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# Novel Epigenetic Therapeutics for the Treatment of Opioid Use Disorder

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University Scholar Thesis

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**Abstract**

Epigenetic-based therapeutics are promising treatment options for Substance Use Disorder (SUD) due to their ability to influence gene expression and reverse behavioral responses associated with addiction. For example, previous studies have shown that epigenetic reader proteins, called Bromodomains and Extra Terminal Domain (BET), are increased in the nucleus accumbens following cocaine self-administration and that pharmacological inhibition of BET proteins reduced cocaine-seeking behaviors in rodents. Although BET inhibitors reduced cocaine-seeking behaviors in animal models, a role for BET proteins in animal models of Opioid Use Disorder (OUD) remains unknown. To identify a role for BET proteins in OUD, we measured time-dependent changes in BET mRNA expression in the prefrontal cortex (PFC), Nucleus Accumbens (NAc), and dorsal striatum (DS) following oral oxycodone self-administration. In these experiments, female and male mice received ad lib home cage water bottles with or without oxycodone (0.5 mg/ml) for 10 consecutive days. The volume consumed and the weight of the mice were recorded at the same time each day. At the end of the 10th day, all mice were given regular drinking water. After 4 h, 24 h, or 7 days of abstinence, expression of BET genes (*Brd2*, *Brd3*, and *Brd4*) were measured via quantitative polymerase chain reaction (qPCR). Results showed that mice consumed more oxycodone water compared to regular water and that oxycodone consumption (mg/kg and ml/g) in females was higher compared to males. In qPCR studies, time-dependent changes in BET mRNA expression in PFC, NAc, and DS was observed following abstinence. In naloxone-precipitated withdrawal behavioral experiments, global withdrawal scores were significantly elevated in mice that consumed oxycodone. Ongoing research will determine if BET inhibitors reduce oxycodone consumption and/or oxycodone-induced withdrawal-like behaviors. Results from these experiments may lead to more effective therapies for the treatment of OUD.

## Introduction

*Opioid Use Disorder*

Opioid Use Disorder (OUD) in the United States remains an immense public health issue. The Substance Abuse and Mental Health (SAMHSA) report estimates that about 1.6 million or 0.6% of the US population in 2019 had OUD [1]. With an increase in illicit drug-related overdoses and limited treatment options, there is a dire need for therapeutic innovations for OUD [2–4]. Oxycodone is a semi-synthetic opioid analgesic and a u-opioid receptor agonist commonly prescribed to treat severe pain [7], and it has played a major role in the opioid crisis and overdose-related deaths [5,6]. Although prescription regulations and guidelines have been improved, over prescribing oxycodone continues to be a risk factor for developing OUD [8]. Currently, most OUD treatments are aimed at preventing overdose, but few are effective at reducing relapse and craving.

*Epigenetic pharmacology for OUD*

To date, there has been little therapeutic innovation in the field of OUD, as most FDA-approved pharmacological treatments for OUD are based on the neurotransmitter receptor agonist/antagonist that was identified over 40 years ago [9–11]. However, OUD is more complex than changes in neurotransmission, and new multifaceted treatment options are urgently needed. Growing evidence indicates that epigenetics, a reversible change in gene expression occurring independently of DNA sequence and often mediated by chromatin structure alterations, play a role in drug-induced neuroadaptations [12]. Epigenetic modifications arise in the nucleosome, which is the fundamental subunit of chromatin and is composed of 147 base pairs of DNA wrapped around an octamer of histone proteins. Multiple sites exist for potential post-translational modifications on the amino-terminal tail of each histone subunit, including acetylation, methylation, phosphorylation, and ubiquitination [13]. Each reversible modification is added ('writers'), removed ('erasers'), or read ('readers') by a particular set of histone-modifying proteins. For example, acetyl groups on histone proteins are added by Histone Acetyltransferases (HATs), removed by Histone Deacetylases (HDACs), or read by bromodomain (BRD) proteins. By altering chromatin structure, these post-translational modifications can dramatically influence gene expression that could ultimately contribute to the etiology of many disorders, such as OUD.

Recent studies indicate that the 'readers' of histone acetylation, known as Bromodomain Extra Terminal (BET) proteins, play an essential role in the rewarding properties of cocaine [12–15]. The BET family of proteins, including somatic bromodomain proteins, BRD2, BRD3, BRD4, and testes-specific BRDT, bind to acetylated histones, recruit protein complexes, and function as co-

activators or co-repressors of gene transcription [16–18]. With the previous development of selective, small-molecule inhibitors of BET proteins (e.g., JQ1) [19–21], there has been increasing interest in their therapeutic utility [22–25], as evidenced by several recent ongoing clinical trials in cancer [26–29]. In cocaine-seeking behaviors, our lab has found that the BET inhibitor JQ1 reduces molecular and behavioral responses to cocaine [30]. However, the role of pharmacological BET inhibition in animal models of OUD remains obscure. The purpose of this proposal is to examine the role of BET proteins in OUD.

*Preclinical models of OUD*

Animal models of opioid addiction are important for understanding the etiology the disease and for testing novel therapeutics. Several models have been developed to study certain aspects of drug addiction processes, including drug intake, withdrawal, abstinence, and relapse [31]. To study opioid-induced withdrawal-like behaviors, researchers typically use a procedure called naloxone-induced precipitated withdrawal. Naloxone is a μ-opioid receptor antagonist which accelerates withdrawal by competing against the opioid drug for the same opioid receptor sites [32]. Treating opioid-dependent animals with naloxone produces somatic signs of withdrawal such as, paw tremors, jumps, generical grooming, wet dog shakes, rearing, and writhing [24,31,33,34]. Using this model, researcher may identify novel therapeutics that reduce withdrawal-like behavior, which could ultimately be translated to opioid dependence in humans.

Many animal models of OUD are focused on intravenous administration; however, few studies have observed the effects of oral opioid intake on addiction-like behaviors. In many instances, opioid dependence is initiated by oral oxycodone prescription, which emphasizes the importance of developing animal models that include this route of administration [35]. For my honors and University Scholar thesis project, I will investigate the role of BET gene expression in oxycodone-induced withdrawal-like behaviors.

## Materials and Methods

*Animals*

Adult male and female C57BL/6 mice (8-10 weeks old, Charles River) were singled housed in a temperature- controlled vivarium under a reverse 12h/12h light/dark cycle (lights off at 9am) and had ad libitum access to food and water. All animal studies have been approved by the UConn Institutional Animal Care and Use Committee (IACUC).

*Drug Treatments*

After a one-week habituation period, half the animals received water with oxycodone hydrochloride (0.5 mg/ml, Sigma) and the other half were maintained on regular drinking water for 10 consecutive days. An empty cage without a mouse was used as a control for bottle leakage. At the same time each day (9 AM), the weight of the mouse and water bottle were recorded. At the end of the 10 days, the oxycodone water was removed and replaced with regular water.

*qRT-PCR*

After 4 hours, 24 hours, and 7 days of forced abstinence, mice were euthanized and *Brd2*, *Brd3* and *Brd4* gene expression were measured in multiple brain regions. Frozen brains were sectioned on a cryostat and the prefrontal cortex (PFC), nucleus Accumbens (NAc), and dorsal striatum (DS) were dissected via tissue punch. RNA was isolated using a Trizol and RNA extraction kit as described by the manufacturer (QIAGEN). RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) and expression was measured using validated Taqman primer probes for *Brd2*, *Brd3*, and *Brd4.* Results were analyzed using $2^{-\Delta\Delta CT}$ method with beta actin as the normalization control. A no template control was used as a negative control.

*Elevated Zero Maze*

The Elevated Zero Maze consisted of a ring-shaped acrylic apparatus elevated 400 mm off the floor. Its outer diameter was 460 mm with an arm width of 55 mm. The ring contains four quadrants of equal lengths, two were closed and surrounded by 110 mm high walls, 5 mm ledges, and the remaining quadrants were open. Each mouse was placed on an open quadrant at the start of their 5 minutes session. Time spent in the open and closed quadrant was recorded using EthoVision video tracking software.

*Open Field*

The open field (Ugo Basile) consisted of a 46 × 46 cm chamber with opaque gray walls (40 cm high). Each mouse was allowed to move freely in the chamber for 30 minutes. Internal time zone and out of zone as well as total distance travelled were recorded and analyzed using EthoVision video tracking software.

*Naloxone-induced Precipitated Withdrawal*

Mice received 10 days of water with or without oxycodone. Following 3 days of abstinence, mice were injected with naloxone (10 mg/kg, i.p.) and placed in a clear plexiglass box where their behavior was recorded for 30 minutes. Weight (before and after injection), defecation, and somatic signs of withdrawal (jumps, wet dog shakes, paw tremors, rearing, genital grooming, face grooming, hind limb scratches, and writhing) were scored by an experimenter blinded to the treatment conditions. The sum of the somatic signs of withdrawal were used to calculate the global withdrawal score as previously described [31].

*Data Analysis*

GraphPad Prism software was used for graph construction and statistical analyses. Relative quantification (Rq) values from qRT-PCR experiments and behavioral data were compared between groups using Student's T-test or Analysis of Variance (ANOVA). When a significant F value is obtained, comparisons will be performed using Bonferroni post hoc analysis; level of significance was set to $P < 0.05$.

