Anxiety-like Behavior in C57BL/6J Mice is Sexually Dimorphic and Altered by Buprenorphine

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Abstract: Buprenorphine is one of a small number of FDA approved drugs used in medication assisted therapy for opioid use disorder (OUD). Recently, buprenorphine has also been suggested to have therapeutic effects for anxiety in humans. Some preclinical studies show support for the anxiolytic effects of systemically administered buprenorphine, however other studies present contrasting evidence. A previous study from our laboratory found that buprenorphine increased anxiety-like behavior in male B6 mice (Thibert, 2022). The current study was designed to test the hypothesis that buprenorphine differentially alters anxiety-like behavior in female mice relative to male mice. Adult female C57BL6/J (B6) mice (n=10) were administered subcutaneous injections of saline (control) and two doses of buprenorphine (1 mg/kg and 10 mg/kg). Mice were tested on an elevated zero maze (EZM) where their behavior was recorded via digital video. The EZM provides reliable and valid measures of anxiety-like behavior using open and closed regions (Tucker & J., 2017). The Deep Lab Cut program (Luxem et al., 2023; Mathis et al., 2018) and cloud-based image processing were used to quantify the dependent measures of latency to enter the open region of the EZM, time spent in the open region, and time spent in the closed region all in seconds. Comparisons of male and female B6 mice on the EZM revealed that buprenorphine caused the male and female mice to differentially exhibit significantly ($p<.05$) decreased time spent in the open region and increased time spent in the closed region. These results support the interpretation that buprenorphine has sexually dimorphic effects on anxiety-like behavior in B6 mice.

INTRODUCTION

Achieving Reliable Pain Management Without Promoting Opioid Addiction: Pain is a phenomenon that has historically been treated in a variety of ways (Meldrum, 2003). The utilization of evidence-based practice has led to significant progress in the treatment of both severe and chronic pain (Aldington & C., 2019). For example, acute pain after surgical procedures is common in the US and opioids are often over-prescribed for the treatment of short-term pain (Serdarevic et al., 2017). Additional data indicate that 20% of Americans suffer from chronic pain which results in an extended use of pain medications (Serdarevic et al., 2017). Acute pain secondary to injury is also adaptive and well managed clinically, whereas chronic pain is less understood and could be considered as a "disease entity in itself, and not merely a symptom" (Clauw et al., 2019). The concept that pain should be regarded as "the fifth vital signal (Lucas et al., 2007) has been reviewed as a well-intended concept but one with unanticipated negative consequences. Over prescription of opioids by physicians, deceptive marketing by pharmaceutical companies, and regulatory failures by the US FDA are all unintended consequences of the initiative to treat pain as a symptom rather than a condition (Kolodny, 2020). As a result of this, more than 564,000 people have died from opioid overdoses from 1999 to 2020, including prescription and illicit opioids, as evidenced by data from the US Centers for Disease Control and Prevention (Hedegaard et al., 2021).

Opioid Use Disorder as an Addiction: Opiate binding in the mesolimbic pathway has been shown to produce a pleasant and rewarding feeling; however, modulation of this intrinsic dopaminergic pathway can cause changes to the brain circuits regulating pleasure and reward (Di Chiara & Imperato, 1988). These changes can result in a psychological and physiological dependence on opioids for the continued experience of the rewarding sensations they produce. Thus, while the immediate painful sensations can be alleviated by opioids, prolonged usage increases the risk of addiction. The desire for opioid-induced

pleasurable sensations can be classified under the category of psychic craving. This craving is commonly characterized by an emotional and psychological drive to continue using a substance that provides effects that the user deems necessary in order for them to maintain an optimal sense of health and well-being (Koob, 2016). Frequently, if these craving sensations are not resolved, then the pattern of usage for the drug increases and can potentially develop into an addiction. This specific type of addiction for this class of drugs has been termed opioid use disorder (OUD) and affects over 2.1 million people in the US (Dydyk et al., 2022). Additionally, while the incidence of OUD varies based on demographic factors, certain regions of the US are significantly more affected than others. The Appalachian region of the US exhibits a significantly higher prevalence of OUD than the non-Appalachian regions. Opioid-related deaths in the Appalachian region are 37% higher than the remainder of the U.S (Schalkoff et al., 2020). Analysis of this increased mortality suggests that socioeconomic conditions, lack of educational opportunities, and higher instances of work-related risks are contributing factors (Moody et al., 2017).

