Buprenorphine Effects on Anxiety-Like Behavior in B6 Mice

Megan K. Thibert

Undergraduate Honors Thesis, Spring 2022

Department of Psychology, The University of Tennessee, Knoxville

Acknowledgements: I would like to express my sincere gratitude to Dr. Ralph Lydic and Dr. Helen Baghdoyan for their consistent support, guidance, and leadership during my undergraduate years and time in the lab. Not only have they supported me throughout the development of my honors thesis, but they have also played a significant role in my decision to pursue my PhD at Vanderbilt after graduation. I also want to extend thanks to all of the lab members, professors, and mentors that have supported and encouraged me along my journey.

Without my loving parents, Caroline and Jason, and the rest of my small but tightknit family, none of this would be possible. They, along with my friends and church community, have been a fortress for me in uncertain times. Thank you for loving me as you do. Most of all, I want to thank my one and only savior and steadfast rock, Jesus Christ, whose word is a lamp to my feet and a light to my path (Psalm 119:105). My accomplishments would mean nothing to me without the knowledge that He has established my steps.

Abstract: Buprenorphine, a semi-synthetic opioid prescribed for the treatment of opioid use disorder (OUD), has been suggested as a potential pharmacological treatment for anxiety. Some preclinical and clinical studies provide support for the anxiolytic effects of buprenorphine, but research in this area is scarce, and findings to date have been mixed. The present study was designed to test the hypothesis that buprenorphine alters anxiety-like behavior in C57BL/IJ (B6) mice measured using the elevated zero maze (EZM). Adult, male mice (n=10) were given subcutaneous injections of saline (control) and three doses of buprenorphine (0.3, 1, and 10 mg/kg). One hour following injection, mice were placed on the EZM for a duration of five minutes. The dependent variables measured were time spent in the open and closed regions of the EZM, and latency to enter the EZM open region. Repeated measures ANOVA revealed that all three dependent measures varied significantly as a function of buprenorphine dose. Dunnett's post-hoc analyses showed for all dependent measures that buprenorphine (10 mg/kg) increased anxiety-like behavior in B6 mice. These results are discussed relative to human studies showing that buprenorphine decreases objective measures of stress and anxiety.

Introduction

Opioids are a class of analgesic drugs that relieve pain by binding to widely distributed opioid receptors in the central and peripheral nervous systems (National Academies of Sciences & Medicine, 2017; Rosenblum et al., 2008). Although effective for pain relief, opioids have unwanted side effects, such as respiratory depression, sleep disruption, constipation, and risk of physical dependence and addiction (Camilleri, 2011; Eacret et al., 2020; Kosten & George, 2002; Zaig et al., 2021). A substantial percentage of people to which opioids are prescribed go on to develop opioid use disorder (OUD). Although incidence is highly variable, population studies report up to 34% of patients prescribed opioids present with OUD symptoms (Klimas et al., 2019). Opioid misuse and abuse are at crisis levels within the United States (US), and the Centers for Disease Control and Prevention (CDC) estimate that approximately 91 deaths due to opioid overdose occur daily (Schiller et al., 2022). Opioid overdose rates have sizably increased in the last two decades, with accidental prescription opioid overdoses having increased nearly fourfold from 2000 to 2010 (Brady et al., 2016). Opioid abuse is highly prevalent in the Appalachian region of the US, and The Appalachian Regional Commission (ARC) reported that deaths due to drug overdose in general were 37% higher in Appalachia than the rest of the US in 2015 (Dasgupta et al., 2018; Schalkoff et al., 2020). Investigations of this trend suggest that poor social and economic conditions, lower education levels, and high work-related injuries make the Appalachian region particularly vulnerable to opioid abuse (Moody et al., 2017). Other important factors highlighted in these studies are the loss of social connection and a diminished sense of hope experienced by people within these regions (Blanco et al., 2020).

Relevant to the opioid epidemic is the ongoing worldwide coronavirus disease 2019 (COVID-19) pandemic. Recent studies have found that individuals suffering from OUD or other

forms of substance use disorder are among the most susceptible to poor outcomes associated with COVID-19 (Volkow, 2020). This is due in part to the respiratory health challenges faced by opioid users, along with indirect factors, such as unstable housing, incarceration, and decreased support and medical care. Regarded as a "disease of despair" (Schalkoff et al., 2020), OUD is also linked to major depressive disorder and anxiety and stress-related disorders (Rosoff et al., 2021). It is plausible that those with OUD have diminished motivation to seek medical treatment and care, which may exacerbate their inherent vulnerabilities to COVID-19. Recent studies have also demonstrated that the pandemic has significantly increased rates of anxiety, depression, and psychological distress amongst the general population in the US (Khubchandani et al., 2021). Already the leading cause of a global health-related burden, diminished mental health has become an even greater cause for concern during the pandemic (Santomauro et al., 2021) .

The endogenous opioid system plays a crucial role in modulating anxiety and other affective states, and some studies suggest that opioids alleviate anxiety-like behavior in rodents and humans (Colasanti et al., 2011). Rodent studies report that mu-opioid receptor agonists, such as morphine (Rezayof et al., 2009), fentanyl (Fujii et al., 2019), and buprenorphine (Almatroudi et al., 2018; Falcon et al., 2015) are anxiolytic, meaning they decrease anxiety-like behavior. Additionally, clinical studies provide support for the antidepressant and anxiolytic effects of buprenorphine in humans (Bershad et al., 2015; Karp et al., 2014; Sher, 2016; Velander, 2018). Researchers have proposed that buprenoprhine could be used as a potential therapeutic for anxiety and depression (Ahmadpanah et al., 2017; Lynch & Benhamou, 2019; Pendergrass et al., 2019). With respect to opioid-dependent patients, studies reported that a single dose of buprenorphine could be an effective tool to treat anxiety (Ahmadi & Jahromi, 2017; Ahmadpanah et al., 2017). Buprenorphine is a unique, semi-synthetic opioid that is clinically

approved for the treatment of OUD and pain syndromes (Sher, 2016). Buprenorphine acts as a high-affinity, partial agonist at mu opioid receptors (MOR) and an antagonist at kappa opioid receptors (KOR) (Falcon et al., 2015). As a *high-affinity* agonist at MORs, buprenorphine blocks the binding of other opioids, thereby decreasing opioid abuse (Velander, 2018). Buprenorphine is a *partial* MOR agonist and full KOR antagonist, and it causes less respiratory depression than full MOR agonists such as morphine and fentanyl. There is a linear relationship between respiratory depression and dose of morphine and fentanyl. By contrast, buprenorphine exhibits a "ceiling effect," for respiratory depression such that respiratory depression is not linearly related to opioid dose (Davis, 2012; Falcon et al., 2015).

