5-2023

Buprenorphine and Anxiety-Like Behavior in C57BL/6J Mice with Diet-Induced Obesity

Nicole Angelina Mickels
nmickels@vols.utk.edu

Buprenorphine and Anxiety-Like Behavior in C57BL/6J Mice with Diet-Induced Obesity

UNIVERSITY OF TENNESSEE

by
Nicole Mickels
May 11, 2023

ACKNOWLEDGEMENTS

I would like to give acknowledgement and appreciation to Dr. Ralph Lydic and Dr. Helen Baghdoyan for the opportunity to work in their lab, as well as for their mentorship and guidance throughout my undergraduate research endeavors. I have learned and grown immensely as an undergraduate student, future scientist, and lifelong learner. I also thank Dr. Elizabeth Fozo for her mentorship regarding my microbiology pursuits and her support in the connection of microbiology with this topic in neuroscience.

Through conducting my research project on the effects of buprenorphine on anxiety-like behavior in diet-induced obese B6 mice, I have developed many key research skills. Reviewing literature, formulating a hypothesis, planning an experiment, and executing the experiments in an ethical and organized way have allowed me to collect data for future critical and statistical analyses. I have also learned the value of collaboration in an academic team setting. The findings from this project I hope can contribute to the progression of scientific discovery regarding opioid use, obesity, the gut microbiome, and anxiety. I believe such findings could eventually develop into clinical studies concerning diet, pharmacology, mental health, and both preventative and curative medicine overall. Because of how much I enjoyed conducting research, as well as my excitement surrounding this subject of interest, I have goals of continuing to learn and research in this subject.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Opioids and obesity are two concurrent health crises in the United States. Buprenorphine is an opioid drug prescribed for treatment of opioid use disorder. Studies in both rodent and human subjects have demonstrated potential for anxiolytic effects of buprenorphine. However, a previous study in the Lydic/Baghdoyan lab suggests that buprenorphine has anxiogenic effects on C57BL/6J (B6) mice. Gut microbiome alteration (concurrent with obesity) has been shown to modulate opioid tolerance in a study using morphine. There also exists a correlation between obesity and anxiety. Considering these multidirectional relationships between opioids, the gut microbiome, obesity, and anxiety, the present study was designed to compare anxiety-like behavior in adult, male B6 mice after a buprenorphine injection to anxiety-like behavior in adult, male B6 mice with diet-induced obesity (DIO) after a buprenorphine injection. DIO mice (n=10) were injected subcutaneously with saline and two doses of buprenorphine (1 mg/kg and 10 mg/kg). One hour post-injection, the mice were placed on the elevated zero maze (EZM) for five minutes. Latency to enter the open region for the first time and the ratio of time spent in the open and closed regions of the EZM were recorded. Experiments were recorded using a GoPro camera. Unpaired t-tests indicated no significant differences in anxiety-like behavior in DIO mice compared to lean B6 mice due to buprenorphine. The results are discussed in the context of various measures of anxiety-like behavior and opioids' relationship with an altered gut microbiome.

INTRODUCTION

Prevalent causes of morbidity and mortality in the United States include obesity and misuse of opioids (Centers for Disease Control and Prevention, 2022a, 2022c). Both disorders are polygenic and driven by complex socio-economic factors. Data from humans and mice show that opioids alter the gastrointestinal (GI) microbiome, an occurrence known as opioid-induced dysbiosis (OID) (reviewed in Mora et al., 2012; reviewed in Thomas et al., 2022; Wang & Roy, 2017). A further complexity is evidence that anxiety-like behavior in rodents is modified by alterations in the GI microbiome (reviewed in Huang & Wu, 2021). Multiple studies over the past few decades have suggested increased anxiety-like behavior in obese human subjects, as well (Cilli et al., 2013; Davis et al., 2005; reviewed in Rajan & Menon, 2017; Scott et al., 2008; Simon et al., 2006). This thesis project was encouraged by two previous studies of C57BL/6J (B6) mice. Previous studies show that morphine differentially altered the composition of the GI microbiome of lean B6 mice but not in B6 mice with diet-induced obesity. Mice with diet-induced obesity rather experienced functional differences of the gut microbiota (Blakeley-Ruiz et al., 2022). A second unpublished study provided preliminary evidence that the opioid buprenorphine increased anxiety-like behavior in normal weight B6 mice (Thibert, 2022). Considered together, these two lines of evidence encouraged the present honors thesis evaluating the hypothesis that buprenorphine would alter anxiety-like behavior in B6 mice with diet-induced obesity.

The first portion of this introduction places the present study within the context of the ongoing epidemic of opioid misuse. The second section describes this epidemic in the light of an altered GI microbiome associated with obesity. Finally, the present study

connects both prevalent disorders to analyze buprenorphine's effects on anxiety-like behavior in diet-induced obese B6 mice.

A Spring 2023 Perspective on Opioid Use Disorder in the U.S.

Opioids are clinically prescribed for management of acute and chronic pain. The addictive qualities of opioids have led to a widespread issue of opioid abuse and overdose in the United States (Centers for Disease Control and Prevention & National Center for Injury Prevention and Control, 2017). About 50,000 deaths in 2019 were related to opioid overdose (reviewed in Sumner et al., 2022). From 2019 to 2020, death by opioid overdose rates rose among all sex, age, race, and Hispanic-origin groups (Hedegaard et al., 2021). From 2020 to 2021, there was a 15% increase in the number of opioid overdose-related deaths, with 80,816 opioid overdose deaths recorded in 2021 in the United States (Centers for Disease Control and Prevention, 2022c). Opioid-induced respiratory depression contributes to about 100,000 deaths per year in the United States (National Center for Health Statistics, 2023). Regional analyses show Tennessee was the eighth highest state in the country for deaths by drug overdose in 2021, and Tennessee is the third highest state in the country for prescription drug abuse (Centers for Disease Control and Prevention, 2022b; Tennessee Bureau of Investigation).

The Opioid Buprenorphine and Its Drug Characteristics

Buprenorphine is an FDA-approved opioid prescribed to treat opioid use disorder (OUD) as part of a comprehensive treatment plan. As an initial medication for OUD that could be prescribed or distributed by a physician office, it has made medicated treatment against OUD more accessible for patients (Substance Abuse and Mental Health Services Administration, 2023). Buprenorphine works as a partial agonist for mu opioid receptors

and as an antagonist at kappa opioid receptors. As only a partial agonist compared to a full agonist, however, the effects of buprenorphine are weaker than those of heroin and morphine (reviewed in Falcon et al., 2015)

If the prescription instructions are followed, buprenorphine is safe and effective in lessening craving and dependency on opioids, increasing safety if overdose occurs, and decreasing the potential for abuse. Abuse can arise in some individuals because of buprenorphine's opioid effects. People who are not dependent on an opioid before using buprenorphine are more likely to abuse buprenorphine (Substance Abuse and Mental Health Services Administration, 2023).