The Role of Buprenorphine in Treating Pain and Opioid Use Disorder: In terms of combatting OUD pharmacologically, a common medication prescribed to treat OUD is buprenorphine which is marked by tradenames including Subcolade™, Probuphine™, Bebluca™, Butrans™, and Buprenex™. Buprenorphine was developed in the 1960s as a synthetic analog of the alkaloid opiate thebaine and is classified as a schedule III drug meaning that it has some potential for low to moderate levels of physical or psychological dependence. Relative to the commonly prescribed opiates such as morphine and oxycodone hydrochloride, buprenorphine carries significantly lower risks for addiction while still being clinically approved to treat a variety of pain syndromes (Sher, 2016). Mechanistically, buprenorphine serves as both a high-affinity partial agonist for Mu Opioid Receptors (MOR) as well as an antagonist for Kappa Opioid Receptors. In contrast, opioids such as fentanyl and morphine act as

full MOR agonists which have been shown to cause higher incidences of respiratory depression (Davis, 2012). Thus, buprenorphine exhibits a non-linear relationship with respect to dosage and respiratory depression. This phenomenon has been coined as the “ceiling effect” (Falcon et al., 2020).

The Role of Buprenorphine in Treating Anxiety and Depression: In addition to the treatment of OUD and pain, buprenorphine has also been suggested to provide therapeutic effects for depression and anxiety (Sher, 2016). This is a potentially significant finding given the high rates of prevalence for co-existing disorders of anxiety and depression that may accompany OUD and heightened experiences of pain. Further, depression and anxiety exhibit a staggeringly high comorbidity rate that has been estimated to be between 45 and 67 percent in diagnosed patients (Kircanski et al., 2017). Thus, having a medication that could potentially treat the symptoms of both anxiety and depression would be a significant development in the field of psychiatry. However, the experimental results acquired from studies testing for anxiolysis and anti-depressive effects in response to the systemic administration of buprenorphine have not been consistent. One such study limited to adult male CD-1 mice utilizing an elevated plus maze and administration of a synthetic analogue of buprenorphine found a reduction in depressive and anxiety-like behavior (Almatroudi et al., 2018). Additional data from previous studies suggests that there are male and female differences in the states of pain and anxiety (Bartley & Fillingim, 2013). The relationship between pain syndromes and opioid dependence could potentially be expanded upon by measuring anxiety-like behavior in both male and female mice. The present study was designed to evaluate the hypothesis that buprenorphine alters anxiety-like behavior in female mice compared to male mice.

METHODS

Animals: The protocol used for this study was established productively and used by a previous study of male C57BL/6J (B6) mice (Thibert, 2022). The protocol was approved by the University of Tennessee's Institutional Animal Care and Use Committee and the study reported here followed the Arrive-2 Guidelines (Percie Du Sert et al., 2020). Adult, female B6 mice (n=10) were purchased from the Jackson Laboratory at the age of 12 weeks and housed individually. When the mice were 18 weeks old, they were anesthetized with isoflurane and implanted with a radio frequency identification chip (RFID-100B 1.4, Microchip ID). Each mouse had *ad libitum* access to food and water and were exposed to an alternating 12-hour light-dark cycle. At 20 weeks, the mice were weighed biweekly and conditioned for handling daily for 3 weeks before beginning baseline data collection.

Measuring Anxiety-Like Behavior in Mice: This study quantified the three dependent variables as 1. Time spent in the open region, 2. Time spent in the closed region, and 3. Latency to enter the open regions as representational measures of anxiety-like behavior in B6 mice. The design of the Elevated Zero Maze (EZM) was engineered based on the knowledge that mice are a prey species that have evolved to spend more time in dark and enclosed areas (Bailey, 2009). As illustrated by Figure 1, the open regions of the maze may be perceived as potentially dangerous for the mice in contrast to the enclosed regions that harbor less risks (Tucker & J., 2017). If the design features of the EZM do evoke feelings of threats in the open segments of the maze and feelings of safety in the walled portion of the EZM, then more time spent in the closed regions and an increased latency to enter the open region of the EZM are interpreted to reflect anxiety-like behavior. Conversely, more time spent in the open regions and shorter latencies to enter the open region would be interpreted as reflecting less anxiety-like behavior (Walf & Frye, 2007).