**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 volume intake were measured at the same time each day **(Figure 1).** Average intake (mL) was higher in the oxycodone group compared to the regular water group (one-way ANOVA main effect: $F_{(2,79)} = 243.8$, $P < 0.0001$) **(Figure 2A)**. Similar findings were observed in mL/g consumption ($t_{68} = 2.18$, $P < 0.05$) (**Figure 2B**). Minimal leakage was observed in water bottles in cages without a mouse (**Figure 2A**). When comparing consumption (mL/g) between sexes, females consumed more water and oxycodone water compared to males. Within females, however, there was no significant differences between conditions, but within males, oxycodone intake was higher compared to regular water (Condition factor: $F_{(1, 66)} = 9.852$, $P < 0.01$; Sex factor: $F_{(1,66)} = 58.03$, $P < 0.0001$; Interaction: $F_{(1, 66)} = 1.344$, $P = 0.2504$) (**Figure 2C)**. For consumption by (mg/kg) females consumed more oxycodone compared to males ($t_{33} = 4.219$, $P < 0.001$) **(Figure 2D)**. Figures 2E – 2G show intake across days in in males and females grouped together and separated. When measuring oral consumption by sex combined each day, we found a significant effect based on drinking (Two-way ANOVA: Condition $F_{(1,677)} = 30.81$, $P < 0.0001$; Day $F_{(9,677)} = 2.437$, $P < 0.01$; Interaction: $F_{(9, 677)} = 1.845$, $P = 0.0573$). Females had a conditional difference between drinking water and

oxycodone (mL/g) each day (Two-way ANOVA: Condition $F_{(1,327)} = 7.736$, $P < 0.01$; Day $F_{(9,327)} = 1.554$, $P = 0.1282$; Interaction: $F_{(9,327)} = 1.493$, $P = 0.1491$) (**Figure 2F)**. Males had a significance interaction, days, and conditional difference between drinking water and oxycodone (mL/g) each day (Two- way ANOVA: Condition: $F_{(1,330)} = 80.51$, $P < 0.0001$; Day: $F_{(9.330)} = 3.838$, $P < 0.0001$; Interaction: $F_{(9,330)} = 1.971$, $P < 0.05$) **(Figure 2G).**

*BET gene expression in the NAc, PFC and DS following oral oxycodone consumption*

To examine the effects of oxycodone on BET gene expression, tissue punches of the NAc were collected at different time points following 10 days of home cage intake**.** *Brd2* expression was significantly increased after 4 h of abstinence ($t_{2.2} = 14$, $P < 0.05$) **(Figure 3A)**, but not after 24-hour ($t_{0.6} = 12$, $P = 0.5769$) **(Figure 3D)** or 7-days of abstinence ($t_{0.8} = 12$, $P = 0.4370$) **(Figure 3G)**. *Brd3* levels in were significantly increased after 24-hour ($t_{2.5} = 12$, $P < 0.05$) (**Figure 3E**) and 7-day ($t_{2.6} = 12$, $P = 0.0213$) (**Figure 3H**) of abstinence but not after 4 hours of abstinence ($t_{0.7} = 14$, $P = 0.4768$) **(Figure 3B)**. *Brd4* expression was not altered in the NAc after 4 hours ($t_{1.4} = 14$, $P = 0.1825$) **(Figure 3C)** and 24-hours of abstinence ($t_{0.6} = 12$, $P = 0.5806$) **(Figure 3F),** but it was significantly increased after 7 days of abstinence in the oxycodone group ($t_{2.3} = 12$, $P < 0.05$) **(Figure 3I).**

The effects of oxycodone on BET gene expression in the NAc by sex was examined (**Figure 4**). After 4 hours of abstinence, *Brd2* was significantly increased in males compared to females that consumed oxycodone ($t_{3.9} = 6$, $P < 0.001$) **(Figure 4A)**. For *Brd2*, gene expression in the 24hr ($t_{1.6} = 6$, $P = 0.1721$) **(Figure 4D)** and 7-day timepoint ($t_{1.3} = 9$, $P = 0.2278$) **(Figure 4G)** showed no significance*.* After 4 hours of abstinence, *Brd3* was significantly increased in males compared to females that consumed oxycodone ($t_{2.5} = 6$, $P < 0.05$) **(Figure 4B)**, but not in the 24-hour ($t_{0.2} = 6$, $P = 0.882$) **(Figure 4E)** and 7-day ($t_{0.9} = 9$, $P = 0.3764$) **(Figure 4H)** time point of abstinence. After 4 hours of abstinence, *Brd4* was significantly increased in males compared to females that consumed oxycodone ($t_{3.3} = 6$, $P < 0.05$) **(Figure 4C),** but not at the 24-hour ($t_{0.3} = 6$, $P = 0.7497$) **(Figure 45)** and 7-day ($t_{0.4} = 9$, $P = 0.7108$) **(Figure 4I)** time point of abstinence.

In the PFC, no significant changes in *Brd2* and *Brd3* were observed at the time points measured (p values > 0.05) (*Brd*2: Figures 5A, 5D, 5G; *Brd*3: Figures 5B, 5E, 5H). Expression of *Brd4* was not changed following 4-hour and 24 hours of abstinence (p values > 0.05) **(Figure 5C)**, but it was significantly elevated following 24 hours of abstinence ($t_{2.3} = 12$, $P = 0.05$) **(Figure 5F)**

When comparing sex-dependent expression of BET in the PFC, *Brd2* expression was not significantly different after 4 hour ($t_{0.8}$ = 4, P < 0.4537) and 7 days of abstinence ($t_{1.9}$ = 5, P = 0.1158) (Figure 6G), **(Figure 6A),** but it was elevated following 24-hours of abstinence ($t_{8.5}$ = 6, P < 0.0001) **(Figure 6D).** After 4 hours of abstinence ($t_{0.1}$ = 4, P = 0.8916) (**Figure 6B)**, and 7 days of abstinence there were no significant changes in *Brd3* expression ($t_{0.8}$ = 5, P = 0.4602) **(Figure 6H)**. However, *Brd3* expression was increased in the males compared to the females after 24 hours of abstinence ($t_{7.0}$ = 6, P < 0.001) **(Figure 6E)**. For *Brd4* no significant change was observed for 4-hour ($t_{0.1}$ = 4, P = 0.9332) **(Figure 6C)**, 24-hour ($t_{0.7}$ = 6, P < 0.5084) **(Figure 6F)**, and 7-day time points ($t_{1.2}$ = 5, P = 0.2765) **(Figure 6I).**

In the DS, no changes in *Brd2* expression were observed at 4 hours ($t_{0.9}$ = 13, P = 0.3916) **(Figure 7A)**, 24-hours ($t_{1.6}$ = 13, P = 0.1422) **(Figure 7D)**, and 7-days ($t_{0.1}$ = 12, P < 0.9041) **(Figure 7G)** of abstinence. *Brd3* expression was increased in the oxycodone group compared to the water group following 4-hours of abstinence ($t_{2.4}$ = 13, P = 0.0322) **(Figure 7B)**, but no significant changes were observed following 24 hour ($t_{0.1}$ = 13, P = 0.9522) **(Figure 7E)**, and 7-days of abstinence ($t_{1.0}$ = 12, P < 0.3565) **(Figure 7H)**. *Brd4* expression was not significantly changed following 4-hour ($t_{1.1}$ = 13, P = 0.2932) **(Figure 7C)**, 24-hour ($t_{1.2}$ = 13, P = 0.2469) **(Figure 7F)**, and 7-days ($t_{0.6}$ = 12, P = 0.5819) **(Figure 7I)** of abstinence.

When comparing males vs. females in the oxycodone group, no significant changes were observed in *Brd2* expression following 4 hours ($t_{2.2}$ = 6, P = 0.0714) **(Figure 8A)**, 24 hours ($t_{2.2}$ = 6, P = 0.0716) **(Figure 8D)**, and 7 days ($t_{1.3}$ = 5, P = 0.2416) **(Figure 8G)** of abstinence. For *Brd3* there were no observed changes in expression following 4 hours ($t_{0.9}$ = 6, P = 0.3883) **(Figure 8B)** and 24 hours ($t_{1.4}$ = 6, P = 0.2138) **(Figure 8E)** of abstinence; however, *Brd3* was increased in the male DS following 7-days of abstinence ($t_{3.4}$ = 5, P < 0.05) **(Figure 8H)**. For *Brd4*, there were no differences in gene expression for the 4-hour ($t_{1.0}$ = 6, P = 0.3482) **(Figure 8C)**, 24-hour ($t_{0.7}$ = 6, P = 0.5271) **(Figure 8F)**, and 7-day ($t_{0.2}$ = 5, P = 0.8811) **(Figure 8I)** timepoint of abstinence.