Preclinical studies provide some support for the potential anxiolytic and antidepressant effects of buprenorphine, but results have been inconsistent to date. One such study reported that buprenorphine had antidepressant and anxiolytic effects on B6 mice in the forced swim test and novelty-induced hypophagia test (Falcon et al., 2015). In a later study by Almatroudi (2018), CD-1 mice were administered a novel buprenorphine analogue (BU10119) and anxiety-like behavior was quantified using an elevated plus maze (EPM). These studies in CD-1 mice also reported antidepressant and anxiolytic effects of the buprenorphine analogue in the forced swim and novelty-induced hypophagia test. However, the behavior of CD-1 mice in the EPM and light dark box test was not significantly different among mice receiving BU10119 and the vehicle (Almatroudi et al., 2018). Table 1 summarizes studies of mice that used the EPM and other tests of anxiety and depressive-like behavior. Mice exposed to fluoride and induced traumatic brain injury displayed anxiety-like behavior in the elevated zero maze (EZM) (Li et al., 2019; Popovitz et al., 2019). Additionally, mice given intraperitoneal injections of oxytocin spent less time in the open regions of the EZM, indicating anxiety-like behavior (Sakamoto et al., 2019). Table 1

illustrates two points relevant to the present study. First, is the wide acceptance in behavioral neuroscience of the EZM for providing an operational measure of anxiety-like behavior in mice. Table 1 also illustrates the novelty of the present studies. Relatively few studies to date have investigated the effects of buprenorphine on anxiety-like behavior in mice using the EPM, and even fewer with the modified EZM. The present study was designed to test the hypothesis that buprenorphine alters anxiety-like behavior in B6 mice as measured using the EZM.

Table 1. Selective summary of data from 2000-2021 on different treatments that influence anxiety-like behavior in mice.

		Test of Anxiety/Depressive-Like Behavior				
		EPM	**EZM**	**FST**	**NIH**	**LDB**
Treatment	**Buprenorphine**	N/A	N/A	B6 mice, antidepressant effects (Falcon et al., 2015)	B6 mice, anxiolytic effects (Falcon et al., 2015)	N/A
	BU10119	CD-1 mice, no significant effects (Almatroudi et al., 2018)	N/A	CD-1 mice, antidepressant effects (Almatroudi et al., 2018)	CD-1 mice, anxiolytic effects (Almatroudi et al., 2018)	CD-1 mice, no significant effects (Almatroudi et al., 2018)
	TBI	B6 mice, anxiogenic effects (Popovitz et al, 2019)	B6 mice, anxiogenic effects (Popovitz et al, 2019)	N/A	N/A	N/A
	Fluoride Exposure	N/A	Kumming mice, anxiogenic effects (Li et al., 2019)	Kumming mice, depressant effects (Li et al., 2019)	N/A	N/A
	IP Oxytocin Injection	N/A	B6 mice, anxiogenic effects (Sakamoto et al., 2019)		N/A	N/A

***BU10119**= Buprenorphine analogue ***TBI**= Traumatic brain injury ***EPM**= Elevated plus maze
***EZM**= Elevated zero maze ***FST**= Forced swim test ***NIH**= Novelty-induced hypophagia test ***LDB**= Light-dark box test

Methods

Animals: The protocol for these studies was reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee. All procedures adhered to The Guide for the Care and Use of Laboratory Animals (Council, 2011) and the Arrive-2 guidelines (Percie Du Sert et al., 2020). Adult, male C57BL/6J (B6) mice (n=10) were purchased from the Jackson Laboratory at age 12 weeks and housed together upon arrival. Each mouse was anesthetized with isoflurane and implanted with a unique radio frequency identification chip (RFID-100B 1.4, Microchip ID). The RFID chips made it possible to identify each mouse using a unique alphanumeric code. Mice had *ad libitum* access to food and water and were housed under a 12:12h light:dark cycle. Mice were weighed and handled daily for 12 days before starting data collection.

Measuring Anxiety-Like Behavior in Mice: This study used the amount of time spent in specific regions of the elevated zero maze (EZM), along with the latency to enter the open region as surrogate measures of anxiety-like behavior in B6 mice.

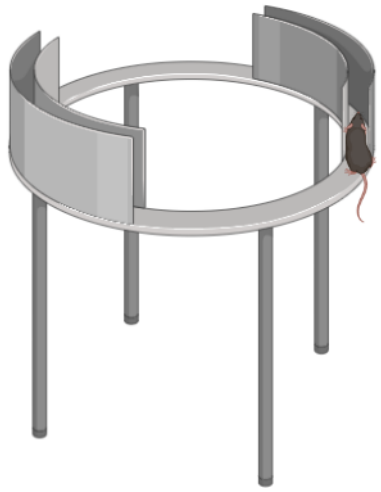


Figure 1. The EZM has alternating open and closed regions that form a circular shape (Shepherd et al., 1994; Walf & Frye, 2007). The EZM used in the present studies has a diameter of 39.5 inches, circumference of 10.3 ft, and height of 25.5 inches. At the start of each experiment a mouse was placed in the position shown in this figure.

Previous studies have suggested that the EZM encourages greater exploratory behavior by B6 mice than the elevated plus maze (EPM) and that the EZM yields more consistent results across trials (Tucker & McCabe, 2017b). The design of EZM was driven by knowledge that as a prey species mice evolved to decrease the risk of predation by staying in darken and closed areas (Bailey & Crawley, 2009).

The open regions of the maze may signal potential danger, whereas the closed regions of the may be perceived as safer and harboring less risk (Tucker & McCabe, 2017a). At the time of testing, mice were placed into the maze at a boundary between an open and closed region facing the closed region (Almatroudi et al., 2018; Tucker & McCabe, 2017a).

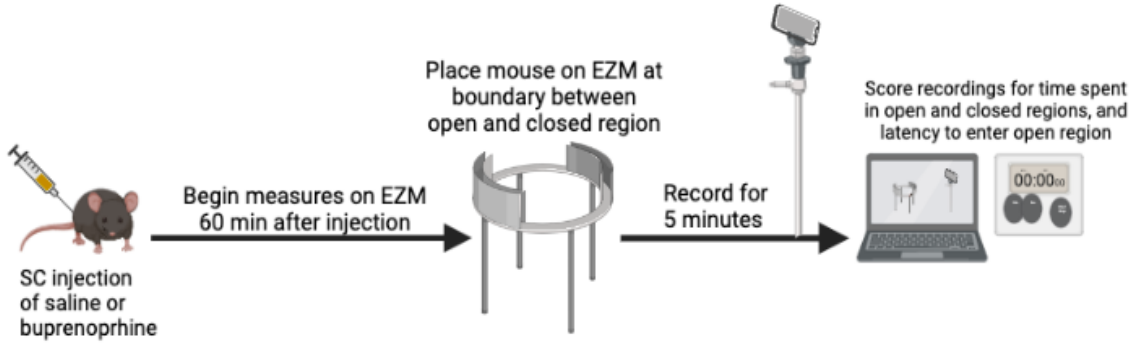


Figure 2. Workflow schematic of steps for measuring buprenorphine effects on anxiety-like behavior in B6 mice. Mice received a subcutaneous (SC) injection of saline (control) or buprenorphine. Sixty min after the injection, mice were placed on the EZM as shown in Fig. 1. During the 5 min after being placed on the EZM, three dependent measures were recorded: time spent in the open or closed regions of the maze, along with the latency to enter the open region.