Additional studies have shown that opioids can help regulate and alleviate symptoms of anxiety in both rodent and human subjects (Colasanti et al., 2011). More specifically, buprenorphine has been reported to mitigate depressive and anxiety-like behavior in mice (Falcon et al., 2015). A novel analogue of buprenorphine (BU10119) resulted in antidepressant behavior in CD-1 mice when tested in a forced swim test and a novelty-induced hypophagia task (Almatroudi et al., 2018). A study done in C57BL/6J mice showed antidepressant behavior in a forced swim test and anxiolytic behavior in a novelty-induced hypophagia test due to buprenorphine (Falcon et al., 2015).

A Spring 2023 Perspective on Obesity in the U.S.

Concurrent with the opioid crisis, clinical and morbid obesity proves to be another major health issue in the United States. From 2017 to March 2020, the obesity rate in the United States was 41.9% (reviewed in Centers for Disease Control and Prevention, 2022a). Type 2 diabetes, cancers, osteoarthritis, sleep apnea, and heart and lung diseases are all associated with obesity and increased risk of preventable and premature death

(Centers for Disease Control and Prevention, 2022a; reviewed in DiBaise et al., 2012; reviewed in Scott et al., 2008). Obesity is often attributed to overconsumption of food and/or lack of exercise. Recent research has demonstrated that gut microbiota composition (which can be caused by a high fat diet) can influence nutrient uptake and energy homeostasis and consequently can promote obesity (Blakeley-Ruiz et al., 2022; reviewed in Bliss & Whiteside, 2018; reviewed in Moran & Shanahan, 2014).

Drawing Connections Between Obesity, Buprenorphine, and Anxiety

Studies in mice fed a high fat diet have shown increased levels of lipopolysaccharide, gut permeability, and a decrease in expression of genes that regulate tight junctions in the intestinal barrier (reviewed in Moran & Shanahan, 2014). The mu, delta, and kappa opioid receptors are found in the gastrointestinal tract, and obesity promotes gut dysbiosis. Chronic use of opioids diminishes the innate immune system, making the gut more susceptible to infection and sensitivity to microbes and bacteria. The exact mechanisms by which opioids alter the gut microbiome have yet to be determined, but one possible mechanism is disruption of the intestinal epithelial barrier. This barrier protects the gut from environmental stimuli and invasion of microbes through activation of the innate immune response (reviewed in Muchhala et al., 2021). Obesity is also associated with chronic pain; obese individuals more likely than lean individuals to report pain (one study found four times more likely), and pain intensity reports are positively associated with body mass index (Hitt et al., 2007; reviewed in Okifuji & Hare, 2015; Stokes et al., 2020).

Reciprocally, the composition of the microbiome affects opioid uptake and tolerance; also, the presence of certain bacterial species can affect mental health

(reviewed in Thomas et al., 2022). One example was a study done using germ-free mice and mice that harbored fecal microbiota from wild type mice. The results show that the germ-free mice did not demonstrate analgesic tolerance to morphine like the mice with the fecal microbiota (reviewed in Muchhala et al., 2021). Additional studies have shown that the gut microbiota affects various players in the generation of anxiety, including the vagus nerve, the hypothalamic-pituitary-adrenal axis, tryptophan metabolism, and neurotransmitter production (Huang & Wu, 2021). The complex multi-directional relationships between gut microbiota, obesity, and opioid use raised questions of obesity and opioids' effects on brain behavior, such as anxiety.

Hypothesis of this Thesis

Previously in the Lydic/Baghdoyan laboratory, the effects of the opioid buprenorphine on anxiety-like behavior in lean male B6 mice have been investigated. That study hypothesized that buprenorphine would decrease levels of anxiety, but the results indicated that buprenorphine induced anxiety-like behavior (Thibert, 2022). The present study was designed to test the hypothesis that male B6 mice with diet-induced obesity (DIO) would also reveal influences on anxiety-like behavior caused by buprenorphine.

MATERIALS AND METHODS

Methods

All procedures in this study were approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) under protocol #2555 and followed ARRIVE guidelines (Percie du Sert et al., 2020). The subjects in this study were adult, male C57BL/6J (B6) mice (n=10) from the Jackson Laboratory. Once received, the

mice were housed together and fed a 60 kcal% fat diet (D12492, Research Diets, Inc., New Brunswick, NJ). Mice are classified by Jackson Laboratory as a model of diet-induced obese (DIO; Stock #38005). Mice weighed an average of 40.1 g (ranging from 32.8 g to 47.1 g) for baseline experiments, an average of 43.3 g (ranging from 34.4 g to 48.7 g) for saline control experiments, an average of 45.9 g (ranging from 38.9 g to 51.1 g) for buprenorphine 1 mg/kg experiments, and an average of 44.9 g (ranging from 37.2 g to 50.3 g) for buprenorphine 10 mg/kg experiments. The mice had *ad libitum* access to food and water, and they were housed in a 12 hour/12 hour light-dark cycle. To identify each mouse, the mice were anesthetized with isoflurane, and a radio frequency identification chip (RFID-100B 14, Trovan, Microchip ID) was inserted subcutaneously at the level of the scapula. Mouse health was observed daily by trained and certified laboratory members. Each mouse was weighed and handled for sixteen days prior to beginning experiments, and each mouse was handled almost daily in between experiment days. This continuous conditioning was performed to mitigate stress caused by handling (Ghosal et al., 2015).

To quantify measures of anxiety-like behavior, time spent in the open and closed regions of an elevated zero maze (EZM), as well as the latency to enter the open region of the EZM, were timed and tested. An image of the EZM is shown below in Figure 1.

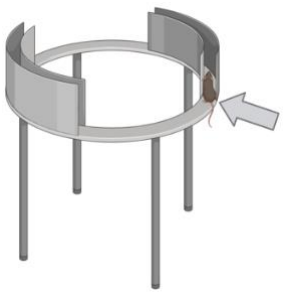

Figure 1. The elevated zero maze (EZM) has dimensions of a diameter of 39.5 inches, circumference of 10.3 ft, and height of 25.5 inches. The arrow in this image illustrates the location on the EZM where a mouse was placed when starting behavioral assessment. Modified from (Thibert, 2022). Image created with BioRender.com.

Mice are a nocturnal prey species and show a preference for the closed portions within the wall of the EZM. Greater time spent in open regions

and/or a decreased latency to enter the open region are indications of anxiolysis (non-anxiety-like behavior). Contrarily, greater time spent in closed regions and/or an increased latency to enter the open region are indications of anxiogenesis (anxiety-like behavior). Many laboratories have shown mouse behavior on the EZM to provide a valid and reliable measure of anxiety-like behavior (reviewed in Braun et al., 2011; Shepherd, 1994; Tucker & McCabe, 2017). At the beginning of each experiment, a mouse was placed at the barrier of the open and closed region, with its head facing into the closed region. Mice were scored on their presence in an open or closed region based on the entirety of the mouse body and tail in that region.