*Locomotor and anxiety-like behaviors following oral oxycodone consumption*

To examine the effects of oxycodone abstinence on anxiety-like behavior, male and female mice were tested in the elevated zero maze (EZM) and open field procedures. In the EZM experiments, time spent in the open and closed arms was not altered (two-way ANOVA: Interaction: $F_{(1,20)}$ = 0.8623, P = 0.3642; Sex: $F_{(1,20)}$ = 5.791, P < 0.05; Condition: $F_{(1,20)}$ = 10.95, P

< 0.01) **(Figure 9A)**, and there were no sex-dependent effects in EZM activity (three-way ANOVA: Condition: $F_{(1,40)} = 0.5006$, P = 0.4834; Sex: $F_{(1,40)} = 0.01234$, P = 0.9121; Arms: $F_{(1,40)} = 280.9$, P < 0.0001) **(Figure 9B).** Locomotor activity in the open field tests was increased in oxycodone group compared to in the water group ($t_{2.2} = 22$, P < 0.0383) **(Figure 9E).** Distance travelled by sex showed treatment differences (two-way ANOVA: Condition: $F_{(1, 20)} = 4.745$, P = 0.0415; Sex: $F_{(1, 20)} = 0.07379$, P = 0.7887; Interaction: $F_{(1, 20)} = 1.415$, P = 0.2482) **(Figure 9F)**, but no significant changes were observed in post hoc analysis.

*Naloxone-induced precipitated withdrawal behaviors following oral oxycodone consumption*

To examine the effects of oxycodone on withdrawal-like behaviors, male and female mice received an i.p. injection of naloxone (10 mg/kg) after 3 days of abstinence. Somatic signs of withdrawal were measured (hind limb scratching, jumps, wet dog shakes, grooming, writhing, rearing, paw tremors) for 30 minutes after naloxone treatment. Hind limb ($t_{0.8} = 22$, P = 0.4287) **(Figure 10A)** jumps ($t_{0.9} = 22$, P = 0.9278) **(Figure 10B)**, wet dog shakes ($t_{1.3} = 22$, P = 0.1930) **(Figure 10C)**, total grooming ($t_{0.2} = 22$, P = 0.8475) **(Figure 10D)**, writhing ($t_{1.4} = 22$, P = 0.1705) **(Figure 10E)** were not significantly changed in the oxycodone group compared to water. However, total rearing ($t_{2.6} = 22$, P < 0.05) **(Figure 10F)** and paw tremors ($t_{2.9} = 22$, P < 0.01) **(Figure 10G)** were significantly elevated in the oxycodone group compared to the water group.

For the somatic signs of withdrawal of hind limb scratching, jumps, wet dog shakes, total grooming, and writhing, there was no significant changes between sex, condition, and interaction (P > 0.05) (hindlimb scratching: **Figure 11A,** jumps: **Figure 11B,** wet dog shakes: **Figure 11C**; total grooming: **Figure 11D**, writhing **Figure 11E).** For total rearing an interaction and condition effect was revealed to be significant (two-way ANOVA: Condition: $F_{(1, 20)} = 8.140$, P < 0.01; Sex: $F_{(1, 20)} = 0.4792$, P = 0.4967; Interaction: $F_{(1, 20)} = 5.669$, P < 0.05). For paw tremors differences condition was noted (two-way ANOVA: Condition: $F_{(1, 20)} = 8.216$, P < 0.01; Sex: $F_{(1, 20)} = 0.5530$, P = 0.4657; Interaction: $F_{(1, 20)} = 0.3840$, P = 0.5425).

The global withdrawal score was calculated by summing all the somatic signs of withdrawal. There was a greater global withdrawal score in the oxycodone group compared to the water group ($t_{2.9} = 22$, P < 0.01) **(Figure 12A)**. When looking at global withdrawal score compared to males there was a significant interaction and condition effect (two-way ANOVA: Condition: $F_{(1, 20)} = 10.95$, P < 0.01; Sex: $F_{(1, 20)} = 0.8454$, P = 0.3688; Interaction: $F_{(1, 20)} = 8.772$, P < 0.01) **(Figure 12B)**. Defecation was also measured and mice in the oxycodone group had greater number of feces compared to water the ($t_{2.5} = 22$, P = 0.0206) **(Figure 13A)**. Number of feces by sex revealed

an interaction effect difference (two-way ANOVA: Condition: $F_{(1, 20)} = 0.2934$, $P = 0.5940$; Sex: $F_{(1, 20)} = 0.05389$, $P = 0.8188$; Interaction: $F_{(1, 20)} = 5.754$, $P < 0.05$) **(Figure 13B)**. Change in weight was measured before receiving the i.p. injection of naloxone and after the 30-minute trial run which revealed that mice in the oxycodone group lost more weight compared to water ($t_{3.2} = 22$, $P < 0.01$) **(Figure 13C)**. Change in weight by sex showed significance for condition effect (two-way ANOVA: Condition: $F_{(1, 20)} = 9.262$, $P < 0.01$; Sex: $F_{(1, 20)} = 0.1983$, $P = 0.6608$; Interaction: $F_{(1, 20)} = 0.08815$, $P = 0.7696$) **(Figure 13D)**.

## Figures

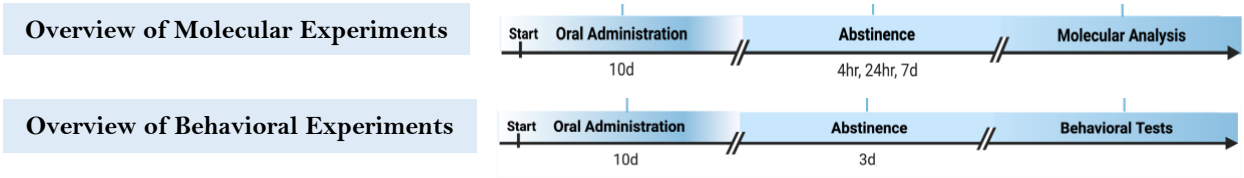


**Figure 1: Overview modules of Experiments**. Mice were assigned regular water or oxycodone water for 10 days (n = 35) (0.5mg/ml) of oral administration. For gene expression experiments, brain tissue was collected after 4hr, 24hr, and 7 days of abstinence. In behavioral studies, mice were tested after 3 days of abstinence.

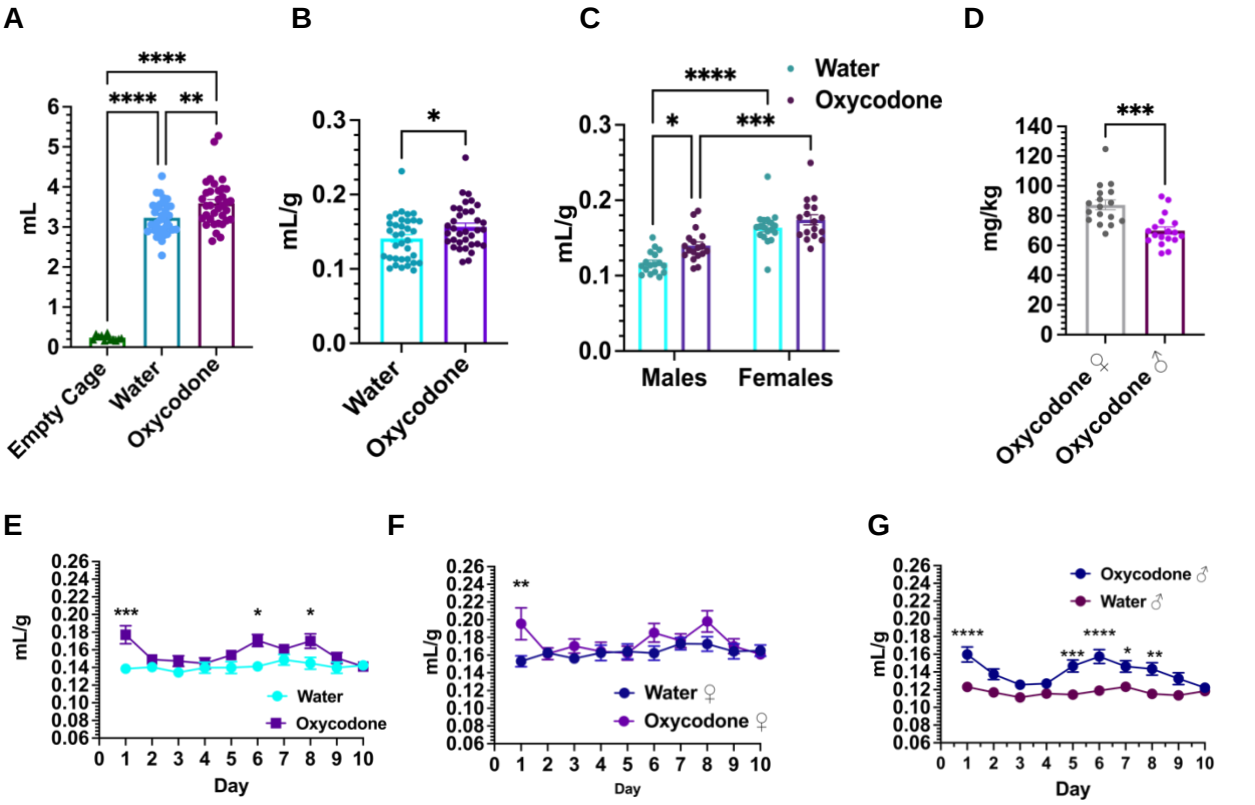


**Figure 2: Oral consumption of oxycodone for 10 days in male and female mice**. (A) Mice were assigned regular water (n = 35) or oxycodone water (n = 35) for 10 days (0.5mg/ml). Changes in volume (mL) were measured in each group and in an empty cage (n = 12). (B) Consumption in mL/g in all mice and (C) in mice separated by sex (n = 16 - 17). (D) Average oxycodone intake (mg/kg) in males and females (n = 16 - 17). (E) Daily intake of oxycodone and water (mL/g) in all mice and in mice separated by (F) females and (G) males.* P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.001 indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM.