The EZM shown in Figs. 1 and 2 was designed with the operational definition that more time in the open regions of the EZM is scored as anxiolysis (decreased anxiety-like behavior) and that anxiolysis would also be expressed as a decreased latency to enter the open region of the maze. Correspondingly, less time spent in the open regions and an increased latency to enter the open region would be representative of anxiogenesis (increased anxiety-like behavior). These dependent variables and the interpretations are based on the previous studies of anxiety-like behavior with the EZM (Braun et al., 2011; Shepherd et al., 1994; Tucker & McCabe, 2017a). Mice were scored as having entered the open region when all four limbs had passed the boundary separating the open and closed regions.

Experimental Design for Baseline and Treatment Conditions: The first step in these studies was to confirm that my use of the EZM provided a reliable measure of anxiety-like behavior in B6 mice. Specifically, my measures of time spent in the open and closed regions, along with the latency to enter the open region of the maze were compared to the previously published measures (Tucker & McCabe, 2017a). Initially, each of the 10 mice was placed on the EZM to provide baseline data, and three measures were obtained from each mouse. In the next phase of the study, all 10 mice were assigned to the control group (0.9% saline 0.3 mL) and their behavior was recorded on the EZM. Following the control trials, mice were assigned to the buprenorphine group (0.3 mg/kg). This dose was chosen because it has been shown to be antinociceptive in B6 mice (Glovak et al., 2017). Two weeks after receiving the 0.3 mg/kg dose, mice were administered 1 mg/kg of buprenorphine. Three weeks after administering 1 mg/kg of buprenorphine, a third series of experiments was performed, whereby mice were administered buprenorphine at 10 mg/kg. For control and buprenorphine conditions, the injections were scheduled to ensure that the same amount of time had elapsed between time of injection and time tested on the EZM. Accordingly, mice received injections in the experiment room 1 h before being placed on the EZM, and mice remained in the room until testing. Figure 2 illustrates the workflow of the study and recording on the EZM.

Results

In Figs. 3-7 each mouse is represented by a single point. The y-axes display time spent in open regions (A) and closed regions (B) in min, and plotted in s, the latency to enter the open regions of the maze (C). The broad horizontal line illustrates the mean, and the two shorter horizontal bars represent the standard deviation. The dependent measures were manually scored by the

investigator at the time of testing and later double scored by a second investigator. Percent similarity was calculated by dividing the sum difference (s) between the two scores of the investigators by the total sum of seconds on the maze. Calculations revealed a percent similarity of 98.8% for time spent in open and closed regions and 99.9% for latency to enter the open region of the EZM.

Baseline Studies: Three baseline measures of time spent in locations on the EZM were conducted prior to the treatment conditions. On average, mice spent 1.01 min in the open regions and 3.99 min in the closed regions with a standard deviation of 0.29 (Fig. 3A, B). The average latency to enter the open region was 13.85 s with a standard deviation of 8.07 (Fig. 3C). In terms of percent time, mice spent an average of 20.3% of their time in the open regions and 79.7% of their time in the closed regions with a standard deviation of 5.8. The three published studies to which these results were compared are summarized in Table 2 (Braun et al., 2011; Shepherd et al., 1994; Tucker & McCabe, 2017a). The numbers in the table cells represent the percent of recording time spent in various portions of the EZM, along with the latency to enter the open region.

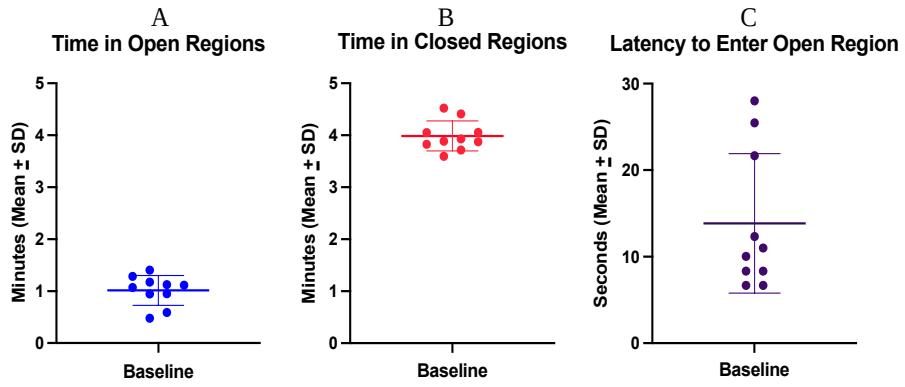


Figure 3. Mean time spent in open regions (A), closed regions (B), and latency to enter open region (C) for each individual mouse during the baseline recordings. Each point represents an individual mouse, and the y-axis displays mean time in minutes (A, B) and seconds (C) along with the standard deviation.

Previous measures on the EZM to which the present study was compared

Study	Percent Time in Open Regions	Percent Time in Closed Regions	Latency to Enter Open Regions (s)
Current Study	20.3	79.7	13.85
Braun et al., 2011	31	69	6.5
Shepherd et al., 1994	30	70	N/A
Tucker & McCabe, 2017	39	61	N/A

Table 2. Within the present study, the percent time in open regions averaged across three baseline trials was 20.3% with a standard deviation of 8.8. The three studies to which the present study was compared averaged 33.3% of time in the open regions with a standard deviation of 4.93.

Saline Injection: Figure 4 summarizes measures taken after mice were administered 0.3 mL of saline one h prior to being placed on the EZM. On average, mice spent 0.83 min in the open regions and 4.17 min in the closed regions of the maze with a standard deviation of 0.52 (Fig. 4A, B). Their average latency to enter the open region was 23.20 s with a standard deviation of 35.36 (Fig. 4C).

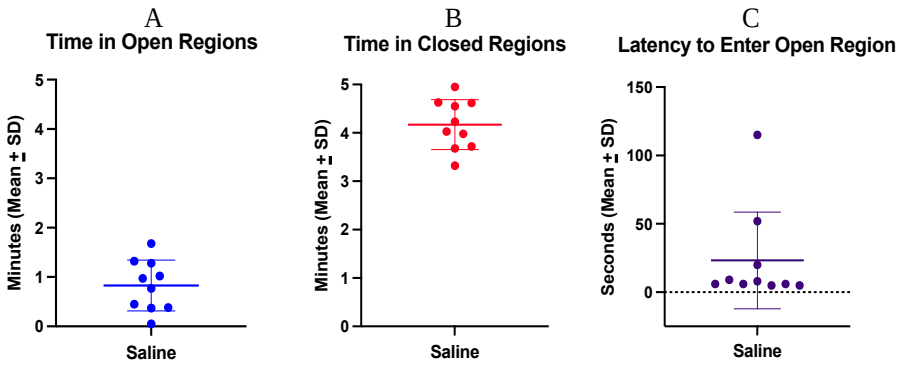


Figure 4. Mean time spent in open regions (A), closed regions (B), and latency to enter open region (C) for each individual mouse during the saline treatment. Each point represents an individual mouse, and the y-axis displays mean time in minutes (A, B) and seconds (C) along with the standard deviation.