The first experiments performed in the study were three baseline measurements. The purpose of these measurements was to condition the mice to the process of the experiment: the mock injection, an hour of waiting, and five minutes spent on the EZM. Conditioning the mice to this process mitigated their anxiety levels as a confounding factor in the saline and buprenorphine experiments. The second reason three baseline measurements were performed was to ensure the EZM would provide consistent results and therefore could act as a reliable measurement of the desired dependent variables. For baseline experiments, each mouse received a mock injection (tip of a pen cap lightly poked to the area of future subcutaneous injection studies). In the next phase of the experiment, each mouse received a 0.3 mL injection of 0.9% saline. This treatment served as the control treatment. For the next phase of the experiment, each mouse received a 0.3 mL injection of 1 mg/kg buprenorphine. In the final phase of experimentation, each mouse received a 0.3 mL injection of 10 mg/kg buprenorphine. For all experiments—baseline, saline, buprenorphine 1 mg/kg, buprenorphine 10

mg/kg— each mouse waited an hour post-injection before being placed on the EZM for five minutes. All injections and the time periods of post-injection waiting took place in the experiment room. Each experiment was recorded with a GoPro Camera that was mounted to the ceiling above the EZM. The digital videos were uploaded to a software cloud and taken for further scoring and data analyses using DeepLabCut and simBA software (Luxem et al., 2023; Mathis et al., 2018; Mathis et al., 2020).

The flow of the experiment methods is portrayed in Figure 2 below.

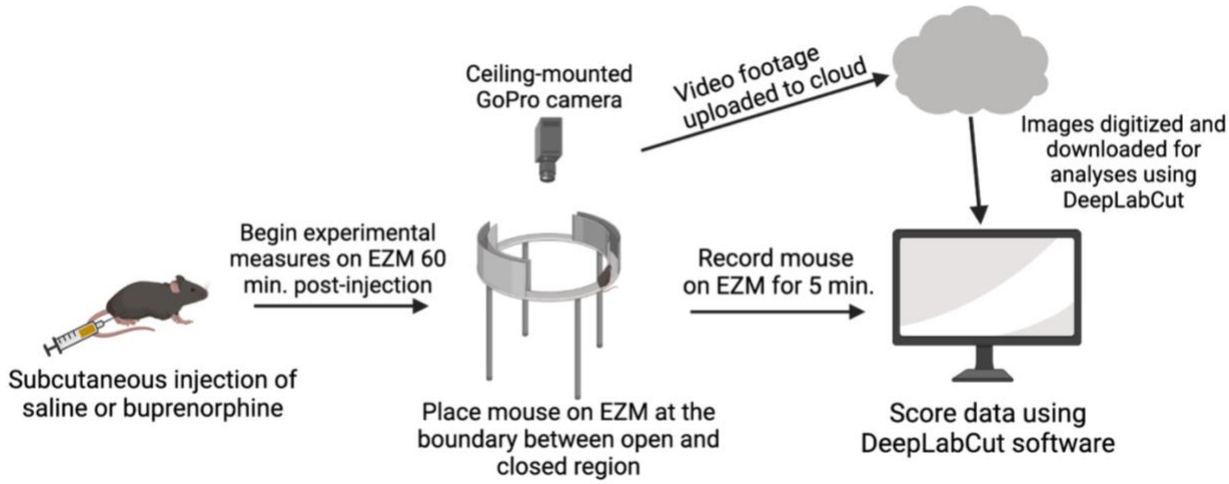

Figure 2. Work flow of experiments measuring the effects of buprenorphine on anxiety-like behavior in DIO mice. Each mouse received a mock injection, saline injection, or buprenorphine injection (all injections were subcutaneous). Sixty minutes after injection, each mouse was placed on the EZM for five minutes. Time spent in the open regions, time spent in the closed regions, and latency to enter the open regions were recorded digitally and via the GoPro camera mounted on the ceiling. DeepLabCut will be used for continued data analysis in the future. Image created with BioRender.com.

RESULTS

Descriptive statistics for DIO mouse performance on EZM

Baseline Studies: Figure 3 displays the results of baseline measurements on the EZM taken one hour post mock-injection. Mice spent an average (± SD) of 48.10 (±12.36) seconds (16%) in the open regions of the EZM, and they spent an average (± SD) of

251.57 (±12.39) seconds (84%) in the closed regions. The mean (± SD) latency to enter the open region for the first transition from closed to open region was 22.54 (±12.35) seconds.

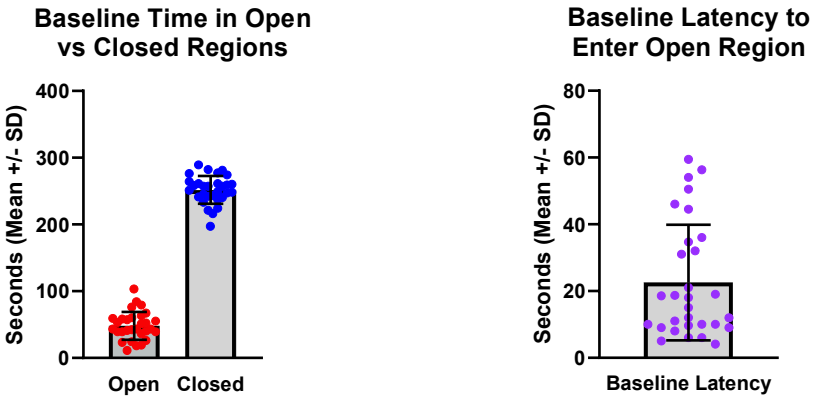

Figure 3. Baseline data across three trials per subject displayed in seconds, with mean shown by the vertical gray bar and standard deviation represented by the vertical black line. The 30 dots on each bar summarize 3 time measures for each of the 10 mice. Seconds spent in the open regions and closed regions (left), and latency to enter the open region for the first time (right) were recorded. The red dots represent time spent in the open regions, blue dots represent time spent in the closed regions, and purple dots represent latency to enter the open region for the first time.

Saline Injection: Figure 4 displays the results of measurements on the EZM taken one hour after a 0.3 mL saline injection. Mice spent an average (± SD) of 30.5 (±17.63) seconds (10%) in the open regions of the EZM, and an average of 269.5 (±17.63) seconds (90%) in the closed regions. The mean (± SD) latency to enter the open region for the first transition from closed to open region was 17.65 (±19.88) seconds.

