids [90–92]. One source of stress in our PoVOCC model is social isolation, as rats were singly-housed for 10 months. However, isolation is unlikely the sole contributor to increased gnawing behavior as no control rats measurably gnawed. It is possible that the combination of oxycodone and isolation increased the likelihood of gnawing, and we might expect that group-housed oxycodone rats would gnaw less than isolated oxycodone rats. Moreover,

because social isolation is more stressful for females than males [93–95], the increased gnawing in particular by experimental females may be explained by an opioid-mediated activation of the dopaminergic mesolimbic system known to induce gnawing behavior [90,91].

PoVOCC model: appetitive and reinforcing behavior, escalation, and incentive salience

In addition, when challenged to drink an aversive tastant added to the oxycodone bottle, most rats, (Experimental and Control) suppressed their drinking from the citric acid adulterated bottle. However, some Experimental rats maintained their preference for oxycodone despite the addition of citric acid. Both female and male experimental rats readily returned to baseline levels when the unadulterated oxycodone bottle was returned, whereas control rats maintained their acquired aversion. These data suggest that the PoVOCC model has high face validity for modelling prescription opioid abuse liability and it provides a broader use of this model to study motivational aspects of prescription opioid abuse, such as individual differences in the development of physical dependence, oxycodone reinforcement, and its motivational salience.

Changes in the gut microbiome in the PoVOCC model

Humans and animals exposed to opiates - typically morphine is studied - have altered gut microbiome [25,96–101]. In general, these opioid-induced alterations manifest in a switch from gram-negative to gram-positive bacteria, where the latter dominates the gut composition. The functional importance of a switch in bacteria or in levels of alpha-diversity remains a hotly-debated issue [102]. However, the opioid-induced switch in bacteria has been postulated to contribute to gut permeability, sustained systemic inflammation, and even opioid tolerance [96,98,103], with duration of morphine exposure appearing to be a key driver of gut microbiome [97]. In contrast to work on experimenter-delivered morphine, little is know

about how oxycodone or any other self-administered opioid influences the gut microbiome in humans or in rodents. With our PoVOCC model in rats, long-term voluntary oral choice access to oxycodone decreased alpha-diversity with minor changes in beta-diversity, primarily driven by a reduction in low abundance, gram-negative bacteria phyla. Relatively high abundant bacteria phyla (Bacteroidetes and Firmicutes) were unaffected. The PoVOCC model results may differ from published work with morphine for many reasons. Exposure duration may be a factor. Immediate changes in the microbiome can occur within hours or days, but to 'permanently' change the composition usually requires more time. Changes in gut microbiome are likely to be drug- and route-of-administration-specific as well, and oral self-administered oxycodone may act differently than systemic, experimenter-delivered morphine on the gut microbiome. Finally, there may be species-specific adaptation of the gut microbiome such that a chronic opioid paradigm rats (such as PoVOCC) may result in different changes than when mice are used. Studies on chronic opioid human users are warranted to explain this discrepancy. Our data overall indicate that changes in gut microbiome composition are mild in our PoVOCC model and that the dysbiosis towards gram-negative-enriched gut microbiota needs further investigation.

Pre-screening for predictive factors

Not all humans become addicted to drugs, and those that do show different degrees of dependence [7,104]. The individual differences that emerge during the PoVOCC model can be harnessed to identify individual vulnerabilities that may be predictive of oxycodone abuse liability. Locomotion, exploratory activity, and anxiety-like phenotypes can be predictive of later drug-seeking behavior [11,105]. In our present work, there were sex differences in the prescreening prediction of later intake and intake-related variables; pre-screened open field behaviors predicted intake and intake-related variables and some components of

dependence in males but not females. A caveat of this is that females had consistently higher levels of oxycodone intake than males. Thus, the fewer predicted intake specific variables for females compared to males may reflect a ceiling effect specifically for intake variables. This ceiling effect is less likely to explain the lack of correlation for non-intake-related variables such as weight loss after precipitated withdrawal.

Conclusions

PoVOCC is a model of voluntary, continuous access, oral oxycodone intake in rats. It mimics oral ingestion of oxycodone in humans, allowing the animal to titrate its own oxycodone levels and the development of physical dependence in a sex-dependent manner. Experimental rats show motivation to drink oxycodone. Future work would address why and how individual rats differ in their intake, pre-existing risk factors that might predict high levels of intake, mechanisms underlying the sex differences, and the gut microbiome alterations found in these studies. The PoVOOC model will be useful for probing treatment options, pharmacological and environmental, and in particular the role of stress and isolation in the motivation to take opioids.