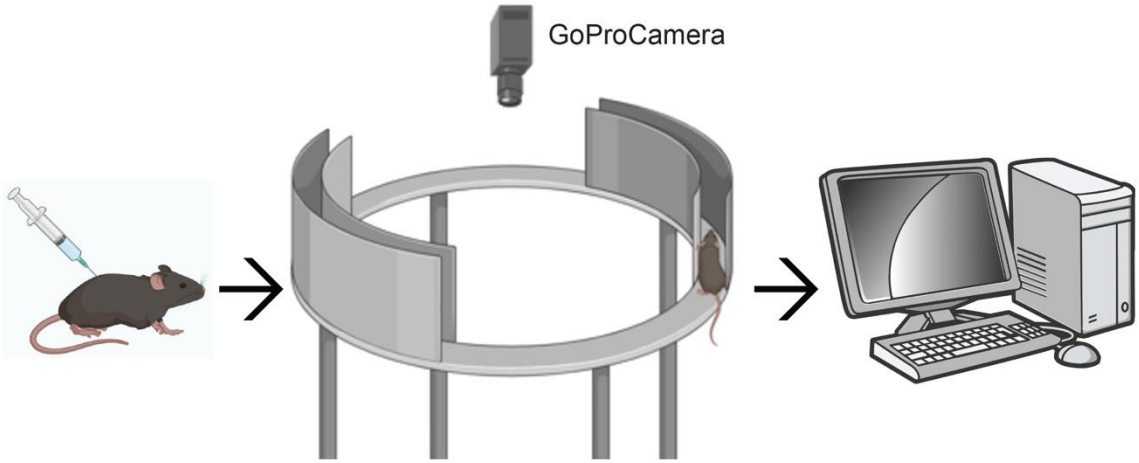


Figure 1: The Elevated Zero Maze (EZM) has two open regions and two closed regions with an overall diameter of 39.5 inches. After injections of saline (control) or buprenorphine, the mouse was placed at the border between the open and closed region as illustrated in the EZM in order to measure anxiety-like behavior in B6 mice. The mice were injected with either saline (0.3 mL) in the control condition or buprenorphine (1mg/kg or 10mg/kg) in the experimental conditions. An hour after the injection, the mice were placed on the EZM at the border between the near open and closed regions as shown above. The mice were allowed to explore the maze for 5 minutes. During this 300 second period, time spent in the open and closed regions as well as latency to enter the open region were recorded digitally and by hand-held timers. The recordings were then scored manually as well as virtually by the Deep Lab Cut and simBA programs (Luxem et al., 2023; Mathis et al., 2018).

Experimental Design for Baseline and Treatment Conditions: The use of the EZM to provide reliable and accurate measures of anxiety-like behavior in B6 mice has been confirmed previously (Tucker & J., 2017). In the initial baseline phase of the study, the mice were administered “mock injections” in the form of pinches to the same area that the injection would later be given. In the control phase of the study, the mice were administered 0.3 mL of 0.9% saline subcutaneously. Following this, the experimental phase of the study consisted of the subcutaneous administration of a 1 milligram per kilogram dose of buprenorphine followed by a 10 milligram per kilogram dose 2 weeks later. The injection volume was 0.3 mL for each condition.

Application of Pose Estimation Software: For all experiments an overhead video recorded each experiment (GoPro Camera in Fig. 1). A novel feature of the data collection for this experiment involved utilizing pose estimation through predictive modeling by the Deep Lab Cut and simBA computer programs (Luxem et al., 2023; Mathis et al., 2018). These programs extracted and quantified timing and position of each mouse in the EZM using computer generated deep-learning algorithms. This allowed for the digital quantification of the three dependent measures: latency to enter the EZM open region, time spent in the open region, and time spent in the closed region. In an effort to ensure reliability and validity, the three dependent measures were also quantified with hand-held timers. The combined utilization of the programs allows for the ability to generate up-to-date heat maps for mice activity on targeted areas of the maze, vector analysis for positional spacing and changes of direction, and body part recognition for advanced motoric analyses. These new visual representations present new trends in the data that open the doors for in-depth statistical analyses as well as further directions for future studies.