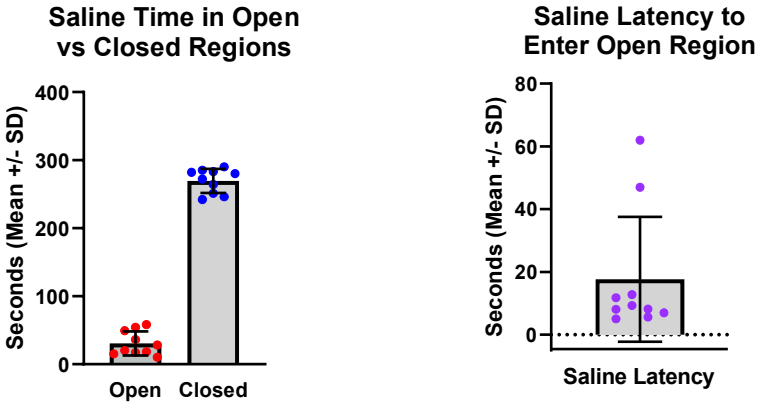

Figure 4. Saline control data displayed in seconds, with mean shown by the vertical gray bar and standard deviation represented by the vertical black line. Each measurement has ten dots displayed, each representing one of ten mice. n=10 in both graphs. Seconds spent in the open regions and closed regions (left), and latency to enter the open region for the first time (right) were recorded. The red dots represent time spent in the open regions, blue dots represent time spent in the closed regions, and purple dots represent latency to enter the open region for the first time.

Buprenorphine (1 mg/kg) Injection: Figure 5 displays the results of measurements on the EZM taken one hour after a 0.3 mL buprenorphine (1 mg/kg) injection. Mice spent an average (± SD) of 40.50 (±36.28) seconds (14%) in the open regions of the EZM, and an average of 259.50 (±36.28) seconds (87%) in the closed regions. The mean (± SD) latency to enter the open region for the first transition from closed to open region was 68.88 (±122.45) seconds. For mice who did not enter the open region, the latency was recorded as the full five minute time period.

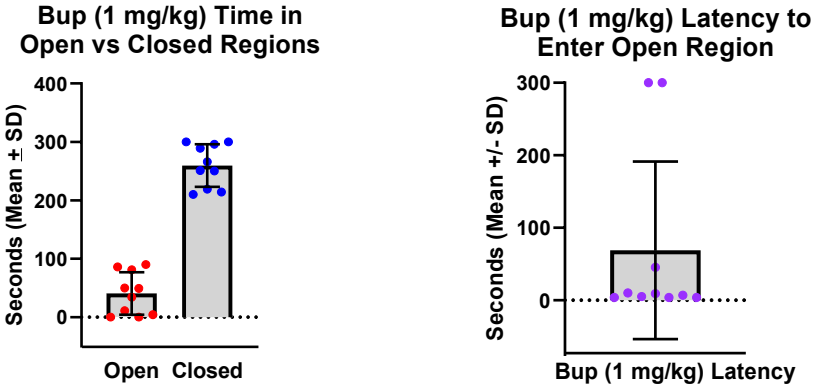

Figure 5. Buprenorphine (1 mg/kg dose) data displayed in seconds, with mean shown by the vertical gray bar and standard deviation represented by the vertical black line. Each measurement has ten dots displayed, each representing one of ten mice. n=10 in both graphs. Seconds spent in the open regions and the closed regions (left), and latency to enter the open region for the first time (right) were recorded. The red dots represent time spent in the open regions, blue dots represent time spent in the closed regions, and purple dots represent latency to enter the open region for the first time.

Buprenorphine (10 mg/kg) Injection: Figure 6 displays the results of measurements on the EZM taken one hour after a 0.3 mL buprenorphine (10 mg/kg) injection. Mice spent an average (± SD) of 44.10 (±46.03) seconds (15%) in the open regions of the EZM, and an average of 255.90 (±46.03) seconds (85%) in the closed regions. The mean (± SD) latency to enter the open region for the first transition from closed to open region was 102.59 (±137.05) seconds. For mice who did not enter the open region, the latency was recorded as the full five minute time period.

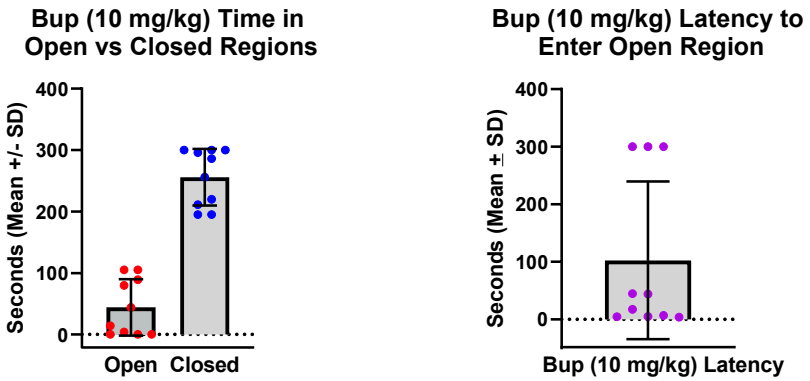

Figure 6. Buprenorphine (10 mg/kg dose) data displayed in seconds, with mean shown by the vertical gray bar and standard deviation represented by the vertical black line. Each measurement has ten dots displayed, each representing one of ten mice. n=10 in both graphs. Seconds spent in the open regions and the closed regions (left), and latency to enter the open region for the first time (right) were recorded. The red dots represent time spent in the open regions, blue dots represent time spent in the closed regions, and purple dots represent latency to enter the open region for the first time.

The following experimental occurrences are noted for the sake of transparency regarding the data. In baseline measure 1, Mouse A was placed on the EZM 1 hour post-injection, but data measures had to restart three minutes later due to timer error. In baseline measure 3, Mouse B was placed on the EZM 1 minute after the 1 hour post-injection time. For mice A-E, the 10 mg/kg buprenorphine solution was forgotten to be filtered. In the 10 mg/kg buprenorphine measure, Mouse E was placed on the EZM 17 minutes after the 1 hour post-injection time due to technological issues with the GoPro camera storage and recording.

Inferential Statistics for Performance on EZM Comparing Lean B6 Mice to DIO B6 Mice

Buprenorphine (1 mg/kg) Injection:

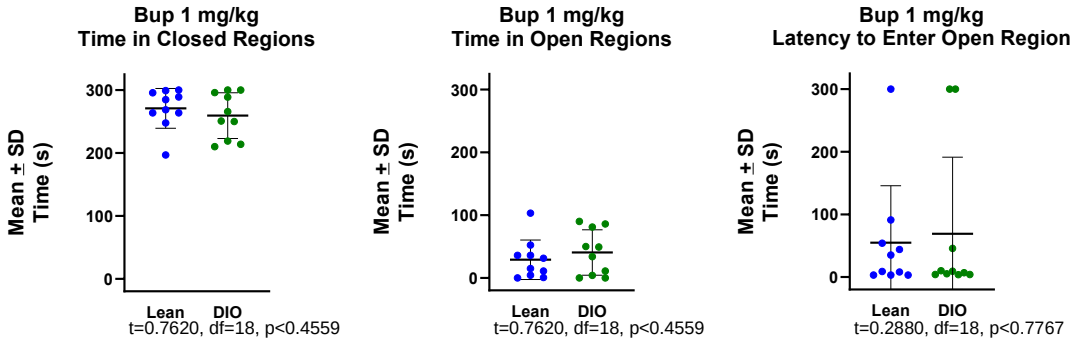

Figure 7. Unpaired t-test comparing measures of anxiety-like behavior between lean B6 mice and DIO B6 mice with a 1 mg/kg injection of buprenorphine. Each measurement has ten dots displayed, each representing one of ten mice. The lean mice are represented with blue dots, and the DIO mice are represented with green dots. These measures include time spent in the closed regions of the EZM, time spent in the open regions of the EZM, and latency to enter the open region.