FUNDING AND DISCLOSURE

This work was supported by the National Institutes of Drug Abuse (AJE, K02DA023555), Development Funds from the Department of Anesthesiology and Critical Care Medicine of CHOP (GAB, AJE), and the James Battaglia Endowed Chair in Pediatric Pain Management (GAB).The authors have nothing to disclose and no conflicts of interest.

FIGURE LEGENDS

<u>Figure 1</u>. Pre-screening behavioral assessment (prior to experimental assignment) revealed no overt group differences.

(A) Experimental time course. Long Evans (**LE**) hooded rats, 12 females (**F**) and 12 males (**M**), were **pre-screened** (Weeks -3 and -2) on the open field **(OF1)** and marble burying **(MB)** tests. After pre-screening, all rats entered the **Baseline** (**Water/Water**) period where they were habituated for two weeks (Weeks -1 and 0) to drink from two water bottles placed either in front of or behind the food hopper positioned on the right side of the cage (**1F**). Starting at Week 1, a two-bottle choice paradigm was initiated for all rats. **Control** rats (3 female and 3 male rats, randomly chosen) continued to receive two bottles containing water (**Water/Water**). **Experimental** rats (9 female and 9 male rats, also randomly chosen) received an oxycodone-containing bottle in the front position and a water-containing bottle in the back position (**Water/Oxycodone**). At the end of Week 11/start of Week 12, the front bottle (containing water or oxycodone) was switched to the back position for both Control and Experimental rats (**Bottle Position Switch**). On one day during weeks 15 and 21, tail vein blood was extracted from all rats. From Weeks 27 to 29, all rats were intermittently exposed to increasing concentrations of citric acid (**CA**) to test for aversion. Week 31, all rats were tested for nociception in the plantar termal test (**PTT**) and open field again (**OF2**) subsequently for signs of withdrawal in the naloxone precipitated withdrawal paradigm (**PW**).

(B) The analysis of total distance travelled over time collapsed across the 30 min of the OF1, analysis of total distance travelled via a two-way ANOVA (Sex x Treatment) revealed no interactions, $F(1,20)=0.05$, $p=0.81$, no main effect of Sex, $F(1,20)=1.41$, $p=0.24$, and no main effect of Treatment, $F(1,20)=1.06$, $p=0.31$.

(C) Analysis of the OF1 entries into the Center using a two-way ANOVA (Sex x Treatment) revealed no interactions, F(1,20)=0.66, *p*=0.42, no main effect of Sex, F(1,20)=1.63, *p*=0.21, and no main effect of Treatment, F(1,20)=1.09, *p*=0.30.

(D) Analysis of the OF1 average time in the Intermediate by a two-way ANOVA (Sex x Treatment) revealed no interactions F(1,20)=0.17, *p*=0.67, a significant effect of Sex F(1,20)=7.14, ***p=0.014**, and no main effect of Treatment F(1,20)=0.06, *p*=0.80

(E) Marble burying test analysis of the number of marbles buried using a two-way ANOVA (Sex x Treatment) revealed no interactions F(1,20)=0.86, *p*=0.36, no main effect of Sex F(1,20)=0.11, *p*=0.73, and no main effect of Treatment F(1,20)=0.02, *p*=0.88.

(F) Schematic of the home cage during the two-bottle habituation (Baseline) period, water (W) bottles placed in the front and the back of the food hopper on the right side of the cage. Rats had access to both bottles 24 hours/day and 7 days/week. Not drawn to scale.

(G) Analysis of total water intake during the Baseline period via two-way ANOVA (Treatment x Sex) revealed no interaction, F(1,20)=0.67, *p*=0.42, no main effect of Sex, F(1,20)=0.71, *p*=0.40, and no main effect of Treatment, F(1,20)=0.45, *p*=0.50.

(H) Analysis of position preference (ratio of intake from one bottle over the total intake) for the front bottle during the Baseline period via two-way ANOVA (Treatment x Sex) revealed no interaction, F(1,20)=0.22, *p*=0.64, no main effect of Sex, F(1,20)=1.22, *p*=0.28, and no main effect of Treatment, F(1,20)=0.61, *p*=0.44.

(I) Analysis of body weight during the Baseline period via two-way ANOVA (Treatment x Sex) revealed no interaction, F(1,20)=1.16, *p*=0.29, a significant main effect of Sex, F(1,20)=503.2, ****p<0.0001**, and no effect of Treatment, F(1,20)=0.06, *p*=0.79.