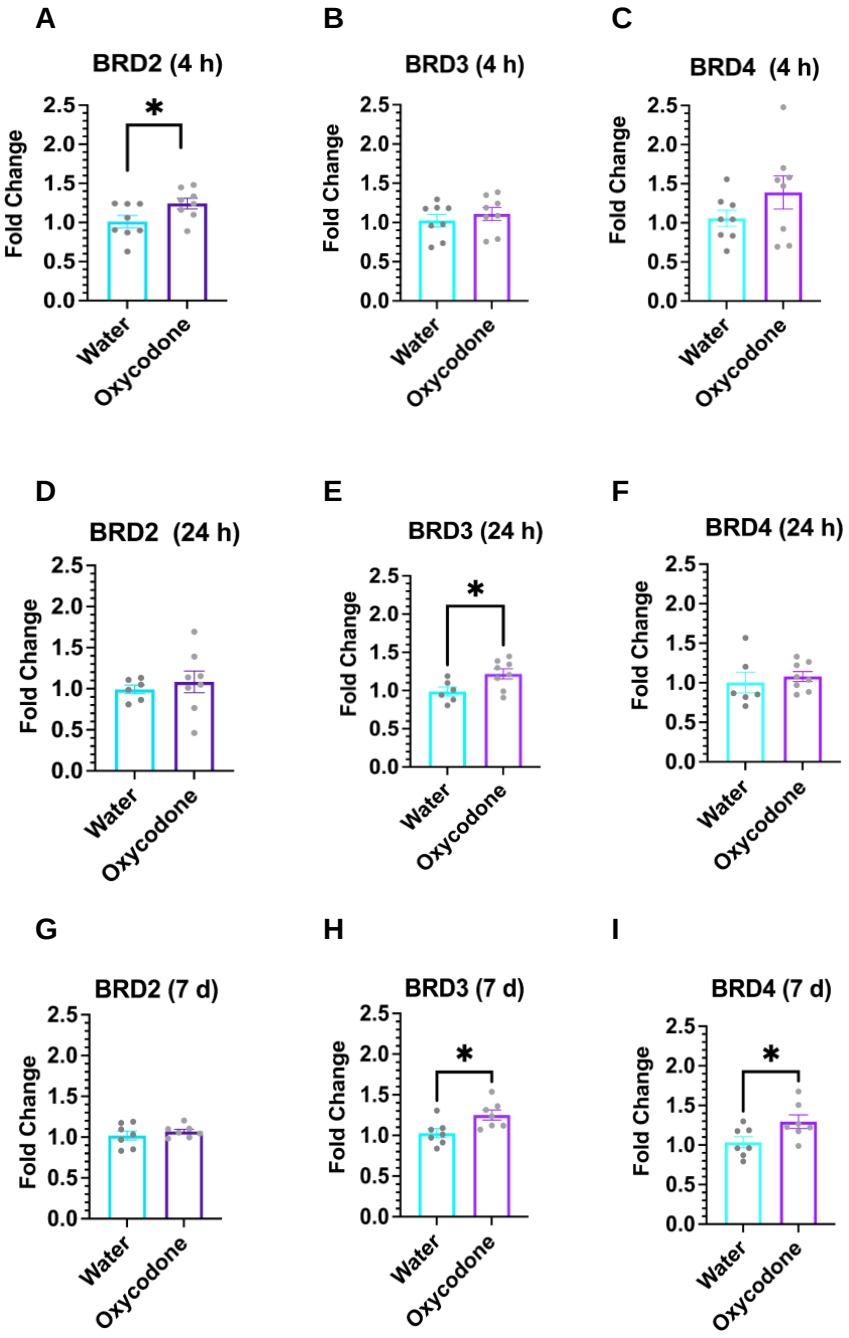


**Figure 3: Time-dependent changes in BET gene expression in the NAc**. Mice received regular water or oxycodone. Following oral administration for 10 days NAc brain tissue was collected and *Brd2, Brd3*, and *Brd4* expression levels were measured after (A-C) 4 h, (D-F) 24 h, and (G-I) 7 d of abstinence. * $P < 0.05$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM, n = 6-8.

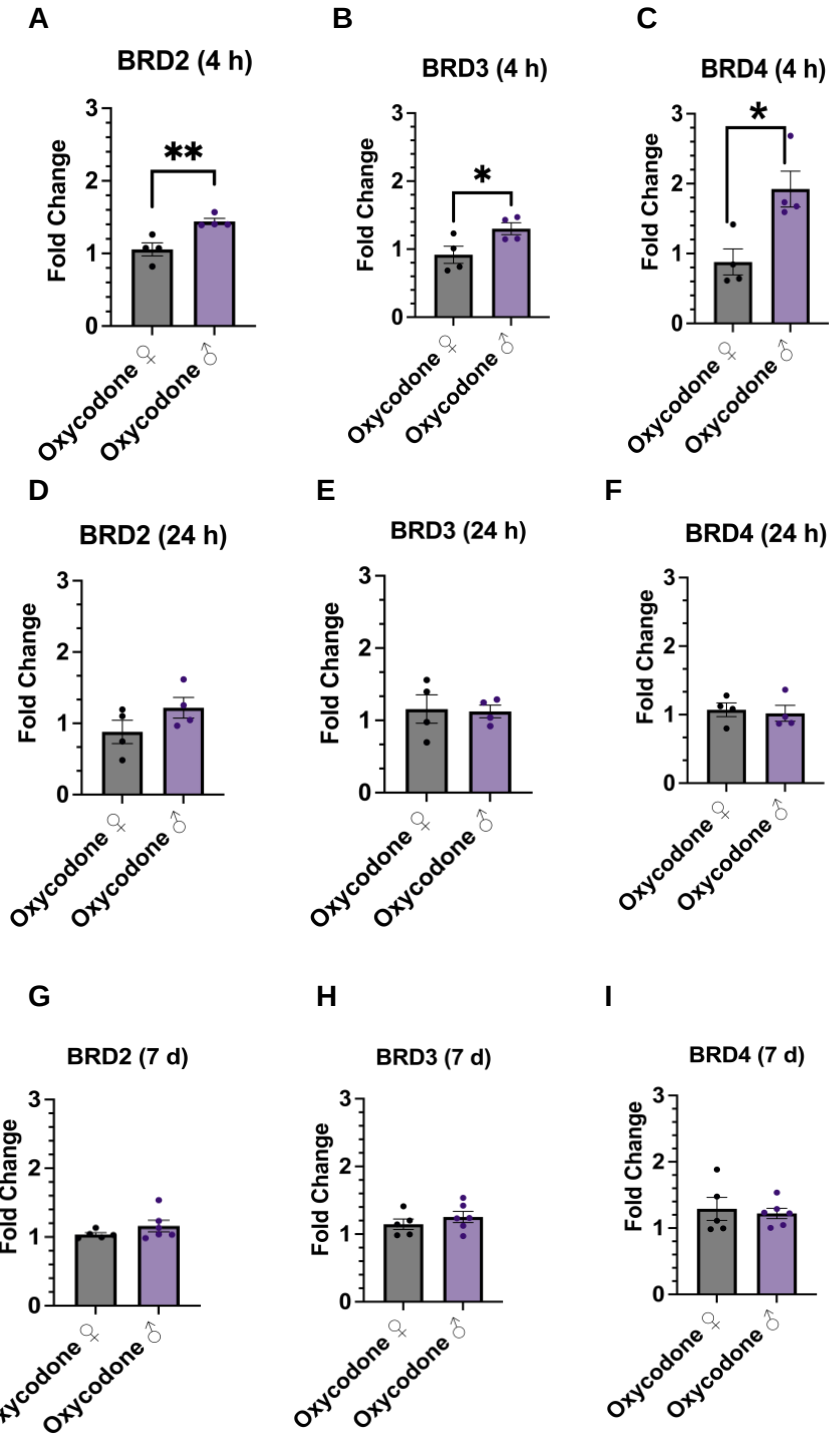


**Figure 4: Time-dependent changes in BET gene expression in the NAc following oxycodone by sex**. Mice received regular water or oxycodone. Following oral administration for 10 days NAc brain tissue was collected and *Brd2*, *Brd3*, and *Brd4* expression levels were measured after (A-C) 4 h, (D-F) 24 h, and (G-I) 7 d of abstinence. * $P < 0.05$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM, n = 3-4.