Buprenorphine (10 mg/kg) Injection:

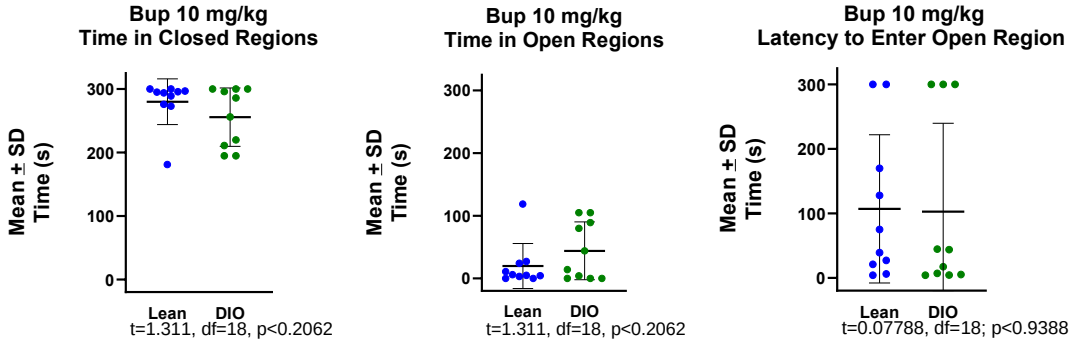

Figure 8. Unpaired t-test comparing measures of anxiety-like behavior between lean B6 mice and DIO B6 mice with a 10 mg/kg injection of buprenorphine. Each measurement has ten dots displayed, each representing one of ten mice. The lean mice are represented with blue dots, and the DIO mice are represented with green dots. These measures include time spent in the closed regions of the EZM, time spent in the open regions of the EZM, and latency to enter the open region.

DISCUSSION/CONCLUSIONS

The experimental results from this study indicate that there is no significant difference in anxiety-like behavior in DIO mice compared to lean B6 mice in response to

buprenorphine. Two doses of buprenorphine were studied: 1 mg/kg and 10 mg/kg. Two dependent measures of anxiety-like behavior were taken for each dose: latency to enter the open region of the EZM for the first time and the ratio of time spent in open versus closed regions of the EZM.

Ethically, studies investigating potentially addictive drugs cannot be performed on human subjects. Therefore, mice are used to address the potential benefits to come from a better understanding of opioid effects on anxiety-like behavior. There are not yet any proven biomarkers to measure anxiety in mice, so methods such as the elevated zero maze are used to quantify anxiety-like behavior. Mice, being a nocturnal prey species, exhibit a decreased latency to enter the open region and an increased time spent in the closed regions of the EZM as demonstrations of anxiety-like behavior (Bailey & Crawley, 2009). The behavioral measures from the present study do not support the hypothesis that there is a difference in anxiety-like behavior between male DIO mice and male B6 mice caused by buprenorphine.

Considering that both the DIO mice and the lean B6 mice are genetically the same, the most prevalent difference between the current study and a similar study of lean B6 mice (Thibert, 2022) is that of diet-induced obesity and a consequently altered gut microbiome. The *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) provides several symptoms and diagnostic criteria on the manifestation of anxiety and anxiety disorders, from chest pain to fear of dying to sleep disturbance to avoidance of social settings (American Psychiatric Association, 2013). This supports the notion that anxiety can present itself in different ways. The lean B6 mice in Thibert's study demonstrated significantly different anxiety-like behavior in measures of both the latency

measure and time spent in the open versus closed regions (both dependent measures) (Thibert, 2022) . DIO mice did not display significantly different anxiety-like behavior in these same measures. However, future studies utilizing other measures of anxiety-like behavior could find significant differences in anxiety-like behavior attributed to obesity and an altered gut microbiome after buprenorphine injection.

Mice with altered gut microbiota exhibited tolerance to morphine when compared to germ-free mice (reviewed in Muchhala et al., 2021). The present study does not indicate a similar tolerance to buprenorphine in mice with obesity and an altered gut microbiome.

Behavior is profoundly altered by epigenetic factors (Brain, 1975). Comparison of the previously conducted study (Thibert, 2022) and the present study reveals procedural differences with potential epigenetic effects on behavior in the EZM. For example, some mice in Thibert's study were housed together, and others were housed individually. All mice in the current study were housed with at least one other mouse in the cage. The possibility of housing differences altering anxiety-like behavior is open to future studies. Another procedural difference between the present study and the previous Thibert study was the number of days between experiments (Thibert, 2022). Whether through epigenetic factors or other unknown factors, these procedural differences could have influenced the results of the present study. The foregoing differences should be considered for future studies in anxiety-like behavior in mice.

Another potential future direction for data analysis and/or future studies is that of correlations between the gradually increasing mouse weight across the duration of the experiments. Figures 9-12 below plot increasing mouse weight versus measures of

anxiety-like behavior. For mice who did not enter the open region, the latency was recorded as the full five minute time period. No statistical analysis has been done, but trends can be seen. Notably, there is a similar pattern between the time spent in the closed regions and the latency measures for the 10 mg/kg buprenorphine dose (Figures 11 and 12.

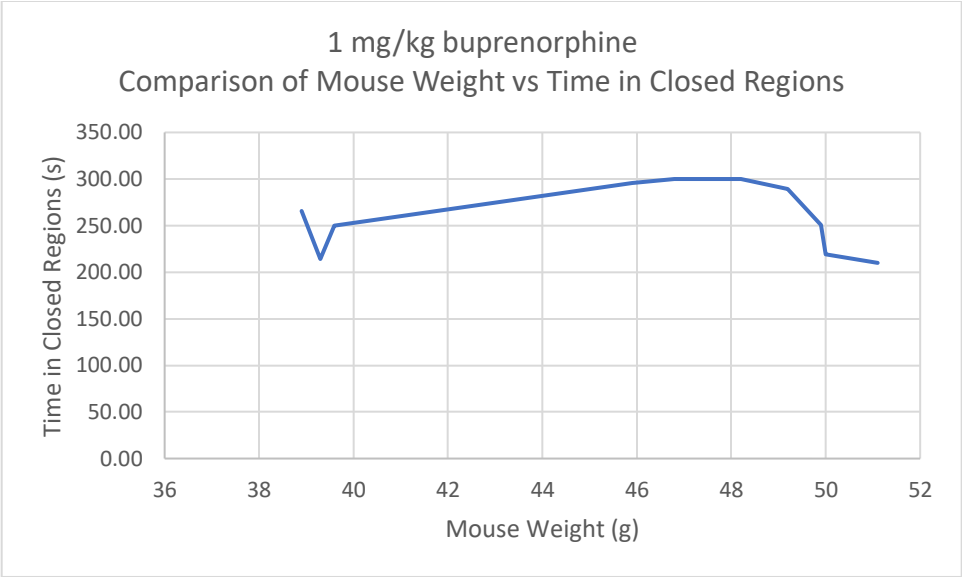

Figure 9. Comparison of mouse weight versus time spent in the closed regions after mice received injection of 1 mg/kg buprenorphine. This line summarizes the weights of 10 mice.