<u>Figure 2.</u> Experimental rats increase liquid intake and oxycodone preference over time and self-administer ascending doses in a sex-dependent manner.

(A) Total liquid intake in Experimental rats (receiving Water/Oxycodone beginning Week 3) analyzed via restricted mixed-effects model (Sex x Time) revealed a significant interaction, F(15,222)=2.942, ***p=0.0003**, no main effect of Sex, F(1,15)=0.7735, p=0.3930, and a significant effect of Time, F(15, 222)=4.153, ****p<0.0001**. Post-hoc analyses within each Sex showed that compared to Week 1, males did not differ over time in their liquid intake, whereas females increased their total intake over time (***a;*** Week 1 vs. Week 18 ***p*=0.0035**, Week 1 vs. Week 19 ***p*=0.0049**, Week 1 vs. Week 20 ***p*=0.0010**, Week 1 vs. Week 21 ***p*=0.0028**, Week 22 ***p*=0.0001**). Post-hoc analysis of total intake between females and males showed no difference at any given week.

(B) Oxycodone preference of Experimental rats analyzed via a restricted mixed-effects model (Time x Sex) revealed a significant interaction F(15,222)=1.990, ***p*=0.00170**, no main effect of Sex F(1,15)=0.6337, p=0.4384, and a significant effect of Time F(15, 222)=22.78, ****p<0.0001**. Post-hoc comparison within Sex showed that both females and males increased their preference by Week 9 relative to Week 1 (females, ***a:*** Week 1 vs. Weeks 9-22 ***p*<0.0001**; males, ***b:*** Week 1 vs. 9-22 ***p*<0.0001**). When the bottle position was switched, post-hoc analyses revealed both females and males maintained their preference for the oxycodone bottle (Week 11 vs. all the other post-bottle switch weeks did not differ). Male rats then escalated their preference for oxycodone by Week 17 relative to Week 11 (***c:*** Week 11 vs. 17 ***p*=0.0112**, vs. 18 ***p*=0.0026**, vs. 19 ***p*=0.0095**, vs. 20 ***p*=0.0007**, vs. 21 ***p*=0.0019**, vs. 22 ***p*=0.0056**). The inset shows the position of water and oxycodone (**Oxy**) bottles in Experimental rat cages until Week 11 and after bottle position switch at Week 12.

(C) Analysis of self-administered dose via a mixed-effects model (Time x Sex) revealed no significant interaction F(15,222)=1.711, p=0.0502, a significant main effect of Sex

F(1,15)=18.72, ***p=**0.0006**, and a significant effect of Time F(15, 222)=10.04, ****p<**0.0001**. Post-hoc analysis within same sex group showed that female rats self-administered oxycodone at a higher dose from Week 9 on compared to Week 1 (***a:*** Week 1 vs 10 p=**0.0002**, Week 1 vs 12 p=**0.0030**, Week 1 vs 13 p=**0.0002**, Week 1 vs 13 p=**0.0001**, and all the other individual weeks compared to Week 1 had p<**0.0001**). Males self-administered oxycodone at a higher dose from Week 14 on compared to Week 1 (***b:*** Week 1 vs 14 p=**0.0140**, week 1 vs 15 p=**0.0065**, week 1 vs 16 p=**0.0041**, week 1 vs 17 p=**0.0015**, week 1 vs 18 p=**0.0012**, week 1 vs 19 p=**0.0003**, week 1 vs 20 p=**0.0026**, week 1 vs 21 p=**0.0067**, week 1 vs 22 p=**0.0013**). Post-hoc analysis of oxycodone dose of female compared to male rats at each time point showed a difference at Weeks 9 to 12 (***c:*** Week 9 p=**0.0004**, Week 10 p=**0.0017**, Week 11 p<**0.0001**, Week 12 p=**0.0500**), Weeks 17 and 18 (***c:*** Week 17 p=**0.0120**, Week 18 p=**0.0062**), and Weeks 20 to 22 (**c:** Week 20 p=**0.0184**, Week 21 p=**0.0204,** Week 22 p=**0.0054**).

(D) Analysis via two-tailed unpaired t-test of oxycodone blood levels between female and male rats averaged over the two blood draws at Weeks 15 and 21 revealed a significant difference between sexes (***p=**0.0003**).