Buprenorphine (0.3 mg/kg) Injection: Figure 5 summarizes measures taken after mice were administered 0.3 mg/kg of buprenorphine (BUP) one h prior to being placed on the EZM. On average, mice spent 0.90 min in the open regions and 4.10 min in the closed regions of the maze with a standard deviation of 0.67 (Fig. 5A, B). Their average latency to enter the open region was 6.0 s with a standard deviation of 3.59 (Fig. 5C).

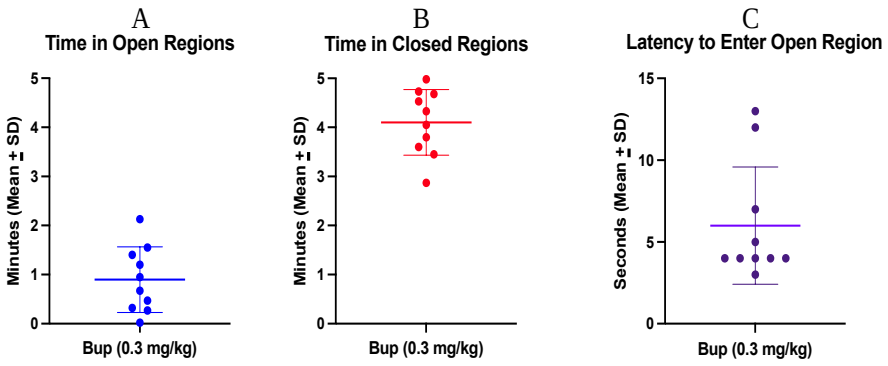


Figure 5. Mean time spent in open regions (A), closed regions (B), and latency to enter open region (C) for each individual mouse during the bup (0.3 mg/kg) treatment. Each point represents an individual mouse, and the y-axis displays mean time in minutes (A, B) and seconds (C) along with the standard deviation.

Buprenorphine (1 mg/kg) Injection: Figure 6 summarizes measures taken after mice were administered 1 mg/kg of buprenorphine one h prior to being placed on the EZM. On average, mice spent .48 min in the open regions and 4.52 min in the closed regions of the maze with a standard deviation of 0.53 (Fig. 6A, B). Their average latency to enter the open region was 55 s with a standard deviation of 90.85 (Fig. 6C).

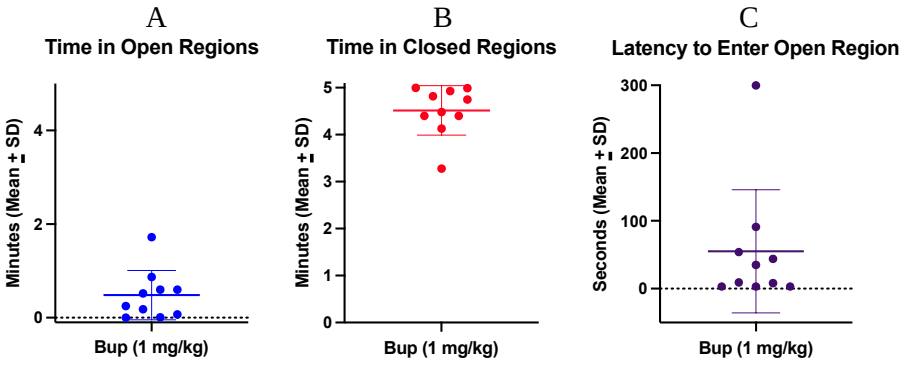


Figure 6. Mean time spent in open regions (A), closed regions (B), and latency to enter open region (C) for each individual mouse during the bup (1 mg/kg) experiment. Each point represents an individual mouse, and the y-axis displays mean time in minutes (A, B) and seconds (C) along with the standard deviation.

Buprenorphine (10 mg/kg) Injection: Figure 7 summarizes measures taken after mice were administered 10 mg/kg of buprenorphine one h prior to being placed on the EZM. On average,

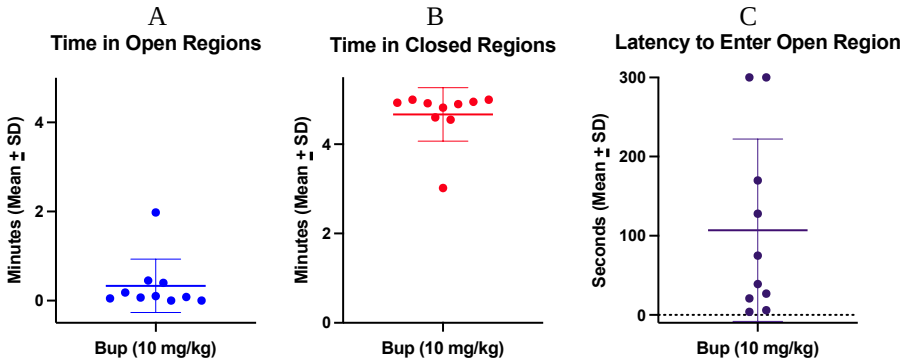


Figure 7. Mean time spent in open regions (A), closed regions (B), and latency to enter open region (C) for each individual mouse during the bup (10 mg/kg) experiment. Each point represents an individual mouse, and the y-axis displays mean time in minutes (A, B) and seconds (C) along with the standard deviation.

mice spent 0.33 min in the open regions and 4.67 min in the closed regions of the maze with a standard deviation of 0.60 (Fig. 7A, B). Their average latency to enter the open region was 107 s with a standard deviation of 115.07 (Fig. 7C).

EZM Behavior Varied with Buprenorphine Dose: Repeated measures ANOVA followed by Dunnett's post-hoc test revealed a significant dose-main effect on each of the three dependent measures. ANOVA revealed a buprenorphine dose effect of time spent in the open regions (F = 6.973; d.f. = 1.999, 17.99; P = 0.0057) and time spent in the closed regions of the EZM (F = 6.973; d.f. = 1.999, 17.99; P = 0.0057), along with the latency to enter the open region (F = 5.501; d.f. = 1.802, 16.22; P = 0.0171). The Dunnett's post-hoc test revealed that the 10 mg/kg dose of buprenorphine caused a statistically significant decrease in time spent in open regions (P = 0.02), increase in time spent in closed regions (P = 0.02), and increase in latency to enter the open region (P = 0.04) as compared to the control.