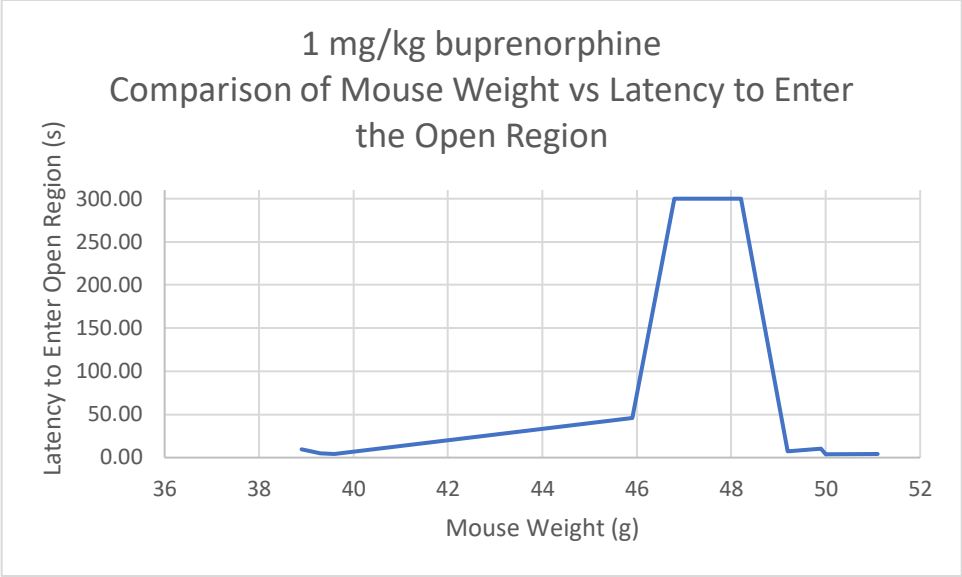

Figure 10. Comparison of mouse weight versus latency to enter the open region after mice received injection of 1 mg/kg buprenorphine. This line summarizes the weights of 10 mice.

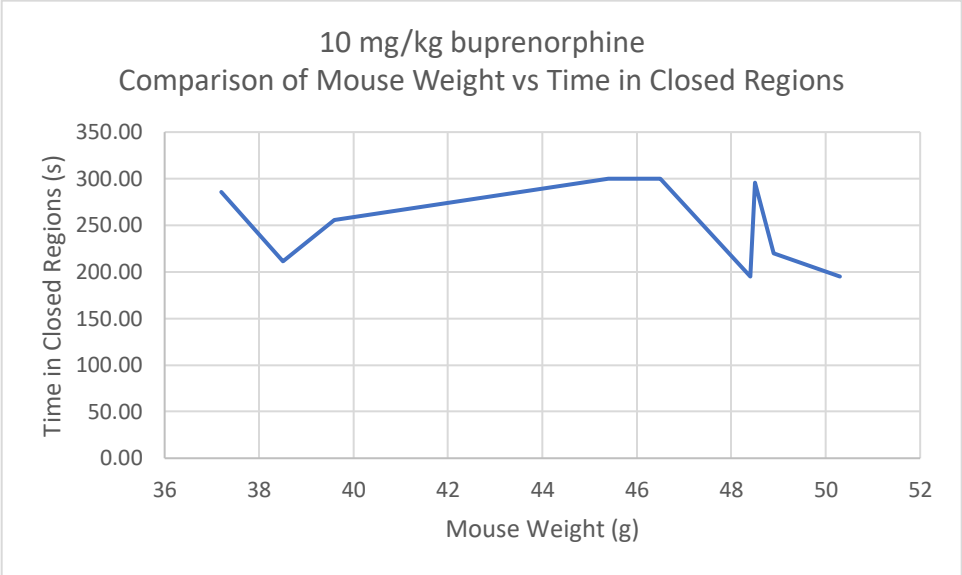

Figure 11. Comparison of mouse weight versus time spent in the closed regions after mice received injection of 10 mg/kg buprenorphine. This line summarizes the weights of 10 mice.

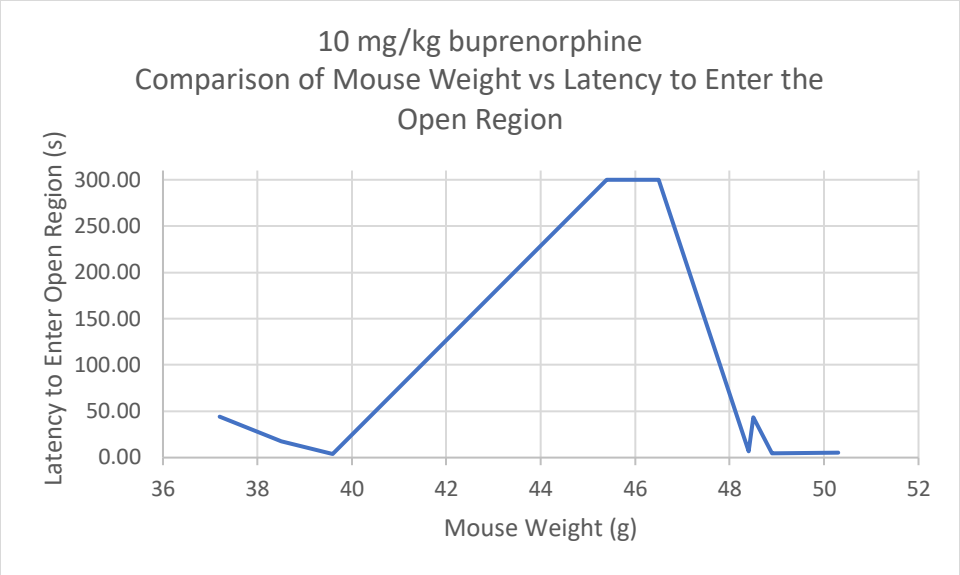

Figure 12. Comparison of mouse weight versus latency to enter the open region after mice received injection of 1 mg/kg buprenorphine. This line summarizes the weights of 10 mice.