(E) Correlation analysis of the blood levels with the intake 12 hours prior blood collection revealed a significant positive correlation in males with the slope of the linear regression of 0.8874, r^2=0.5738, and p=**0.0181**, whereas for females the slope of the linear regression was -4.458, r^2=0.1965, and p=0.2320.

<u>Figure 3.</u> Gnawing behavior and withdrawal signs are increased in experimental rats - particularly in females - relative to control rats

(A) Individual spontaneous withdrawal sign analysis via two-way ANOVA (Sex x Treatment) revealed no interaction and no effect of Treatment or Sex for frequently occurring behaviors:

Burrow (interaction, F(1,19)=3.935^{e-006}, *p*=0.9984; Sex, F(1,19)=1.537, *p*=0.2302; Treatment, F(1,19)=3.396, *p*=0.0810), **Teeth chatter** (interaction, F(1,19)=1.547, *p*=0.2287; Sex, F(1,19)=0.05031, *p*=0.8249; Treatment, F(1,19)=0.01040, *p*=0.9199), **Stretching** (interaction, F(1,19)=4.006, *p*=0.0598; Sex, F(1,19)=0.2305, *p*=0.6366; Treatment, F(1,19)=0.1310, *p*=0.7214), **Jumping** (interaction, F(1,19)=0.001045, *p*=0.9745; Sex, F(1,19)=0.1455, *p*=0.7071; Treatment, F(1,19)=3.161, *p*=0.0914). Two-way ANOVA of the **Total** spontaneous withdrawal signs also showed no interaction F(1,19)=0.1533, *p*=0.6997 or effect of Sex F(1,19)=0.5439, *p*=0.4698 or Treatment F(1,19)=3.651, *p*=0.0712.

(B) The sum of total amount in grams of chewed nylabone and wooden block averaged over 11 weeks (Weeks 12 to 22). Two-way ANOVA (Treatment x Sex) revealed an interaction F(1,19)=5.106, ***p=0.0358**, an effect of Sex F(1,19)=5.499, ***p=0.0300**, and an effect of Treatment F(1,19)=14.39, ****p=0.0012**. Post-hoc analysis within and between Sex revealed experimental females gnawed more than control females ****p=0.0026**, control males ****p=0.0023** and experimental males ****p=0.0015**.

(C) Individual precipitated withdrawal signs analysis via two-way ANOVA (Sex x Treatment) revealed no interaction and no effect of Sex but a significant effect of Treatment for the following behaviors: **Burrow** (interaction, F(1,19)=0.2538, *p*=0.6202; Sex, F(1,19)=1.821, *p*=0.1931; Treatment, F(1,19)=7.000, ***p=0.0159**); **Teeth chatter** (interaction, F(1,19)=0.0002038, *p*=0.9645; Sex, F(1,19)=0.1217, *p*=0.7311; Treatment, F(1,19)=10.06, ****p=0.0050**); **High walking** (interaction, F(1,19)=1.797, *p*=0.1959; Sex, F(1,19)=1.797, *p*=0.1959; Treatment, F(1,19)=6.634, ****p=0.0185**); **Diarrhea** (interaction, F(1,19)=1.121, *p*=0.3030; Sex, F(1,19)=1.121, *p*=0.3030; Treatment, F(1,19)=12.10, ****p=0.0025**). Between treatment group post-hoc analysis showed more diarrhea in experimental females compared to control females (****p=0.0097**). However, no interaction and no effect of Sex or Treatment was found in **Stretching** (interaction, F(1,19)=0.4660, *p*=0.5031; Sex, F(1,19)=3.775,

p=0.0670; Treatment, F(1,19)=0.7970, *p*=0.3832). Analysis of the **Total** withdrawal signs via Two-way ANOVA showed no interaction F(1,19)=0.1784, *p*=0.6805, no effect of Sex F(1,19)=1.248, *p*=0.2779 but a significant effect of Treatment F(1,19)=21.73, ******p*0.0002**. Post-hoc analysis between treatment groups showed a higher total withdrawal score in experimental females and males compared to their respective same sex controls (****p=0.0041** and ***p=0.0139**, respectively).

(D) Weight loss after precipitated withdrawal analyzed via two-way ANOVA (Sex x Treatment) revealed no interaction F(1,19)=0.5872, *p*=0.4529, no Sex effect F(1,19)=0.01726, *p*=0.8969, but a Treatment effect F(1,19)=4.664, ***p=0.0438**.