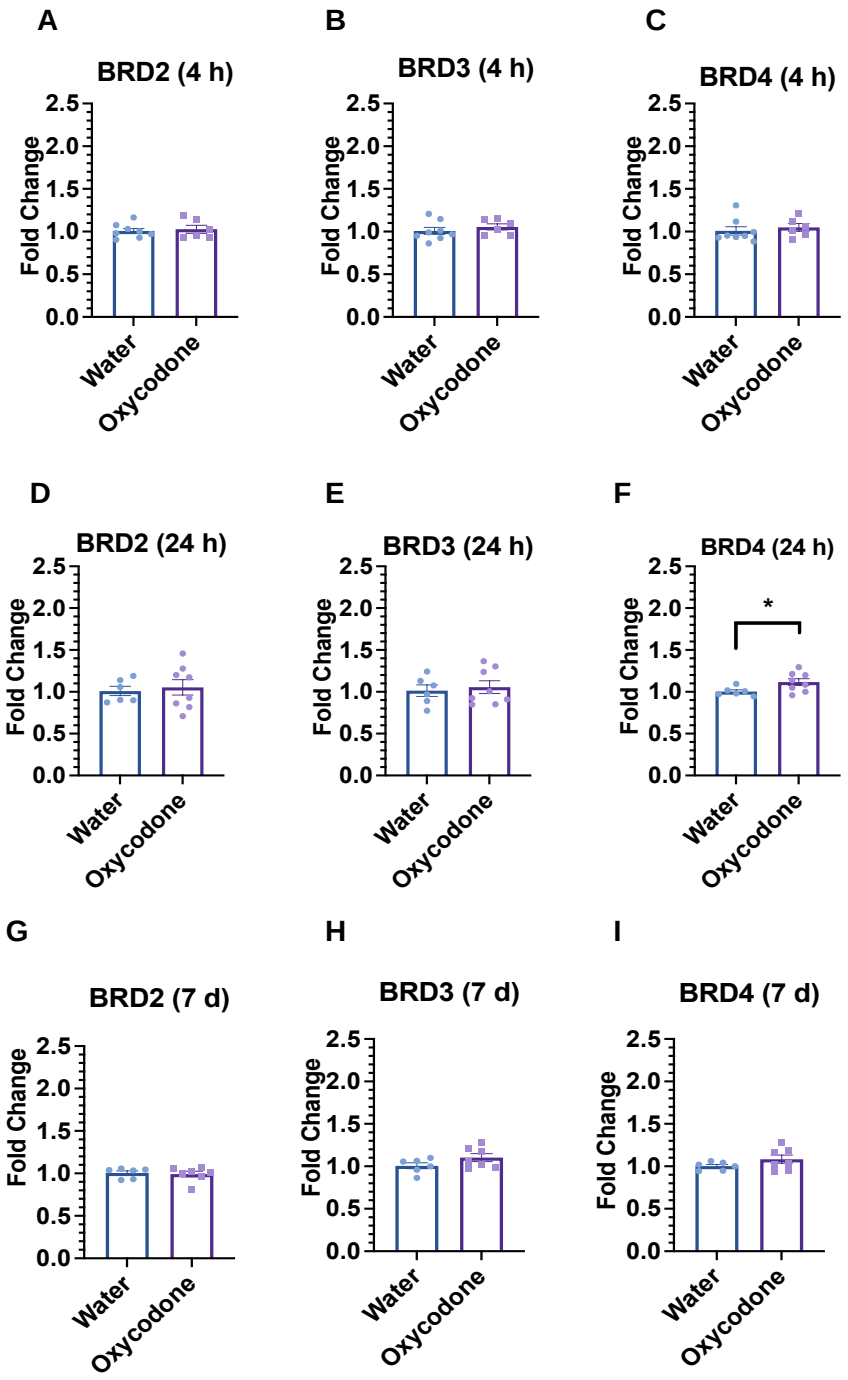


**Figure 5: Time-dependent changes in BET gene expression in the PFC**. Mice received regular water or oxycodone. Following oral administration for 10 days NAc brain tissue was collected and *Brd2*, *Brd3*, and *Brd4* expression levels were measured after (A-C) 4 h, (D-F) 24 h, and (G-I) 7 d of abstinence. * $P < 0.05$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM, n = 4-7.

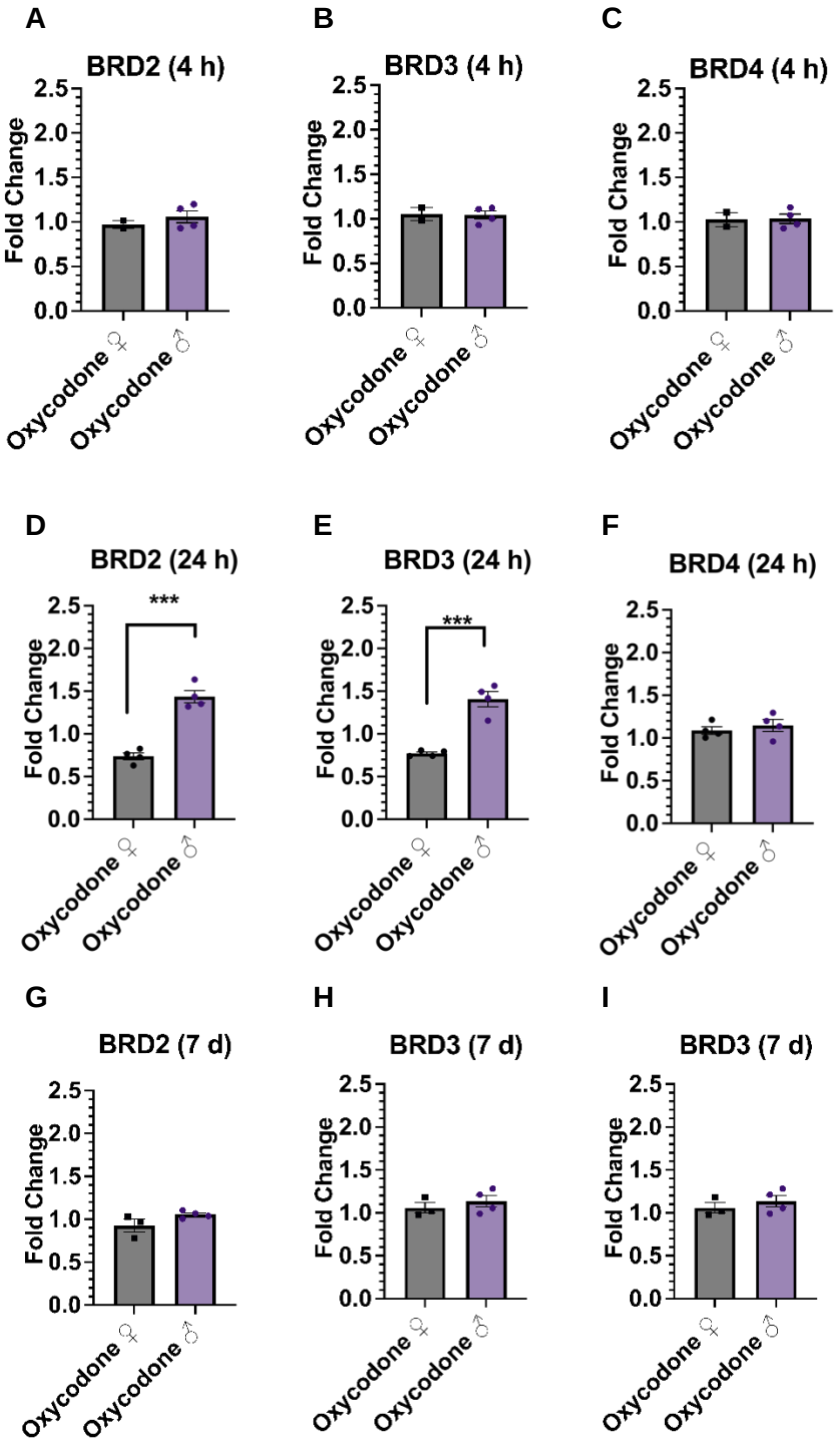


**Figure 6: Time-dependent changes in BET gene expression in the PFC following oxycodone by sex**. Mice received regular water or oxycodone. Following oral administration for 10 days NAc brain tissue was collected and *Brd2*, *Brd3*, and *Brd4* expression levels were measured after (A-C) 4 h, (D-F) 24 h, and (G-I) 7 d of abstinence. *** $P < 0.001$, indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM, n = 2-4.