RESULTS

Each of the following figures represents each mouse by a single point on the graph. The horizontal bars represent the average of the 10 mice along with error bars to indicate the standard deviation (S.D.). The y axes represent time spent in the open regions, time spent in the closed region, and latency to enter the open region all in seconds. The dependent measures were manually scored using handheld timers, double scored by digital film, and triple scored by the Deep Lab Cut and simBA analyses programs.

EZM Behavior after Saline Administration: Figure 2 displays the dependent measures taken one hour after the mice were injected with 0.3 mL of 0.9% saline solution and placed on the maze. The average latency to enter the open region was 48.95 seconds with an S.D. of 94.65 seconds. In the open region, the mice averaged 13.82 seconds with an S.D. of 5.98 seconds. In the closed region, the mice averaged 286.89 seconds with an S.D. of 5.55 seconds. Independent T-Test results revealed a significant* sex-dependent difference in the time spent in the open region (f=3.61;, d.f.=18;p= .002) and closed region (f= 3.69;, d.f.=18 ;p= .0017). These measures provide an inverse measure of the same behavior.

EZM Behavior after Buprenorphine (1mg/kg) Administration: Figure 2 displays the dependent measures taken one hour after the mice were injected with 0.3 mL of a 1.0 milligram per kilogram solution of buprenorphine and placed on the maze. The average latency to enter the open region was 88.98 seconds with an S.D. of 128.22 seconds. In the open region, the mice averaged 7.12 seconds with an S.D. of 5.98 seconds. In the closed region, the mice averaged 292.98 seconds with an S.D. of 7.61 seconds. Independent T-Test results revealed a significant* sex-dependent difference in the time spent in the open region (f=2.13;, d.f.=18;p= .0475) and closed region (f= 2.14;, d.f.=18 ;p= .0466). These measures provide an inverse measure of the same behavior.

EZM Behavior after Buprenorphine (10mg/kg) Administration: Figure 2 displays the dependent measures taken one hour after the mice were injected with 0.3 mL of a 10.0 milligrams per kilogram solution of buprenorphine and placed on the maze. The average latency to enter the open region was 113.49 seconds with an S.D. of 119.43 seconds. In the open region, the mice averaged 5.95 seconds with an S.D. of 6.11 seconds. In the closed region, the mice averaged 294.05 seconds with an S.D. of 6.11 seconds. Independent T-Test results revealed that there were no significant* sex-dependent difference in the time spent in the open region (f=1.20;, d.f.=18;p= .2442) and closed region (f= 1.20;, d.f.=18 ;p= .2442) in this condition. These measures provide an inverse measure of the same behavior.

Anxiety-like Behavior Varied by Sex: An unpaired t-test between the male and female mice data sets revealed that there was a baseline difference in anxiety-like behavior across sex in the saline condition (control) for the same two dependent measures: time spent in the open region (P=.0027) and time spent in the closed region (P=.0020). There was also a significant difference between the saline and low dose buprenorphine condition (1mg/kg) for time spent in the open region (P=.0475) and time spent in the closed region (P=.0466).