<u>Figure 4.</u> Citric acid suppressed drinking in all rats, but experimental - but not control - rats returned to baseline preference

(A) Schematic of home cage layout during the citric acid (**CA**) test. Control rats (left panel) received a water bottle in the front and a water bottle with increasing concentrations of CA every third day (1, 3, and 5 mM) in the back. Experimental rats (right schematic, shaded) received a water bottle in the front and an oxycodone bottle (**Oxy**) with increasing concentrations of CA every third day (1, 3, and 5 mM) in the back. The 5mM concentration of CA was repeated twice (5 and 5'). CA days were interspersed with "baseline" days (**BL0, BL1, BL3, BL5, BL5'**).

(B) Preference for the back bottle of Control and Experimental rats analyzed via two-way RM ANOVA (CA concentration x Treatment) revealed a significant interaction F(24,152=1.621, ***p=0.0432**, significant effect of CA concentration F(8,152)=26.5, ********p<0.0001**, and a significant effect of Treatment F(3, 19)=4.806, ***p=0.0118**. Post-hoc analysis of the preference changes over increasing concentrations of CA revealed Experimental males suppressed at 3mM CA (**b: *p=0.0275**) compared to the initial preference when there was no

citric acid (CA concentration=0). All animals significantly suppressed intake at both presentations of the 5mM concentration: Experimental females (***a*:** 5, ****p*=0.0018** and 5' ******p*<0.0001**); Control females (***c*:** 5, ****p*=0.0041** and 5' ******p*=0.0022**); Control males (***d*:** 5, *****p*=0.0003** and 5'**, ******p*=0.0041**); Experimental males (***b*:** 5 and 5' ******p*<0.0001**).

(C) Analysis of change from initial baseline (**BL0**) via a two-way RM ANOVA (Return to BL_0 x Treatment) revealed a significant interaction F(12,76)=3.851, ********p*=0.0001**, an effect of Return to BL_0 F(4, 76)=9.591, ********p*<0.0001**, and no effect of Treatment F(3,20)=1.784, *p*=0.1825. Post-hoc analysis showed that Control female and male rats maintained their aversion (***a*:** Control females BL5, ****p*=0.0151**; BL5' ******p*=0.0046**; **b:** Control males BL5',******** ***p*<0.0001**).

<u>Figure 5.</u> Microbiome comparison showed differences in relative abundance of bacterial composition and alpha diversity between treatment groups and the gut microbiome composition were more similar within treatment groups than between treatment groups.

(A) Relative abundance of bacterial phylum within individual rats. Each bar represents an individual and colors represent relative abundance of bacterial phylum.

(B) Analysis of relative abundance in control and oxycodone gut microbiota at Week 28 via a Mann-Whitney U test with false discovery rate method for adjustment of p-values revealed differences between Deferribacteres (**p=0.0012**), Proteobacteria (**p=0.011**) and Turnicutes (**p=0.013**) and no differences between Actinobacteria (**p=0.22**), Bacteroidetes (**p=0.81**), Cyanobacteria (**p=0.13**), Firmicutes (**p=0.07**), Lentisphaerae (**p=0.48**) and Verrucomicrobia (**p=0.55**) .

(C) Kruskall-Wallis analyses comparing alpha diversity among Week -1 vs. Week 28 in Control and Oxycodone rats showed a significant difference (********p*=0.0001743**). Post-hoc analysis showed that alpha diversity was similar between Week -1 Control and Week -1

Oxycodone (**p=0.6631**), Week -1 Control and Week 28 Control (**p=0.5569**), Week -1 Oxycodone and Week 28 Control (**p=0.9192**) and differed between Week -1 Control and Week 28 Oxycodone (**p=0.0026**), Week 28 Control and Week 28 Oxycodone (**p=0.0563**) and Week -1 Oxycodone and Week 28 Oxycodone (**p=0.0005**).

(D) Bray Curtis Dissimilarity and principal coordinates analysis (PCoA) revealed gut microbial composition are more similar within treatment groups than between treatment groups (PERMANOVA R^2 = 0.19098; **p = 2e-04 ***).