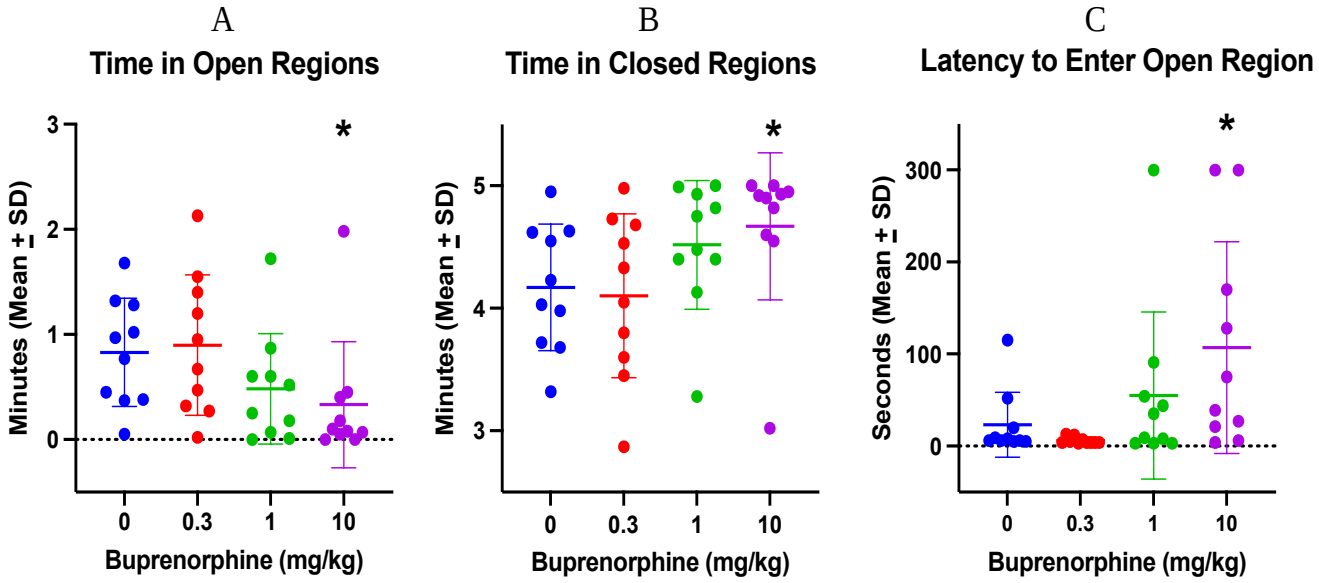


Figure 8. Summary of the three dependent variables measured across the control and three treatment groups (Bup 0.3, 1, and 10 mg/kg). Figure 8A represents the mean time spent in the open regions in minutes, Figure 8B represents the mean time spent in the closed regions in minutes, and Figure 8C represents the mean latency to enter the open region in seconds. The y-axis displays time in minutes (A, B) and seconds (C) along with the standard deviation. Asterisks indicate significant differences by Dunnett's test comparing measures after saline and 10 mg/kg buprenorphine.

A. Time in Open Regions

		Buprenorphine (mg/kg)		
	Saline	**0.3**	**1**	**10**
Minutes (Mean ± SD)	0.83 ± 0.52	0.90 ± 0.67	0.48 ± 0.53	0.33 ± 0.60

B. Time in Closed Regions

		Buprenorphine (mg/kg)		
	Saline	**0.3**	**1**	**10**
Minutes (Mean ± SD)	4.17 ± 0.52	4.10 ± .0.67	4.52 ± 0.53	4.67 ± 0.60

C. Latency to Enter Open Region

		Buprenorphine (mg/kg)		
	Saline	**0.3**	**1**	**10**
Seconds (Mean ± SD)	23.20 ± 35.36	6.00 ± 3.59	55 ± 90.85	107 ± 115.07

Table 3. Representation of the mean and standard deviation (SD) of time in open regions and closed regions (min) (A, B) and latency to enter the open region (s) (C) for each dependent variable.

Discussion

The novel finding to emerge from the present study is that all three dependent measures indicate that buprenorphine (10 mg/kg) increased anxiety-like behavior in this sample of B6 mice. Three doses of buprenorphine (0.3, 1, and 10 mg/kg) were studied, and the 10 mg/kg dose was the only dose to have a significant effect on time spent in the open and closed regions, along with the latency to enter the open region. Given that previous papers report buprenorphine-induced anxiolysis, what factors might explain the present results? Methodological factors and potential confounding variables should be addressed as results from this study are interpreted within the context of previous literature.

Potential methodological reasons for buprenorphine-induced anxiogenesis: To the best of my knowledge, this is the first study to investigate the effects of buprenorphine on anxiety-like behavior in B6 mice as measured using the EZM. Previous studies have reported anxiolytic and antidepressant effects of buprenorphine on rodents using alternative measures of anxiety-like behavior, such as the forced swim and novelty-induced hypophagia test. One such study included in Table 1 reported anxiolysis using the novelty-induced hypophagia test, but did not achieve these same effects using the EPM (Almatroudi et al., 2018). As discussed in the introduction, the

EZM is a modified version of the EPM, and use of the EZM has been demonstrated to be a reliable measure of anxiety-like behavior in different rodent species. The 5-min recording time on the maze and the dependent variables measured were selected based on previous literature employing the EZM (Braun et al., 2011; Shepherd et al., 1994; Tucker & McCabe, 2017b). Differences between the present effects of buprenorphine on anxiety-like behavior may be due in part to the different measures used to operationally measure anxiety-like behavior. The EZM relies on the inherent apprehension of the mouse to approach the open regions of the maze, which may be perceived as more dangerous than the dark, enclosed regions. The novelty-induced hypophagia test relies on hyponeophagia (novelty-suppressed feeding), and the dependent variable of interest is the latency to enter the center of an arena and engage in eating behavior. Both tests appear to provide a reliable measure of anxiety-like behavior in rodents based on previous literature. However, inconsistent findings suggest a need for further comparison of and refinement of paradigms used to assess anxiety-like behavior in rodents. They also highlight the complexity of anxiety-like behavior and the inherent difficulty in characterizing this behavior in animals.

Also worthy of discussion is the present experimental design to administer three doses of buprenorphine (0.3, 1, and 10 mg/kg). Buprenorphine 10 mg/kg was the only dose to cause a statistically significant effect on all three dependent variables. The 0.3 mg/kg dose was chosen, because it has been shown to be antinociceptive in B6 mice (Glovak et al., 2017), and the two higher doses are half a log unit and a whole log unit greater than the first dose, respectively. Buprenorphine is unique from other MOR agonists in that analgesia and respiratory depression are not linearly related to dosage. One study characterized the dose-response effects of buprenorphine on nociception, reporting a bell-shaped curve for antinociception, whereby 1.0

mg/kg caused significantly greater antinociception than the 3.0 mg/kg dose (Lutfy et al., 2003). A dose-response curve for the effects of buprenorphine on anxiety-like behavior has yet to be reported. The buprenorphine-induced anxiogenesis observed in the current study encourages future dose-response studies.