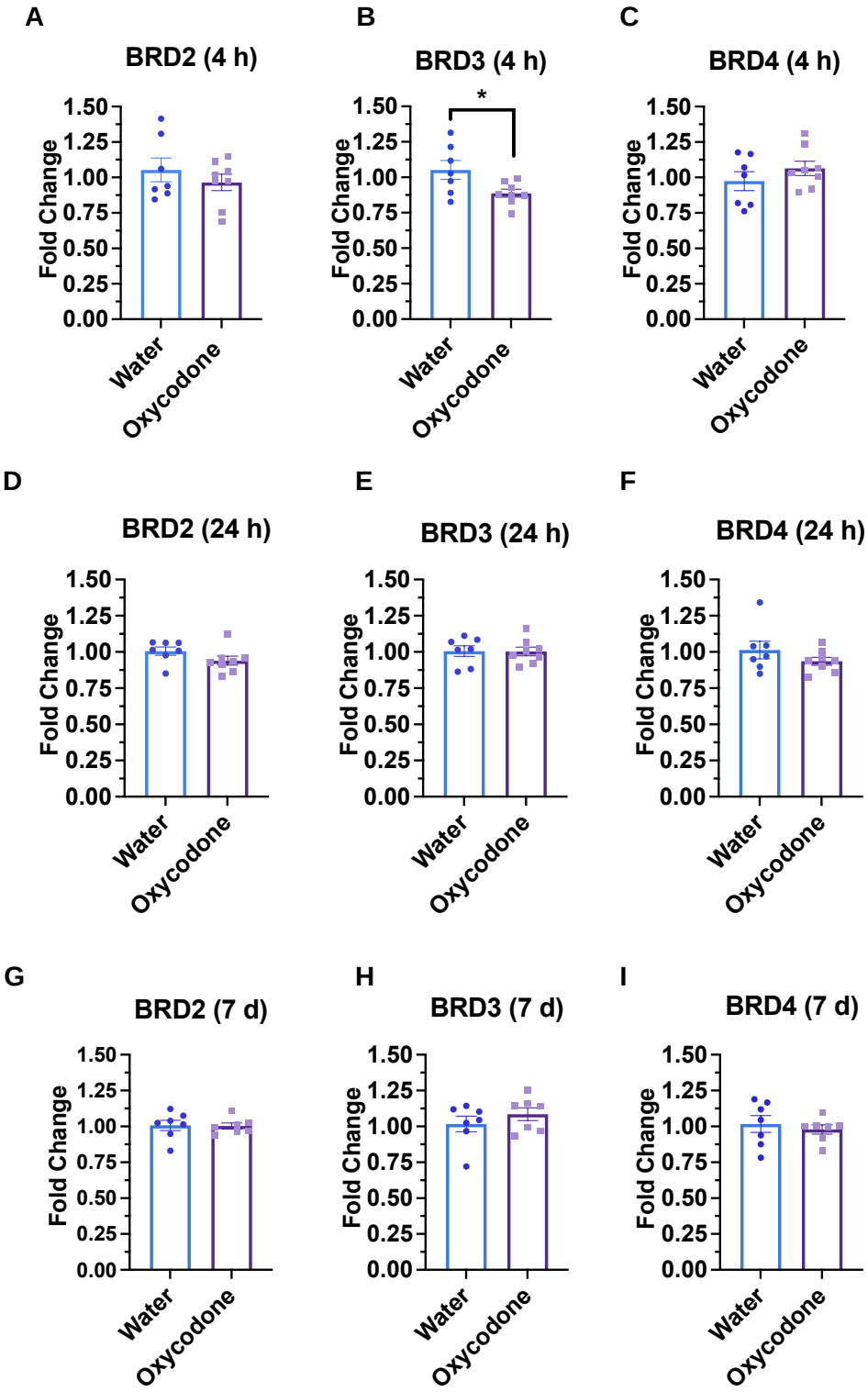


**Figure 7: Time-dependent changes in BET gene expression in the DS.** Mice received regular water or oxycodone. Following oral administration for 10 days, NAc brain tissue was collected and *Brd2*, *Brd3*, and *Brd4* expression levels were measured after (A-C) 4 h, (D-F) 24 h, and (G-I) 7 d of abstinence. * $P < 0.05$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM, n = 7-8.

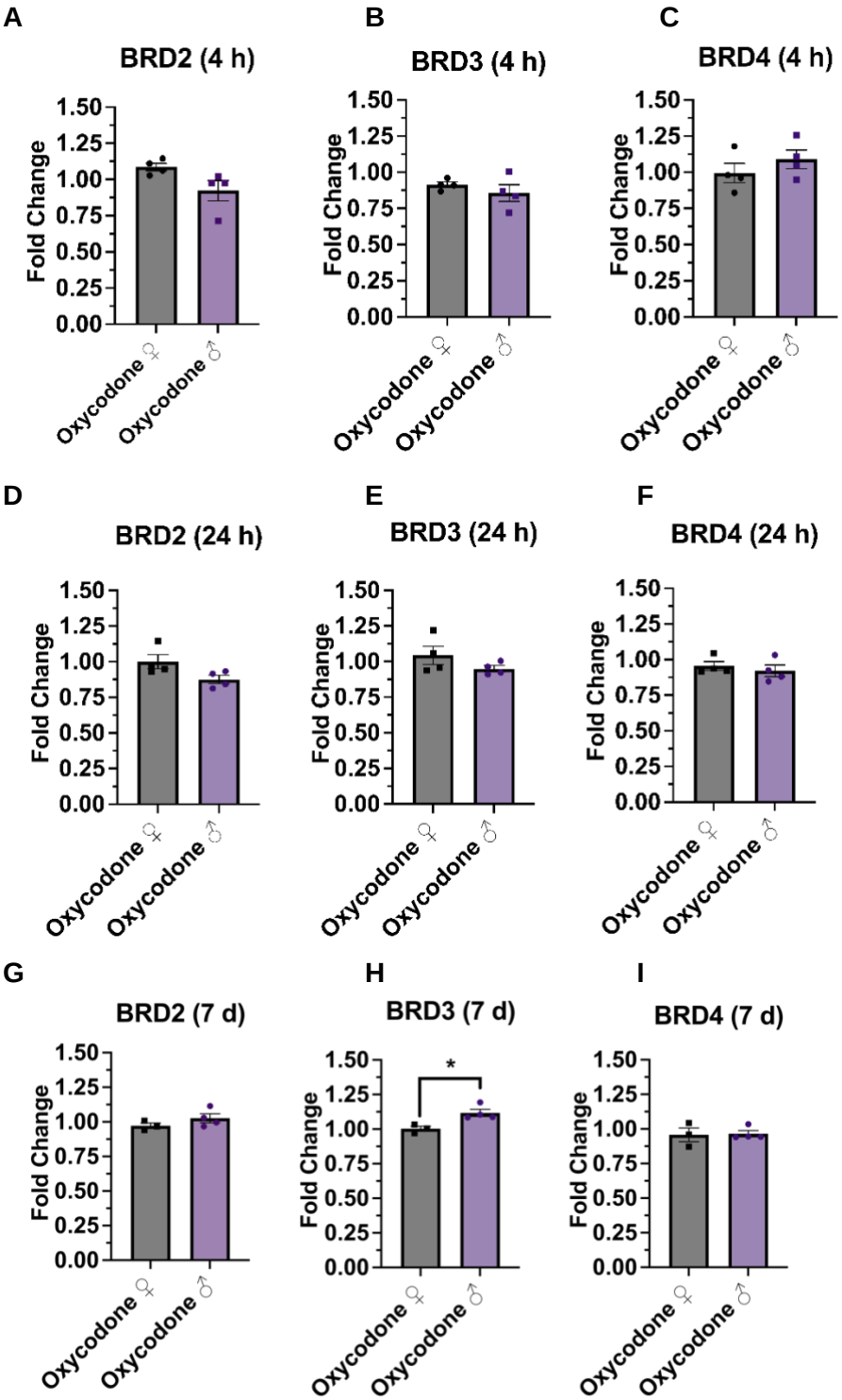


**Figure 8: Time-dependent changes in BET gene expression in the DS following oxycodone by sex.** Mice received regular water or oxycodone. Following oral administration for 10 days, NAc brain tissue was collected and *Brd2*, *Brd3*, and *Brd4* expression levels were measured after (A-C) 4 h, (D-F) 24 h, and (G-I) 7 d of abstinence. * $P < 0.05$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM, n = 3-4.