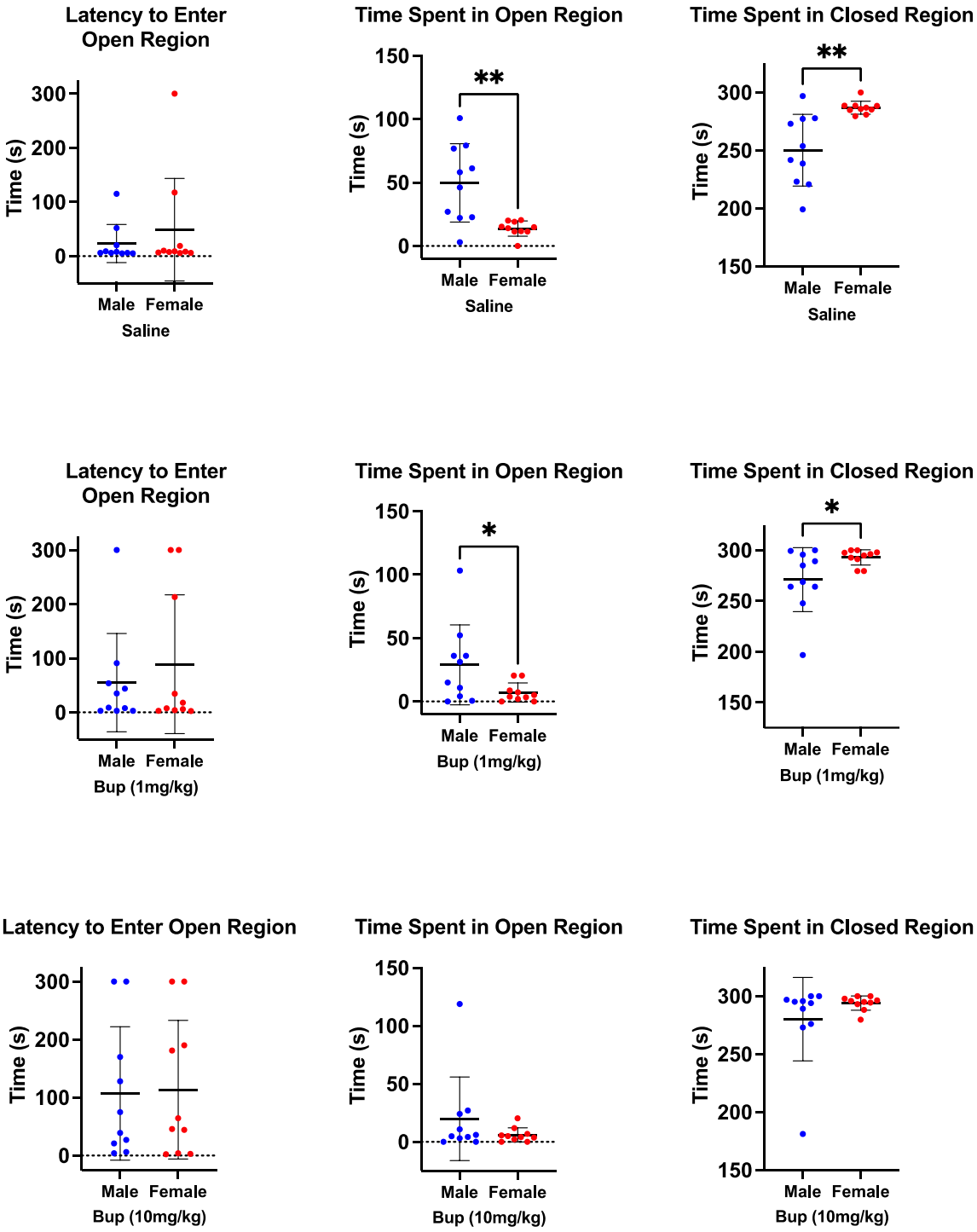


Figure 2 quantifies the dependent measures for the saline, low dose, and high dose of buprenorphine conditions. Unpaired t-test results present the mean latency ± S.D. to enter the open region, mean time spent in the open region ± S.D, and mean time spent in the closed region ± S.D in seconds. The x axis displays sex and the y axis displays latency to enter the open region, time spent in the open region, and time spent in the closed regions in seconds. A single asterisk denotes ($p<.05$) and a double asterisk denotes ($p<.01$).

Descriptive Statistics Showing Mean ± S.D. for the 3 Dependent Measures for Female B6 Mice:

The average latency to enter the open region in the saline condition was 48.95 seconds with an S.D. of 94.65 seconds, in the low dose condition (1mg/kg) it was 88.98 seconds with a standard deviation of 128.22 seconds, and in the high dose condition (10mg/kg) it was 113.49 seconds with a standard deviation of 119.43 seconds. The average time spent in the open region in the saline condition was 13.82 seconds with an S.D. of 5.98 seconds, in the low dose condition it was 7.12 seconds with an S.D. of 5.98 seconds, and in the high dose condition it was 5.95 seconds with an S.D. of 6.11 seconds. The average time spent in the closed region in the saline condition was 286.89 seconds with an S.D. of 5.55 seconds, in the low dose condition it was 292.98 seconds with an S.D. of 7.61 seconds, and in the high dose condition it was 294.05 seconds with an S.D. of 6.11 seconds.