Opioid-induced motor activity and sleep disruption as potential confounds for anxiety-like behavior: Motor activity is another variable that is directly impacted by opioid administration in rodents. Previous studies report that opioids induce rapid and repetitive bouts of motor activity (circling), which are followed by periods of complete immobility (Andrew Mickley et al., 1990; Brase et al., 1977; Murphy et al., 2001). As opioid doses increase above analgesic ranges, these periods of locomotion become more visible and are correlated with respiratory variables, such as ventilatory depression (Haouzi et al., 2021). Some studies have reported a biphasic effect of opioids on locomotion, whereby low doses decrease locomotion and higher doses increase it (Patti et al., 2005). Falcon et. al, 2015 demonstrated that buprenorphine doses ranging from 0.065-2 mg/kg all caused significant increases in locomotor activity as relative to saline. Based on these findings, it is expected that high doses of buprenorphine would increase bouts of locomotion in mice. Total motor activity was not measured in the present study but should be taken into consideration when interpreting the results.

Relevant to motor activity, our lab has previously shown that opioids significantly disrupt the temporal organization of sleep/wake states. We have shown that fentanyl and morphine both disrupt sleep in a dose-dependent manner, with higher doses eliminating sleep altogether (Zebadua Unzaga et al., 2021). Notably, our lab demonstrated that opioid-induced sleep disruption was independent of enhanced motor activity (Zebadua Unzaga et al., 2022). Additionally, antinociceptive doses of buprenorphine eliminated NREM and REM sleep and

significantly altered electroencephalogram frequency bands (O'Brien et al., 2021). These findings are relevant to the present study in that enhanced levels of arousal in a novel environment could promote anxiety-like behavior. As opioids have been shown to disrupt sleep and promote wakefulness in a dose-dependent manner, it is conceivable that the 10 mg/kg dose of buprenorphine had the greatest effect on arousal. Buprenorphine-induced anxiogenesis, then, could be secondary to increased arousal in the novel environment of the EZM. Although not quantified in the present study, the locomotor behavior of mice receiving 10 mg/kg buprenorphine was not noticeably different from the locomotor behavior associated with lower doses of buprenorphine.

Limitations, Conclusions, and Future Directions: The major finding of the present study is that buprenorphine at 10 mg/kg induced anxiogenesis in B6 mice as measured using the EZM. This dose caused a statistically significant decrease in time spent in the open regions, increase in time spent in the closed regions, and increase in the latency to enter the open region. Anxiety is known to be sexually dimorphic (Kokras et al., 2012) and to vary as a function of age (Borodovitsyna et al., 2022). The present results encourage future studies of the effects of buprenorphine on anxiety-like behavior in female B6 mice and in male and female mice across a range of ages. The buprenorphine-induced anxiogenesis observed in the current study encourages a future dose-response study of anxiety-like behavior that spans 5 to 7 log units of buprenorphine concentration. The present results also raise questions regarding the role of endogenous opioids as potential contributors to anxiety-like behavior in B6 mice. This question could be addressed by future studies on the effects of naloxone on anxiety-like behavior.

Both opioid abuse (Mauro et al., 2022) and anxiety-related disorders (Daly & Robinson, 2022) continue to be major public health concerns in the US, and the COVID-19 pandemic has

significantly heightened these concerns (Alexander et al., 2020; Asmundson et al., 2022). Continued basic research on the relationship between opioids and anxiety-like behavior has potential relevance for mental health (Miller, 2022). The results from my honors thesis provide a novel finding that will inform future studies aiming to better understand opioid-induced changes in behavior.

References

Ahmadi, J., & Jahromi, M. S. (2017). Anxiety Treatment of Opioid Dependent Patients with Buprenorphine: A Randomized, Double-blind, Clinical Trial. *Indian journal of psychological medicine*, *39*(4), 445-449. https://doi.org/10.4103/0253-7176.211765

Ahmadpanah, M., Reihani, A., Ghaleiha, A., Soltanian, A., Haghighi, M., Jahangard, L., Sadeghi Bahmani, D., Holsboer-Trachsler, E., & Brand, S. (2017). Buprenorphine augmentation improved symptoms of OCD, compared to placebo - Results from a randomized, double-blind and placebo-controlled clinical trial. *J Psychiatr Res*, *94*, 23-28. https://doi.org/10.1016/j.jpsychires.2017.06.004

Alexander, G. C., Stoller, K. B., Haffajee, R. L., & Saloner, B. (2020). An Epidemic in the Midst of a Pandemic: Opioid Use Disorder and COVID-19. *Annals of Internal Medicine*, *173*(1), 57-58. https://doi.org/10.7326/M20-1141

Almatroudi, A., Ostovar, M., Bailey, C. P., Husbands, S. M., & Bailey, S. J. (2018). Antidepressant-like effects of BU10119, a novel buprenorphine analogue with mixed κ/μ receptor antagonist properties, in mice. *British Journal of Pharmacology*, *175*(14), 2869-2880. https://doi.org/10.1111/bph.14060

Andrew Mickley, G., Mulvihill, M. A., & Postler, M. A. (1990). Brain μ and δ opioid receptors mediate different locomotor hyperactivity responses of the C57BL/6J mouse. *Psychopharmacology*, *101*(3), 332-337. https://doi.org/10.1007/BF02244050

Asmundson, G. J. G., Rachor, G., Drakes, D. H., Boehme, B. A. E., Paluszek, M. M., & Taylor, S. (2022). How does COVID stress vary across the anxiety-related disorders? Assessing factorial invariance and changes in COVID Stress Scale scores during the pandemic. *Journal of Anxiety Disorders*, *87*, 102554. https://doi.org/https://doi.org/10.1016/j.janxdis.2022.102554

Bailey, K. R., & Crawley, J. N. (2009). Anxiety-related behaviors in mice. In *Methods of behavioral analysis in neuroscience, 2nd ed.* (pp. 77-101). CRC Press/Routledge/Taylor & Francis Group.

Blanco, C., Wiley, T. R. A., Lloyd, J. J., Lopez, M. F., & Volkow, N. D. (2020). America's opioid crisis: the need for an integrated public health approach. *Translational Psychiatry*, *10*(1). https://doi.org/10.1038/s41398-020-0847-1

Borodovitsyna, O., Tkaczynski, J. A., Corbett, C. M., Loweth, J. A., & Chandler, D. J. (2022). Age- and Sex-Dependent Changes in Locus Coeruleus Physiology and Anxiety-Like Behavior Following Acute Stressor Exposure [Original Research]. *Frontiers in behavioral neuroscience*, *16*. https://doi.org/10.3389/fnbeh.2022.808590

Brady, K. T., McCauley, J. L., & Back, S. E. (2016). Prescription Opioid Misuse, Abuse, and Treatment in the United States: An Update. *American Journal of Psychiatry*, *173*(1), 18-26. https://doi.org/10.1176/appi.ajp.2015.15020262

Brase, D. A., Loh, H. H., & Way, E. L. (1977). Comparison of the effects of morphine on locomotor activity, analgesia and primary and protracted physical dependence in six mouse strains. *J Pharmacol Exp Ther*, *201*(2), 368-374.