LIMITATIONS OF THE STUDY AND FUTURE DIRECTIONS

The next step in the present study is utilization of DeepLabCut and simBA software. These tools will allow for additional data scoring and analysis using pose estimation. Various other measures of anxiety-like behavior can be analyzed, as well (Luxem et al., 2023; Mathis et al., 2018; Mathis et al., 2020).

Previous dose-response studies have shown that antinociception in DIO mice is caused by buprenorphine doses as low as 0.3 mg/kg (Glovak et al., 2017). Those results encouraged the present study to bracket the low and high therapeutic window for buprenorphine doses that reliably produce antinociception. This allowed the present study to be adequately powered by a sample size of 10 DIO mice to be compared to previous results obtained from 10 lean B6 mice. These limitations on sample size while achieving statistical power are in accord with recommendations of the ARRIVE guidelines (Percie du Sert et al., 2020). The small number of drug concentrations tested is a limitation of

this study. An increased number of dosage concentrations of buprenorphine would enable dose-dependent measures of anxiety-like behavior in DIO mice.

The present study was limited to male mice, yet obesity is known to be sexually dimorphic in humans and in mice (Casimiro et al., 2021; Palmer & Clegg, 2015). Anxiety is likewise sexually dimorphic (Kokras et al., 2012). A concurrent study (Sharma 2023, in progress) in the Lydic/Baghdoyan laboratory is investigating anxiety-like behavior on the EZM using female B6 mice for comparison to data from male B6 mice (Thibert, 2022). The emerging results encourage future studies to determine if antinociceptive doses of buprenorphine alter mouse behavior on the EZM that vary significantly as a function of body weight, sex, and sex by weight interaction.

Additionally, variability in weight among each respective mouse throughout the months of experimentation could be a contributing factor to the present results. This points to a potential future analysis of correlations between the weight of the mice at the time of each buprenorphine injection and the resulting anxiety-like behavior. Although the present study was stimulated by the discovery that opioids altered intestinal microbiome composition and function differentially in lean versus obese mice, the present study involved no direct measures of the microbiome. Such studies in mice are feasible as recently demonstrated by microbiome samples from feces, urine, colon tissue, and plasma analyzed using targeted LC-MS/MS quantification of metabolites (Blakeley-Ruiz et al., 2022). Additionally, the present study was not designed to investigate the underlying mechanism by which anxiety-like behavior is modulated in DIO mice.

POTENTIAL HEALTH-RELATEDNESS OF THE STUDY

This honors thesis also was stimulated by continually emerging data that human behavior traits and behavioral states are modulated by the microbiome. Directly related to the present focus on anxiety-like behavior in mice is evidence that human anxiety and social anxiety behavior are altered by the composition of the gut microbiome (Butler et al., 2023; reviewed in Yang et al., 2019). Increased knowledge regarding the interaction between prescription opioids, human obesity, and anxiety has the potential to enhance clinical practice regarding diagnoses and treatment of affective disorders.

REFERENCES

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 kappa/mu receptor antagonist properties, in mice. *Br J Pharmacol*, *175*(14), 2869-2880. https://doi.org/10.1111/bph.14060

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders: DSM-5* (5 ed.). American Psychiatric Association. https://doi.org/10.1176/appi.books.9780890425596

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

Blakeley-Ruiz, J. A., McClintock, C. S., Shrestha, H. K., Poudel, S., Yang, Z. K., Giannone, R. J., Choo, J. J., Podar, M., Baghdoyan, H. A., Lydic, R., & Hettich, R. L. (2022). Morphine and high-fat diet differentially alter the gut microbiota composition and metabolic function in lean versus obese mice. *ISME Communications*, *2*(1). https://doi.org/10.1038/s43705-022-00131-6

Bliss, E. S., & Whiteside, E. (2018). The gut-brain axis, the human gut microbiota and their integration in the development of obesity. *Front Physiol*, *9*, 900. https://doi.org/10.3389/fphys.2018.00900

Brain, P. (1975). What does individual housing mean to a mouse? *Life Sciences*, *16*(2), 187-200. https://doi.org/10.1016/0024-3205(75)90017-X

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. *Pharmacol Biochem Behav*, *97*(3), 406-415. https://doi.org/10.1016/j.pbb.2010.09.013

Butler, M. I., Bastiaanssen, T. F. S., Long-Smith, C., Morkl, S., Berding, K., Ritz, N. L., Strain, C., Patangia, D., Patel, S., Stanton, C., O'Mahony, S. M., Cryan, J. F., Clarke, G., & Dinan, T. G. (2023). The gut microbiome in social anxiety disorder: evidence of altered composition and function. *Transl Psychiatry*, *13*(1), 95. https://doi.org/10.1038/s41398-023-02325-5

Casimiro, I., Stull, N. D., Tersey, S. A., & Mirmira, R. G. (2021). Phenotypic sexual dimorphism in response to dietary fat manipulation in C57BL/6J mice. *J Diabetes Complications*, *35*(2), 107795. https://doi.org/10.1016/j.jdiacomp.2020.107795

Centers for Disease Control and Prevention. (2022a). *Adult obesity facts*. Centers for Disease Control and Prevention. Retrieved March 11 from https://www.cdc.gov/obesity/data/adult.html

Centers for Disease Control and Prevention. (2022b). *Drug overdose mortality by state*. Centers for Disease Control and Prevention. Retrieved March 11 from https://www.cdc.gov/nchs/pressroom/sosmap/drug_poisoning_mortality/drug_poisoning.htm

Centers for Disease Control and Prevention. (2022c). *U.S. overdose deaths in 2021 increased half as much as in 2020 - but are still up 15%. .* Centers for Disease Control and Prevention. Retrieved February 8 from https://www.cdc.gov/nchs/pressroom/nchs_press_releases/2022/202205.htm

Centers for Disease Control and Prevention, & National Center for Injury Prevention and Control. (2017). *Prescription opioids*. Centers for Disease Control and Prevention. Retrieved May 10 from https://www.cdc.gov/opioids/basics/prescribed.html

Cilli, M., De Rosa, R., Pandolfi, C., Vacca, K., Cugini, P., Ceni, Z., & Bella, S. (2013). Quantification of sub-clinical anxiety and depression in essentially obese patients and normal-weight healthy subjects. *Eating and Weight Disorders - Studies on Anorexia, Bulimia and Obesity 8*, 319-320. https://doi.org/10.1007/BF03325033

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

Davis, E. M., Rovi, S., & Johnson, M. S. (2005). Mental health, family function and obesity in African-American women. *Journal of the National Medical Association*, *97*(4), 478-182.