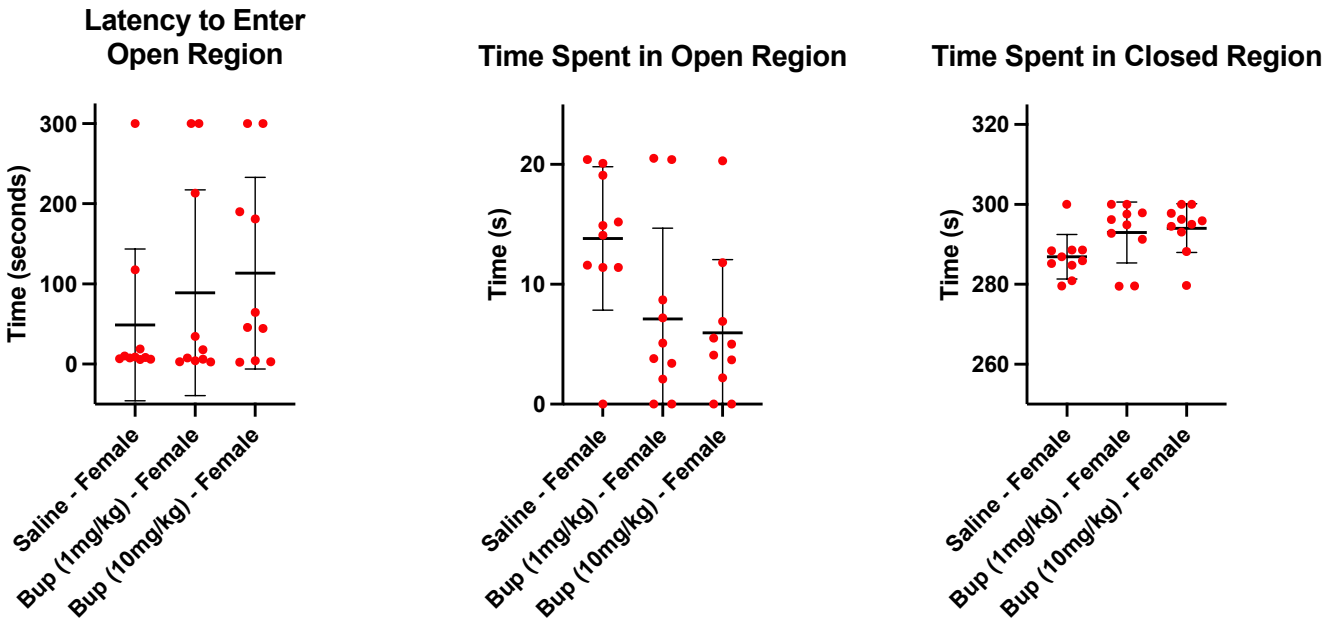


Figure 3 quantifies the effects of buprenorphine (1mg/kg & 10mg/kg) administration compared to the saline (control) condition. One-way ANNOVA analysis results revealed that there were no significant differences between the experimental groups.

Discussion

The novel results produced by this study are that two of the three dependent measures indicated the high dose of buprenorphine (10mg/kg) increased anxiety-like behavior in this sample of female B6 mice. Previous studies have shown that in male B6 mice there was no statistically significant difference in anxiety like behavior at.3 mg/kg and 1mg/kg dose compared to the baseline across the three dependent measures (Thibert, 2022). However, this study found that there no significant differences in any of the dependent variables via one-way ANNOVA analysis of the three experimental conditions. Additionally, comparisons between the current female sample and the previous study on male mice revealed that there was a statistically significant difference in the saline (baseline) condition and the low dose (1mg/kg) condition for the same two dependent variables: time spent in the open region and time spent in the closed region.

Sex and Anxiety-like Behavior: When analyzing the findings of this study, it can be observed that there are similar trends reported in human behavior as well. One such study found that there are notable differences in the rates of human anxiety across sex with female prevalence rates for anxiety disorders being reported at 30.5 percent whereas for males it was 19.2(McLean et al., 2011). Given that human females exhibit higher rates of dysfunctional forms of anxiety, they could also experience a higher predisposition towards baseline feelings of anxiety. This would be consistent with the findings summarized by Fig. 2 which revealed that there were statistically significant differences in the time spent in the open and closed regions of the EZM in the saline condition. The present study was not designed to investigate the underlying biological mechanisms that produce the observed sex-dependent differences in behavior. Population research studies have found that there is a greater clinical pain prevalence among females in conditions such as fibromyalgia, migraines, bowel syndromes, and temporal mandibular disorders (Bartley & Fillingim, 2013).