Braun, A. A., Skelton, M. R., Vorhees, C. V., & Williams, M. T. (2011). Comparison of the elevated plus and elevated zero mazes in treated and untreated male Sprague–Dawley rats: Effects of anxiolytic and anxiogenic agents. *Pharmacology Biochemistry and Behavior*, *97*(3), 406-415. https://doi.org/10.1016/j.pbb.2010.09.013

Camilleri, M. (2011). Opioid-Induced Constipation: Challenges and Therapeutic Opportunities. *Official journal of the American College of Gastroenterology | ACG*, *106*(5), 835-842. https://doi.org/10.1038/ajg.2011.30

Colasanti, A., Rabiner, E. A., Lingford-Hughes, A., & Nutt, D. J. (2011). Opioids and anxiety. *J Psychopharmacol*, *25*(11), 1415-1433. https://doi.org/10.1177/0269881110367726

Council, N. R. (2011). *Guide for the Care and Use of Laboratory Animals: Eighth Edition*. The National Academies Press. https://doi.org/doi:10.17226/12910

Daly, M., & Robinson, E. (2022). Depression and anxiety during COVID-19. *The Lancet*, *399*(10324), 518. https://doi.org/10.1016/s0140-6736(22)00187-8

Dasgupta, N., Beletsky, L., & Ciccarone, D. (2018). Opioid Crisis: No Easy Fix to Its Social and Economic Determinants. *American Journal of Public Health*, *108*(2), 182-186. https://doi.org/10.2105/ajph.2017.304187

Davis, M. P. (2012). Twelve reasons for considering buprenorphine as a frontline analgesic in the management of pain. *J Support Oncol*, *10*(6), 209-219. https://doi.org/10.1016/j.suponc.2012.05.002

Eacret, D., Veasey, S. C., & Blendy, J. A. (2020). Bidirectional Relationship between Opioids and Disrupted Sleep: Putative Mechanisms. *Molecular Pharmacology*, *98*(4), 445-453. https://doi.org/10.1124/mol.119.119107

Falcon, E., Maier, K., Robinson, S. A., Hill-Smith, T. E., & Lucki, I. (2015). Effects of buprenorphine on behavioral tests for antidepressant and anxiolytic drugs in mice. *Psychopharmacology (Berl)*, *232*(5), 907-915. https://doi.org/10.1007/s00213-014-3723-y

Fujii, K., Koshidaka, Y., Adachi, M., & Takao, K. (2019). Effects of chronic fentanyl administration on behavioral characteristics of mice. *Neuropsychopharmacology Reports*, *39*(1), 17-35. https://doi.org/10.1002/npr2.12040

Glovak, Z., Mihalko, S., Baghdoyan, H. A., & Lydic, R. (2017). Leptin status alters buprenorphine-induced antinociception in obese mice with dysfunctional leptin receptors. *Neurosci Lett*, *660*, 29-33. https://doi.org/10.1016/j.neulet.2017.09.012

Haouzi, P., McCann, M., & Tubbs, N. (2021). Respiratory effects of low and high doses of fentanyl in control and β-arrestin 2-deficient mice. *Journal of Neurophysiology*, *125*(4), 1396-1407. https://doi.org/10.1152/jn.00711.2020

Khubchandani, J., Sharma, S., Webb, F. J., Wiblishauser, M. J., & Bowman, S. L. (2021). Post-lockdown depression and anxiety in the USA during the COVID-19 pandemic. *Journal of Public Health*, *43*(2), 246-253. https://doi.org/10.1093/pubmed/fdaa250

Klimas, J., Gorfinkel, L., Fairbairn, N., Amato, L., Ahamad, K., Nolan, S., Simel, D. L., & Wood, E. (2019). Strategies to Identify Patient Risks of Prescription Opioid Addiction

When Initiating Opioids for Pain. *JAMA Network Open*, *2*(5), e193365. https://doi.org/10.1001/jamanetworkopen.2019.3365

Kokras, N., Dalla, C., Sideris, A. C., Dendi, A., Mikail, H. G., Antoniou, K., & Papadopoulou-Daifoti, Z. (2012). Behavioral sexual dimorphism in models of anxiety and depression due to changes in HPA axis activity. *Neuropharmacology*, *62*(1), 436-445. https://doi.org/https://doi.org/10.1016/j.neuropharm.2011.08.025

Kosten, T., & George, T. (2002). The Neurobiology of Opioid Dependence: Implications for Treatment. *Science & Practice Perspectives*, *1*(1), 13-20. https://doi.org/10.1151/spp021113

Li, X., Zhang, J., Niu, R., Manthari, R. K., Yang, K., & Wang, J. (2019). Effect of fluoride exposure on anxiety- and depression-like behavior in mouse. *Chemosphere*, *215*, 454-460. https://doi.org/https://doi.org/10.1016/j.chemosphere.2018.10.070

Lutfy, K., Eitan, S., Bryant, C. D., Yang, Y. C., Saliminejad, N., Walwyn, W., Kieffer, B. L., Takeshima, H., Carroll, F. I., Maidment, N. T., & Evans, C. J. (2003). Buprenorphine-Induced Antinociception Is Mediated by μ-Opioid Receptors and Compromised by Concomitant Activation of Opioid Receptor-Like Receptors. *The Journal of Neuroscience*, *23*(32), 10331-10337. https://doi.org/10.1523/jneurosci.23-32-10331.2003

Lynch, S., & Benhamou, O.-M. (2019). Depression: What's Buprenorphine Got to Do With It? *American Journal of Psychiatry Residents' Journal*, *14*(2), 5-7. https://doi.org/10.1176/appi.ajp-rj.2019.140202

Mauro, P. M., Gutkind, S., Annunziato, E. M., & Samples, H. (2022). Use of Medication for Opioid Use Disorder Among US Adolescents and Adults With Need for Opioid Treatment, 2019. *JAMA Network Open*, *5*(3), e223821-e223821. https://doi.org/10.1001/jamanetworkopen.2022.3821

Miller, B. F. (2022). Making progress on mental illness. *Science*, *375*(6582), 728-728. https://doi.org/doi:10.1126/science.abn8143

Moody, L. N., Satterwhite, E., & Bickel, W. K. (2017). Substance use in rural Central Appalachia: Current status and treatment considerations. *Journal of Rural Mental Health*, *41*(2), 123-135. https://doi.org/10.1037/rmh0000064