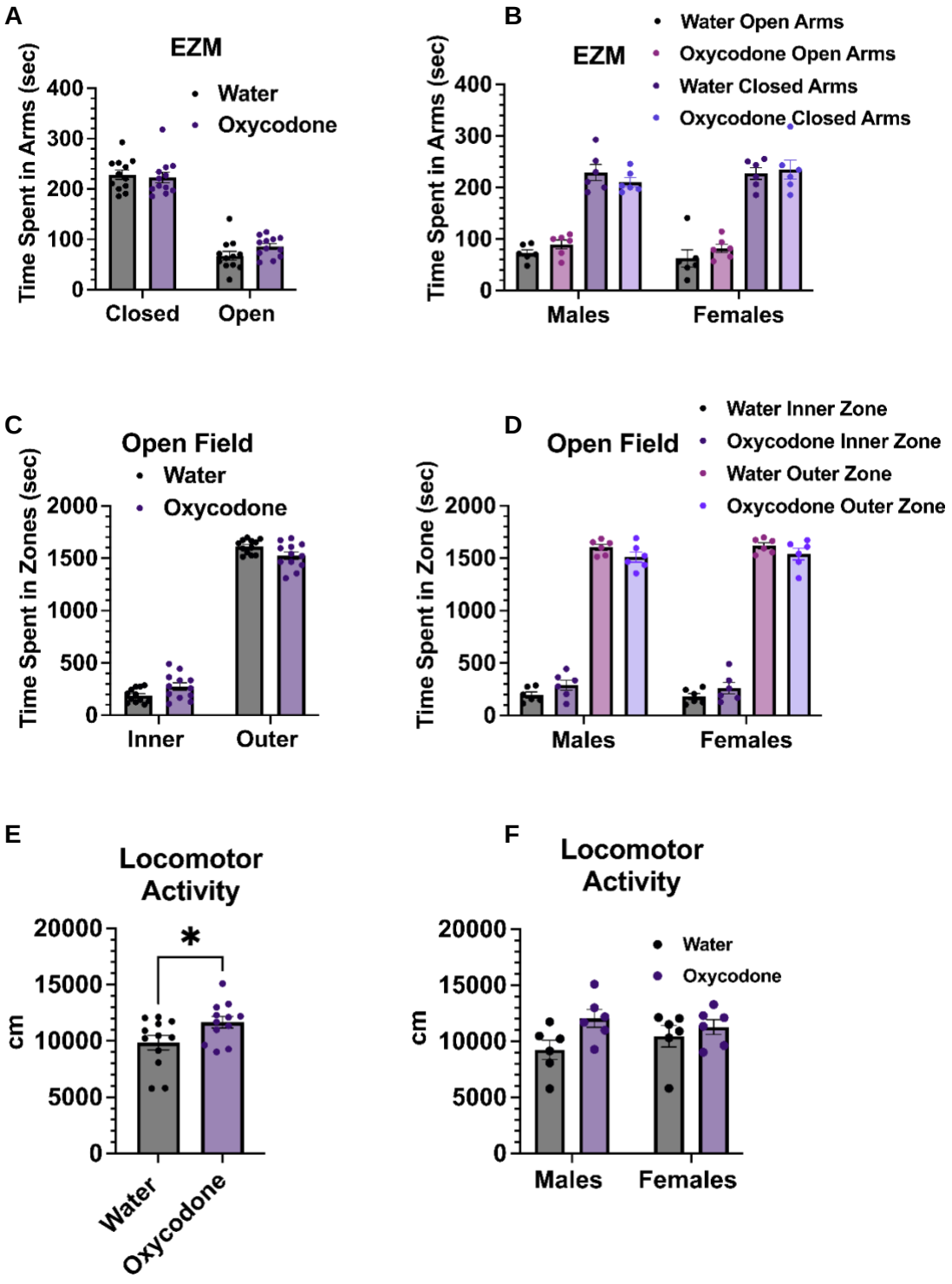


**Figure 9: Anxiety-like effects after 3 days of abstinence.** Mice received regular water or oxycodone (n = 6 males and 6 females). Following oral administration for 10 days and after 3 days of abstinence mice proceeded with behavioral tests for (A-B) elevated zero maze, (C-D) open field, and (E-F) locomotor activity. * $P < 0.05$, ** $P < 0.01$ indicate a significant difference using Bonferroni *post hoc* test. Data are mean ± SEM.

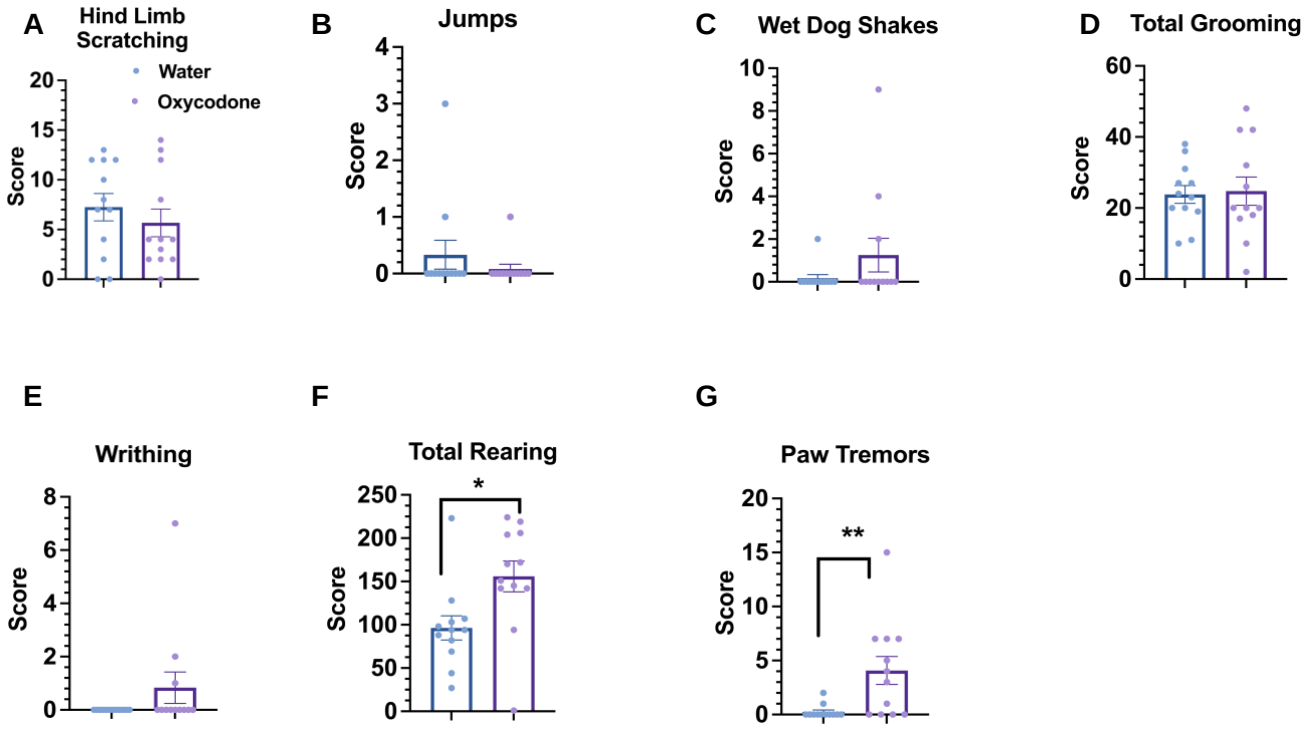


**Figure 10: Withdrawal-like effects after 3 days of abstinence.** Naloxone Precipitated Withdrawal symptoms of (A) hind limb scratching (B) jumps, (C) wet dog shakes, (D) total grooming, (E) writhing, (F) total rearing, (G) paw tremors by males and females combined (n = 12). Data analyzed via Student's t-test, * $P < 0.05$, ** $P < 0.01$. Data are expressed as means (±SEM).

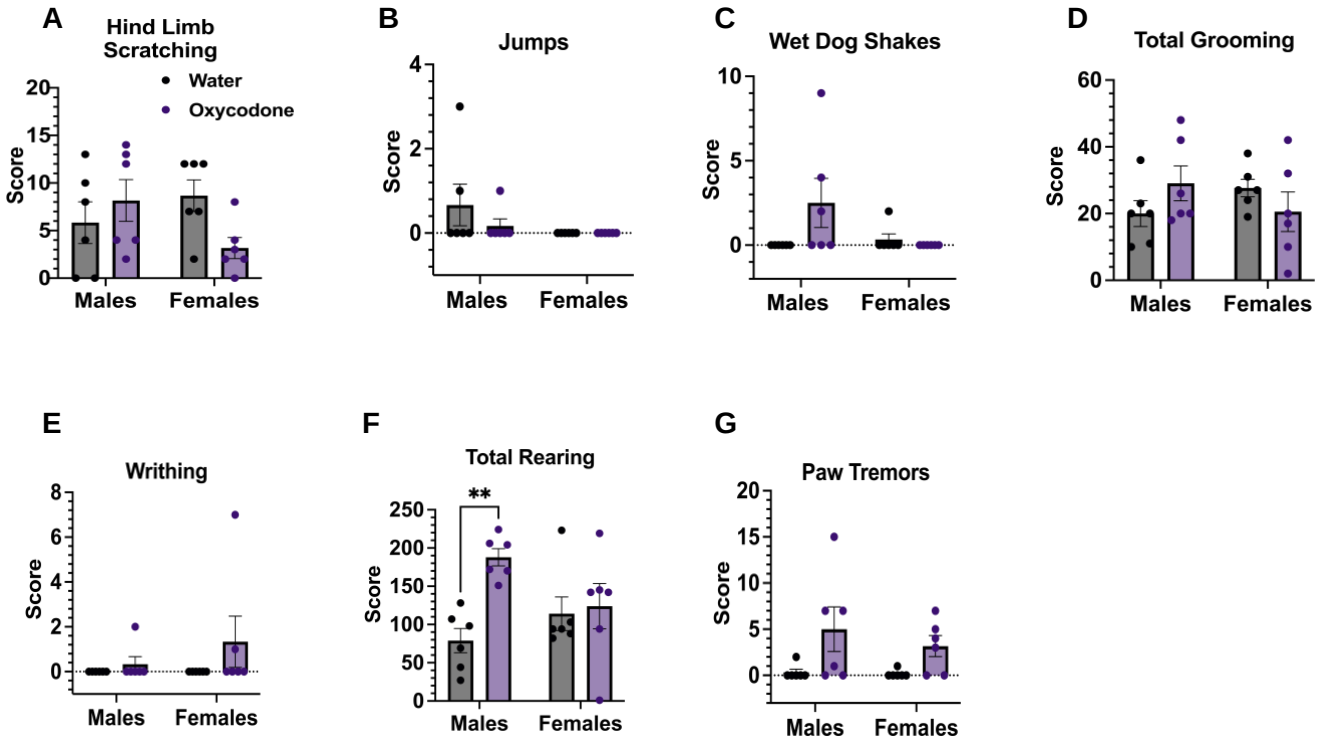


**Figure 11: Sex-dependent withdrawal-like effects after 3 days of abstinence.** Naloxone Precipitated Withdrawal symptoms of (A) hind limb scratching (B) jumps, (C) wet dog shakes, (D) total grooming, (E) writhing, (F) total rearing, (G) paw tremors by males and females (n = 6 males and 6 females). Data analyzed via analysis of variance followed by Bonferroni post-hoc, * $P < 0.05$, ** $P < 0.01$. Data are expressed as means (±SEM).