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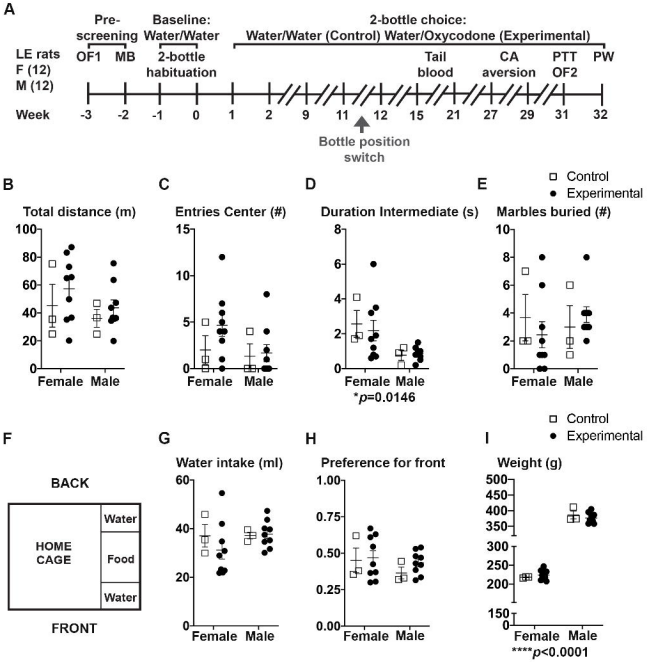
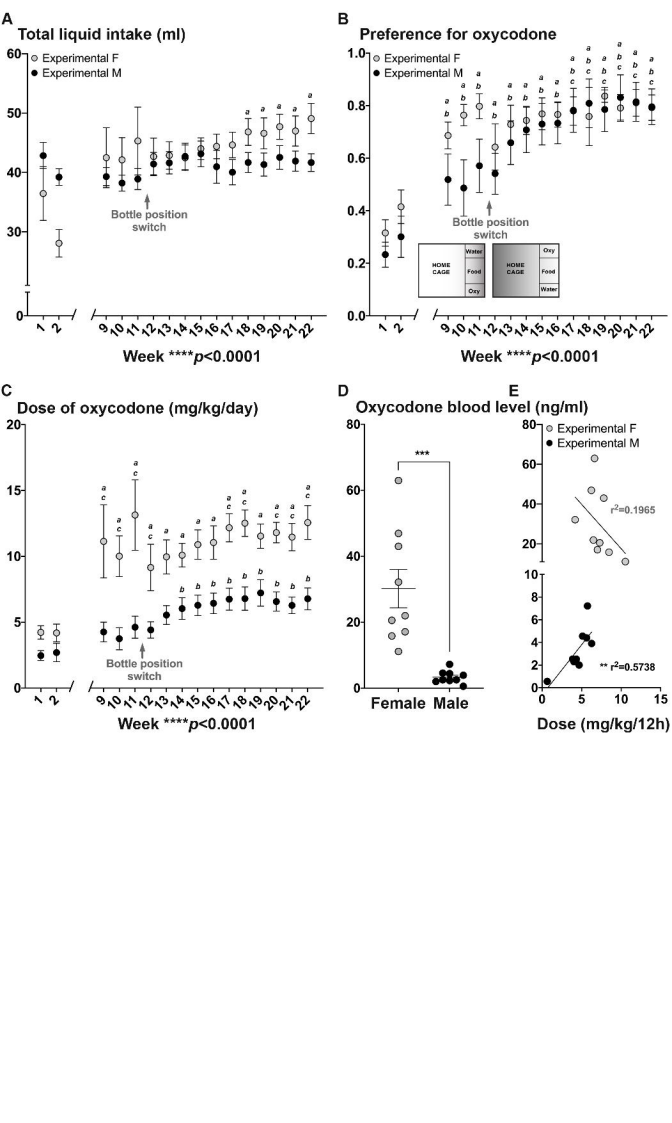