Murphy, N. P., Lam, H. A., & Maidment, N. T. (2001). A comparison of morphine-induced locomotor activity and mesolimbic dopamine release in C57BL6, 129Sv and DBA2 mice. *Journal of Neurochemistry*, *79*(3), 626-635. https://doi.org/https://doi.org/10.1046/j.1471-4159.2001.00599.x

National Academies of Sciences, E., & Medicine. (2017). *Pain Management and the Opioid Epidemic: Balancing Societal and Individual Benefits and Risks of Prescription Opioid Use*. The National Academies Press. https://doi.org/doi:10.17226/24781

O'Brien, C. B., Locklear, C. E., Glovak, Z. T., Unzaga, D. Z., Baghdoyan, H. A., & Lydic, R. (2021). Opioids cause dissociated states of consciousness in C57BL/6J mice. *Journal of Neurophysiology*, *126*(4), 1265-1275. https://doi.org/10.1152/jn.00266.2021

Patti, C. L., Frussa-Filho, R., Silva, R. H., Carvalho, R. C., Kameda, S. R., Takatsu-Coleman, A. L., Cunha, J. L. S., & Abílio, V. C. (2005). Behavioral characterization of morphine

effects on motor activity in mice. *Pharmacology Biochemistry and Behavior*, *81*(4), 923-927. https://doi.org/https://doi.org/10.1016/j.pbb.2005.07.004

Pendergrass, S. A., Crist, R. C., Jones, L. K., Hoch, J. R., & Berrettini, W. H. (2019). The importance of buprenorphine research in the opioid crisis. *Molecular Psychiatry*, *24*(5), 626-632. https://doi.org/10.1038/s41380-018-0329-5

Percie Du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., Maccallum, C. J., Macleod, M., . . . Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLOS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Popovitz, J., Mysore, S. P., & Adwanikar, H. (2019). Long-Term Effects of Traumatic Brain Injury on Anxiety-Like Behaviors in Mice: Behavioral and Neural Correlates. *Frontiers in behavioral neuroscience*, *13*, 6-6. https://doi.org/10.3389/fnbeh.2019.00006

Rezayof, A., Hosseini, S.-S., & Zarrindast, M.-R. (2009). Effects of morphine on rat behaviour in the elevated plus maze: The role of central amygdala dopamine receptors. *Behavioural Brain Research*, *202*(2), 171-178. https://doi.org/10.1016/j.bbr.2009.03.030

Rosenblum, A., Marsch, L. A., Joseph, H., & Portenoy, R. K. (2008). Opioids and the Treatment of Chronic Pain: Controversies, Current Status, and Future Directions. *Experimental and clinical psychopharmacology*, *16*(5), 405-416. https://doi.org/10.1037/a0013628

Rosoff, D. B., Smith, G. D., & Lohoff, F. W. (2021). Prescription Opioid Use and Risk for Major Depressive Disorder and Anxiety and Stress-Related Disorders. *JAMA Psychiatry*, *78*(2), 151. https://doi.org/10.1001/jamapsychiatry.2020.3554

Sakamoto, T., Sugimoto, S., & Uekita, T. (2019). Effects of intraperitoneal and intracerebroventricular injections of oxytocin on social and emotional behaviors in pubertal male mice. *Physiology & Behavior*, *212*, 112701. https://doi.org/https://doi.org/10.1016/j.physbeh.2019.112701

Santomauro, D. F., Mantilla Herrera, A. M., Shadid, J., Zheng, P., Ashbaugh, C., Pigott, D. M., Abbafati, C., Adolph, C., Amlag, J. O., Aravkin, A. Y., Bang-Jensen, B. L., Bertolacci, G. J., Bloom, S. S., Castellano, R., Castro, E., Chakrabarti, S., Chattopadhyay, J., Cogen, R. M., Collins, J. K., . . . Ferrari, A. J. (2021). Global prevalence and burden of depressive and anxiety disorders in 204 countries and territories in 2020 due to the COVID-19 pandemic. *The Lancet*, *398*(10312), 1700-1712. https://doi.org/10.1016/s0140-6736(21)02143-7

Schalkoff, C. A., Lancaster, K. E., Gaynes, B. N., Wang, V., Pence, B. W., Miller, W. C., & Go, V. F. (2020). The opioid and related drug epidemics in rural Appalachia: A systematic review of populations affected, risk factors, and infectious diseases. *Substance Abuse*, *41*(1), 35-69. https://doi.org/10.1080/08897077.2019.1635555

Schiller, E. Y., Goyal, A., & Mechanic, O. J. (2022). Opioid Overdose. In *StatPearls*. StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC.

Shepherd, J. K., Grewal, S. S., Fletcher, A., Bill, D. J., & Dourish, C. T. (1994). Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology*, *116*(1), 56-64. https://doi.org/10.1007/bf02244871

Sher, L. (2016). Buprenorphine and the treatment of depression, anxiety, non-suicidal self-injury, and suicidality. *Acta Psychiatr Scand*, *134*(1), 84-85. https://doi.org/10.1111/acps.12577

Tucker, L. B., & McCabe, J. T. (2017a). Behavior of Male and Female C57BL/6J Mice Is More Consistent with Repeated Trials in the Elevated Zero Maze than in the Elevated Plus Maze. *Frontiers in behavioral neuroscience*, *11*, 13-13. https://doi.org/10.3389/fnbeh.2017.00013

Tucker, L. B., & McCabe, J. T. (2017b). Behavior of Male and Female C57BL/6J Mice Is More Consistent with Repeated Trials in the Elevated Zero Maze than in the Elevated Plus Maze. *Frontiers in Behavioral Neuroscience, 11*. https://doi.org/10.3389/fnbeh.2017.00013

Velander, J. R. (2018). Suboxone: Rationale, Science, Misconceptions. *The Ochsner journal*, *18*(1), 23-29. https://pubmed.ncbi.nlm.nih.gov/29559865

Volkow, N. D. (2020). Collision of the COVID-19 and Addiction Epidemics. *Annals of Internal Medicine*, *173*(1), 61-62. https://doi.org/10.7326/M20-1212

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*, *2*(2), 322-328. https://doi.org/10.1038/nprot.2007.44

Zaig, S., Da Silveira Scarpellini, C., & Montandon, G. (2021). Respiratory depression and analgesia by opioid drugs in freely behaving larval zebrafish. *eLife, 10*. https://doi.org/10.7554/elife.63407

Zebadua Unzaga, D., Bustamante, C., Thibert, M., & Baghdoyan, H. (2021). Fentanyl and morphine cause dose-dependent sleep disruption in C57BL/6J (B6) mice. *The FASEB Journal*, *35*(S1). https://doi.org/https://doi.org/10.1096/fasebj.2021.35.S1.01706

Zebadua Unzaga, D., Zhu, X., Price, J., Lydic, R., Baghdoyan, H. (2022). Fentanyl-induced sleep disruption in C57BL/6J (B6) mice is not secondary to increased motor activity or hypothermia. *The FASEB Journal, 36.* (In Press).