Sex and Response to Systemically Administered Opioids: Analysis of the low dose buprenorphine (1mg/kg) experimental condition revealed that there was a statistically significant difference in the time spent in the open and closed regions between the male and female mice. This supports the interpretation of a sex-dependent difference in buprenorphine effects on anxiety-like behavior in B6 mice. A meta-analysis conducted on the response to opioids for pain relief similarly found that men and women differ significantly in response to opioids for pain relief, however factors such as age, type of administration, body weight, and comorbid mental disorders significantly affected the results (Pisanu et al., 2019). With respect to the current study, these factors have been controlled for given that the mice were all the same age, had similar body weights, and were injected subcutaneously. Although the mental state of the mice is unknown, each mouse was housed, handled, and tested identically. Thus, the similarities also support the interpretation that the buprenorphine effects on anxiety-like behavior varied due to sex. Interestingly, additional human studies found that females were significantly more likely to exhibit lifetime use of prescription opioids relative to males (Serdarevic et al., 2017). This further suggests that opioids are metabolized differentially and promote varying patterns of usage based on sex. While this finding was limited to chronic opioid users, it also suggests that repeated administration of opioids can promote sexually dimorphic dependencies. This may, in part, be explained by the role of the mesolimbic pathway in providing rewarding sensations that can promote increased opioid usage. Additionally, a brain dialysis study that found that opiates preferentially increased synaptic dopamine concentrations and altered the mesolimbic system in freely moving rats (Di Chiara & Imperato, 1988). The study also found that opiate administration at low doses elicited hypermotility in the rats – a pattern also observed in the current study.

Sex and Motoric Activity: While specific motoric activity was not quantitatively recorded, observations of the mice in each experimental condition revealed a general trend of increased locomotion in the 60-minute period between the injection and when the mice were placed on the EZM.

In the saline condition the mice displayed average levels of motoric activity, however in the experimental conditions there was a noticeable difference in the activity of the mice during this incubation period. This finding is supported by the pilot study which also found increased levels of motoric activity following the subcutaneous administration of buprenorphine (Thibert, 2022). Additionally, another study that observed the effects of repeated opioid administration in rats and similarly found increased levels of locomotion following intraperitoneal injections of buprenorphine (Smith et al., 2009). This supports the current studies finding that motoric activity was increased with higher doses of opioid administration.

Dosing and Metabolism Relative to Buprenorphine: A surprising finding from this study was that the high dose of buprenorphine (10mg/kg) did not elicit significant differences in any of the three dependent measures when compared to the male cohort. While the general trends observed in the data from the saline to the experimental conditions remained consistent, the high dose condition was the only condition that did not display statistically significant differences in any dependent measures with respect to sex. Rather, the male and female cohorts were incredibly similar with the specific latency measures differing by only 5.7% after receiving the 10 mg/kg dose of buprenorphine. This potentially suggests that the behavioral effects are the same regardless of sex once a certain buprenorphine dose is administered. While there was an established difference in the baseline levels of anxiety-like behavior, the high dose administration could potentially induce an altered neural mechanism that remains consistent across sex. This could be an example of an evolutionarily conserved mechanism given the role of endogenous opiates in the stress response. While stress and anxiety are two distinct states, studies have found direct positive correlations between the two and this would explain the increase in anxiety-like behavior following administration of the high doses of buprenorphine observed in the current study (Daviu et al., 2019).

Limitations, Conclusions, and Future Directions: One potential limitation of the present study is that the experimental design did not measure ovulation of the female mice. While the trials were conducted on the same day and time of each week during the 3-month experimental period, the estrous cycle for female B6 mice is typically 4-5 days long. Thus, at each week interval, the female mice were likely at different stages of their ovulation period. Such ovulatory variability would be anticipated to work against a unified hormonal effect on anxiety-like behavior caused by buprenorphine. Another obvious limitation of the present study was the use of only the 1.0 mg/kg and 10.0 mg/kg doses of buprenorphine. However, previous studies of B6 mice from out laboratory showed that systemic buprenorphine doses in this range cause significant changes in breathing (Glovak, 2022). Additional studies report that even lower doses of buprenorphine (0.3mg/kg) administered to B6 mice significantly altered fundamental behaviors such as nociception (Glovak, 2017) and states of sleep and wakefulness (O'Brien, 2021). The present finding that lower instead of higher doses of buprenorphine significantly altered anxiety-like behavior in a sex-dependent manner encourages future studies of buprenorphine ranging from 0.3 to 1.0 mg/kg.

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