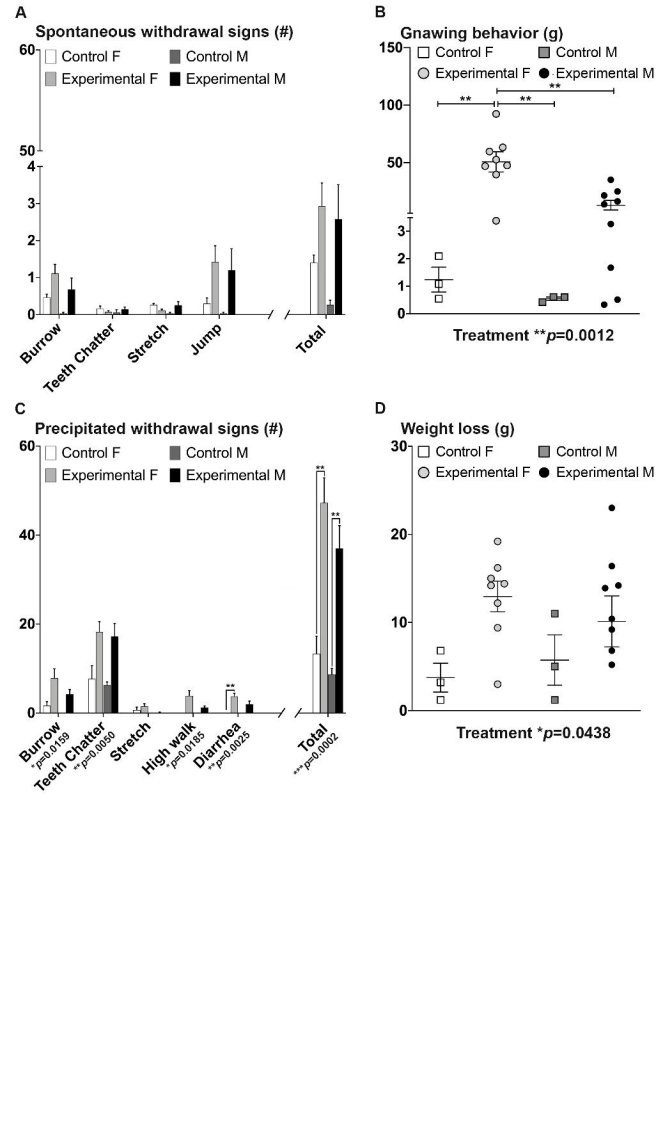
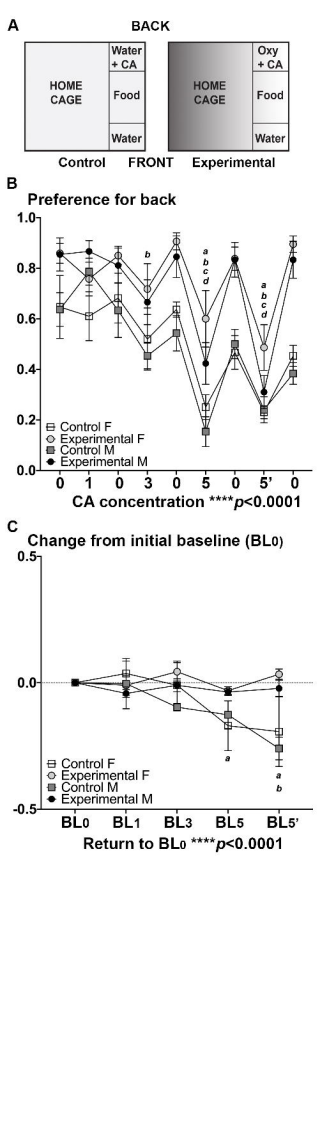
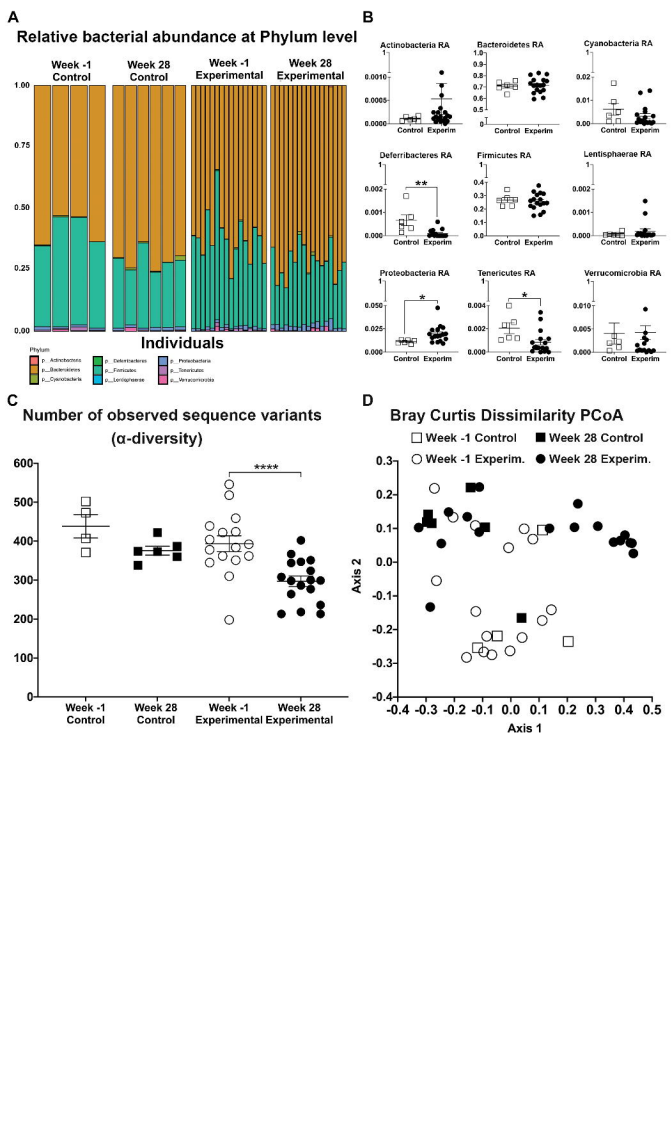