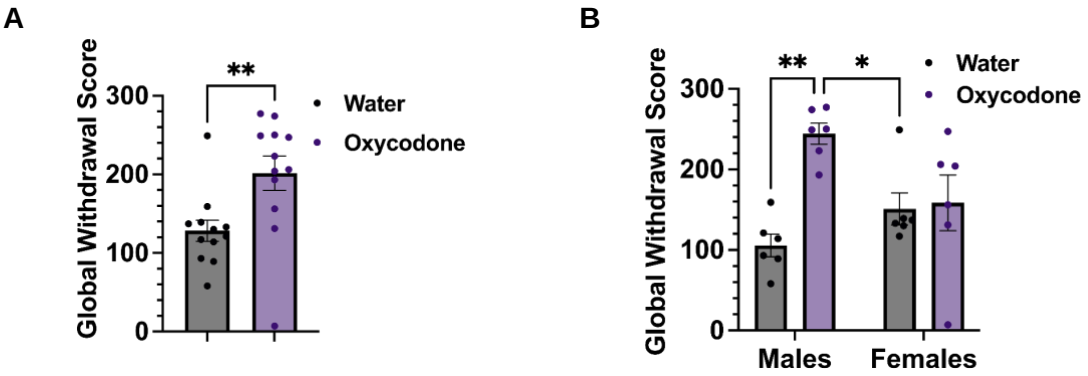


**Figure 12: Differences in global withdrawal score after 3 days of abstinence.** (A-B) Global withdrawal score following naloxone precipitated withdrawal in all mice and in mice separated by sex (n = 6 males and 6 females). Data analyzed via analysis of variance followed by Bonferroni post-hoc, * $P < 0.05$, ** $P < 0.01$. Data are expressed as means (±SEM).

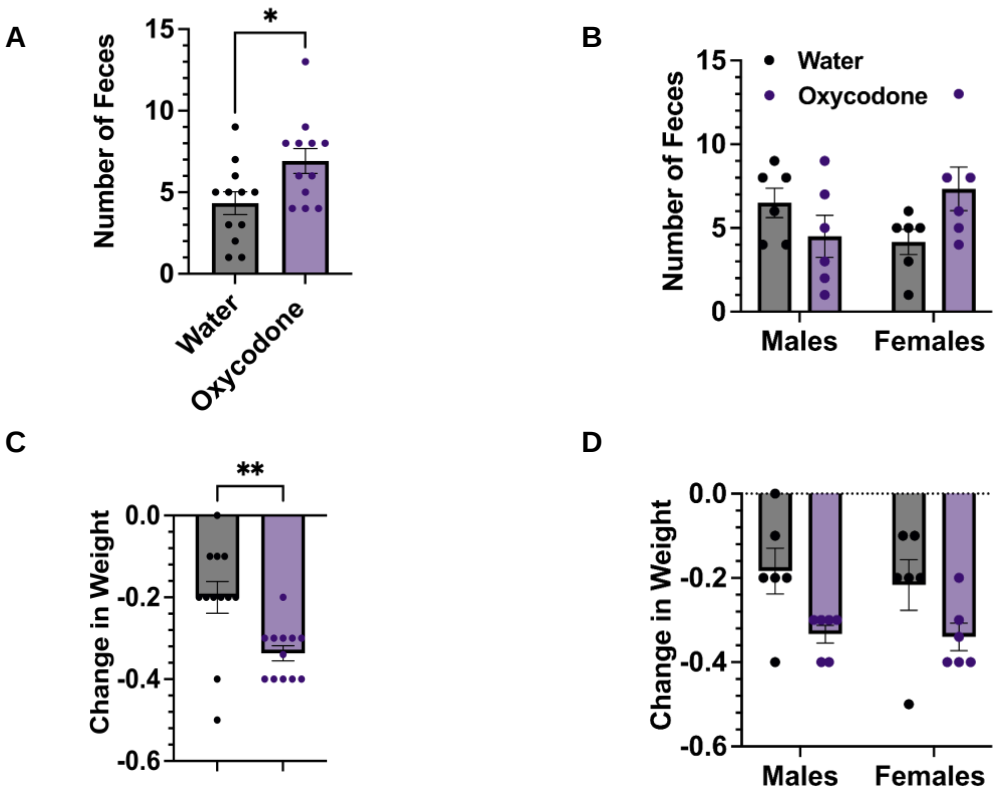


**Figure 13: Changes in defecation and in weight in mice after 3 days of abstinence**. (A-B) Number of feces during 30-minute naloxone precipitated withdrawal and (C-D) change in weight after naloxone precipitated withdrawal by sex combined (n = 12) and by males and females (n = 6). Data analyzed via analysis of variance followed by Bonferroni post-hoc, * $P < 0.05$, ** $P < 0.01$. Data are expressed as means (±SEM).

## Discussion

Animal models of opioid addiction are important for understanding the etiology of the human disease. Many animal models of addiction are focused on intravenous administration; however, few studies have investigated oral intake of opioids. Dependency on oxycodone usually starts via oral ingestion, which emphasizes the importance of developing animal models that include this method. Here, I observed that mice consumed more oxycodone compared to regular water. These results are consistent with a previous study showing that rats prefer oxycodone and escalate their consumption in a voluntary drinking model [36]. I also observed that females consumed more oxycodone than males (mL/g), but their water intake was also higher than males. Other experiments found that females rats preferred oxycodone more than males in a chronic two-bottle oral voluntary choice paradigm [36]. Although females consumed more oxycodone than males, greater difference in consumption is shown amongst the males between oxycodone and regular water. This may be due to the way oxycodone is being metabolized differently by males and female mice, as sex differences in oxycodone metabolism has been shown in rats [37].

Time-dependent changes in BET mRNA expression were observed during abstinence. Change in BET expression was mostly found in the NAc, in which mice consuming oxycodone had a greater increase in expression compared to regular water. In previous studies, the NAc was shown to be an important brain region for BET activity in a cocaine conditioned placed preference model[14]. Greater *Brd4* gene expression in the oxycodone group was commonly observed at multiple time-points in the NAc and PFC. BRD4 has been shown to play a role in substance use disorder [38–40]. In the NAc, males had a greater fold change than females in the oxycodone group for all BET proteins during the 4-hour timepoint of abstinence, an indication that BETs may also regulate oxycodone-induced neuroadaptations. Overall, these results indicate that BET gene expression is elevated more in addiction-related brain regions in males compared to females.

In behavioral studies, oral oxycodone produced weak withdrawal- and anxiety-like effects after three days of abstinence. Relative to the water control group, a significant increase in global withdrawal scores was observed in mice that consumed oxycodone and males showed greater signs of withdrawal. Mice in the oxycodone group also had greater defecation and change in weight compared to the water control group. Based on our animal model of study, males exhibited greater withdrawal-like behaviors compared to females. Consistent with these findings, a study investigating sex differences in responsiveness to prescription opioid oxycodone in mice showed that males experienced greater analgesia compared to females [41].

In the open field test and elevated zero maze, no significant changes in anxiety-like behaviors were observed. However, when looking at locomotor activity in the open field, mice in

the oxycodone group had increase in distance travelled compared to the water control group. These findings are also similar to a recent study using oral administration of oxycodone in mice in which mice that volitionally consumed oxycodone showed hyperlocomotion during an open field test [42]. For the few somatic signs of withdrawal, significant increase in paw tremors were found. Previous studies have also demonstrated that wet dog shakes, grooming, and writhing showed minor changes and were categorized as miscellaneous signs of withdrawal following heroin consumption in rats [31].

Some limitations of these experiments are also noted. For example, mice did not have a choice between regular water vs. oxycodone water. In a different study where rats were given the voluntary and free-choice access to oxycodone and water, results showed that rats had a preference for oxycodone and that both sexes became dependent on oxycodone [36]. Also, in our animal model, the frequency, and the time of oxycodone consumption over a 24-hour window is unknown. In ongoing experiments, the blood concentration of oxycodone after abstinence is being explored using liquid chromatography mass spectrometry. These studies are important as mice may quickly metabolize oxycodone following oral intake [43], which may contribute to the weak withdrawal-like behaviors that were observed. Another limitation of this study is that we only observed behavior at one time-point of abstinence (3 days). Future studies should include same days of abstinence for both gene expression and behavioral studies to investigate the role of BET proteins in OUD. Further studies can also explore BET gene and BET protein expression in other brain regions, as we only examined BET gene expression in the prefrontal cortex, nucleus accumbens, and dorsal striatum. The ventral tegmental area (VTA) is a brain region that can be explored and has been investigated in different animal models associated with opioid use disorders [44–46]. Lastly, future studies will explore the therapeutic effects of BET inhibitors on oxycodone-induced withdrawal-like behaviors and oxycodone consumption. Overall, our oral administration model of oxycodone was a promising start to investigating the role of BET proteins in OUD, and ongoing experiments will determine their functional role in opioid dependence.

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