A
Pre-screening
Baseline: Water/Water
2-bottle choice: Water/Water (Control) Water/Oxycodone (Experimental)
LE rats
F (12)
M (12)
OF1
MB
2-bottle habituation
Tail blood
CA aversion
PTT OF2
PW
Week
-3
-2
-1
0
1
2
9
11
12
15
21
27
29
31
32
Bottle position switch
B
Total distance (m)
Female
Male
C
Entries Center (#)
D
Duration Intermediate (s)
*p=0.0146
E
Control
Experimental
Marbles buried (#)
F
BACK
HOME CAGE
Water
Food
Water
FRONT
G
Water intake (ml)
H
Preference for front
I
Weight (g)
****p<0.0001


A
Total liquid intake (ml)
Experimental F
Experimental M
Bottle position switch
Week ****p<0.0001
B
Preference for oxycodone
Experimental F
Experimental M
Bottle position switch
HOME CAGE
Water
Food
Oxy
Week ****p<0.0001
C
Dose of oxycodone (mg/kg/day)
Bottle position switch
Week ****p<0.0001
D
Oxycodone blood level (ng/ml)
***
Female
Male
E
Experimental F
Experimental M
r2=0.1965
** r2=0.5738
Dose (mg/kg/12h)


A
Spontaneous withdrawal signs (#)
Control F
Control M
Experimental F
Experimental M
Burrow
Teeth Chatter
Stretch
Jump
Total
B
Gnawing behavior (g)
Control F
Control M
Experimental F
Experimental M
Treatment **p=0.0012
C
Precipitated withdrawal signs (#)
Control F
Control M
Experimental F
Experimental M
Burrow
*p=0.0159
Teeth Chatter
**p=0.0050
Stretch
High walk
*p=0.0185
Diarrhea
**p=0.0025
Total
***p=0.0002
D
Weight loss (g)
Control F
Control M
Experimental F
Experimental M
Treatment *p=0.0438


A
BACK
HOME CAGE
Water + CA
Food
Water
HOME CAGE
Oxy + CA
Food
Water
Control
FRONT
Experimental
B
Preference for back
1.0
0.8
0.6
0.4
0.2
0.0
b
a b c d
a b c d
Control F
Experimental F
Control M
Experimental M
0 1 0 3 0 5 0 5' 0
CA concentration ****p<0.0001
C
Change from initial baseline (BL0)
0.5
0.0
-0.5
a
a b
Control F
Experimental F
Control M
Experimental M
BL0 BL1 BL3 BL5 BL5'
Return to BL0 ****p<0.0001


A
Relative bacterial abundance at Phylum level
Week -1 Control
Week 28 Control
Week -1 Experimental
Week 28 Experimental
1.00
0.75
0.50
0.25
0.00
Individuals
Phylum
p__Actinobacteria
p__Bacteroidetes
p__Cyanobacteria
p__Deferribacteres
p__Firmicutes
p__Lentisphaerae
p__Proteobacteria
p__Tenericutes
p__Verrucomicrobia
B
Actinobacteria RA
Bacteroidetes RA
Cyanobacteria RA
Deferribacteres RA
Firmicutes RA
Lentisphaerae RA
Proteobacteria RA
Tenericutes RA
Verrucomicrobia RA
Control
Experim
**
*
*
C
Number of observed sequence variants
(α-diversity)
600
500
400
300
200
100
0
****
Week -1 Control
Week 28 Control
Week -1 Experimental
Week 28 Experimental
D
Bray Curtis Dissimilarity PCoA
Week -1 Control
Week 28 Control
Week -1 Experim.
Week 28 Experim.
Axis 2
Axis 1
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.4 -0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4 0.5