DiBaise, J. K., Frank, D. N., & Mathur, R. (2012). Impact of the gut microbiota on the development of obesity: current concepts. *The American Journal of Gastroenterology Supplements*, *1*(1), 22-27. https://doi.org/10.1038/ajgsup.2012.5

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

Ghosal, S., Nunley, A., Mahbod, P., Lewis, A. G., Smith, E. P., Tong, J., D'Alessio, D. A., & Herman, J. P. (2015). Mouse handling limits the impact of stress on metabolic endpoints. *Physiol Behav*, *150*, 31-37. https://doi.org/10.1016/j.physbeh.2015.06.021

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

Hedegaard, H., Miniño, A. M., Spencer, M. R., & Warner, M. (2021). Drug overdose deaths in the United States, 1999–2020. *Centers for Disease Control and Prevention; National Center for Health Statistics*(428). https://doi.org/10.15620/cdc:112340

Hitt, H. C., McMillen, R. C., Thornton-Neaves, T., Koch, K., & Cosby, A. G. (2007). Comorbidity of obesity and pain in a general population: results from the Southern Pain Prevalence Study. *J Pain*, *8*(5), 430-436. https://doi.org/10.1016/j.jpain.2006.12.003

Huang, F., & Wu, X. (2021). Brain neurotransmitter modulation by gut microbiota in anxiety and depression. *Front Cell Dev Biol*, *9*, 649103. https://doi.org/10.3389/fcell.2021.649103

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/10.1016/j.neuropharm.2011.08.025

Luxem, K., Sun, J. J., Bradley, S. P., Krishnan, K., Yttri, E., Zimmermann, J., Pereira, T. D., & Laubach, M. (2023). Open-source tools for behavioral video analysis: setup, methods, and best practices. *Elife*, *12*. https://doi.org/10.7554/eLife.79305

Mathis, A., Mamidanna, P., Cury, K. M., Abe, T., Murthy, V. N., Mathis, M. W., & Bethge, M. (2018). DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. *Nat Neurosci*, *21*(9), 1281-1289. https://doi.org/10.1038/s41593-018-0209-y

Mathis, A., Schneider, S., Lauer, J., & Mathis, M. W. (2020). A primer on motion capture with deep learning: principles, pitfalls, and perspectives. *Neuron*, *108*(1), 44-65. https://doi.org/10.1016/j.neuron.2020.09.017

Mora, A. L., Salazar, M., Pablo-Caeiro, J., Frost, C. P., Yadav, Y., DuPont, H. L., & Garey, K. W. (2012). Moderate to high use of opioid analgesics are associated with an increased risk of Clostridium difficile infection. *Am J Med Sci*, *343*(4), 277-280. https://doi.org/10.1097/MAJ.0b013e31822f42eb

Moran, C. P., & Shanahan, F. (2014). Gut microbiota and obesity: role in aetiology and potential therapeutic target. *Best Pract Res Clin Gastroenterol*, *28*(4), 585-597. https://doi.org/10.1016/j.bpg.2014.07.005

Muchhala, K. H., Jacob, J. C., Kang, M., Dewey, W. L., & Akbarali, H. I. (2021). The guts of the opioid crisis. *Physiology (Bethesda)*, *36*(5), 315-323. https://doi.org/10.1152/physiol.00014.2021

National Center for Health Statistics. (2023). *Provisional drug overdose death counts*. National Vital Statistics System. Retrieved March 11 from https://www.cdc.gov/nchs/nvss/vsrr/drug-overdose-data.htm

Okifuji, A., & Hare, B. D. (2015). The association between chronic pain and obesity. *J Pain Res*, *8*, 399-408. https://doi.org/10.2147/JPR.S55598

Palmer, B. F., & Clegg, D. J. (2015). The sexual dimorphism of obesity. *Mol Cell Endocrinol*, *402*, 113-119. https://doi.org/10.1016/j.mce.2014.11.029

Percie du Sert, N., 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., Hurst, V., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., Pearl, E. J., Petersen, O. H., Rawle, F., Reynolds, P., Rooney, K., Sena, E. S., Silberberg, S. D., Steckler, T., & Wurbel, H. (2020). Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biol*, *18*(7), e3000411. https://doi.org/10.1371/journal.pbio.3000411

Rajan, T., & Menon, V. (2017). Psychiatric disorders and obesity: a review of association studies. *Journal of Postgraduate Medicine*, *63*(3), 182. https://doi.org/10.4103/jpgm.JPGM_712_16

Scott, K. M., McGee, M. A., Wells, J. E., & Oakley Browne, M. A. (2008). Obesity and mental disorders in the adult general population. *J Psychosom Res*, *64*(1), 97-105. https://doi.org/10.1016/j.jpsychores.2007.09.006

Shepherd, J. K. G., 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*, 56-64. https://doi.org/10.1007/bf02244871

Simon, G. E., Von Korff, M., Saunders, K., Miglioretti, D. L., Crane, P. K., van Belle, G., & Kessler, R. C. (2006). Association between obesity and psychiatric disorders in the US adult population. *Arch Gen Psychiatry*, *63*(7), 824-830. https://doi.org/10.1001/archpsyc.63.7.824

Stokes, A., Lundberg, D. J., Hempstead, K., Berry, K. M., Baker, J. F., & Preston, S. H. (2020). Obesity and incident prescription opioid use in the U.S., 2000-2015. *Am J Prev Med*, *58*(6), 766-775. https://doi.org/10.1016/j.amepre.2019.12.018

Substance Abuse and Mental Health Services Administration. (2023). *Buprenorphine*. Substance Abuse and Mental Health Services Administration. Retrieved Feburary 8 from https://www.samhsa.gov/medications-substance-use-disorders/medications-counseling-related-conditions/buprenorphine

Sumner, S. A., Bowen, D., Holland, K., Zwald, M. L., Vivolo-Kantor, A., Guy, G. P., Jr., Heuett, W. J., Pressley, D. P., & Jones, C. M. (2022). Estimating weekly national opioid overdose deaths in near real time using multiple proxy data sources. *JAMA Netw Open*, *5*(7), e2223033. https://doi.org/10.1001/jamanetworkopen.2022.23033

Tennessee Bureau of Investigation. *Opioids*. Tennessee State Government. Retrieved March 23 from https://www.tn.gov/tbi/crime-issues/crime-issues/opioids.html

Thibert, M. K. (2022). Buprenorphine effects on anxiety-like behavior in B6 mice. *Tennessee Research and Creative Exchange*. https://trace.tennessee.edu/utk_selectug/16

Thomas, K. R., Watt, J., Wu, C. M. J., Akinrinoye, A., Amjad, S., Colvin, L., Cowe, R., Duncan, S. H., Russell, W. R., & Forget, P. (2022). Pain and opioid-induced gut microbial dysbiosis. *Biomedicines*, *10*(8). https://doi.org/10.3390/biomedicines10081815

Tucker, L. B., & McCabe, J. T. (2017). 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. *Front Behav Neurosci*, *11*, 13. https://doi.org/10.3389/fnbeh.2017.00013

Wang, F., & Roy, S. (2017). Gut homeostasis, microbial dysbiosis, and opioids. *Toxicol Pathol*, *45*(1), 150-156. https://doi.org/10.1177/0192623316679898

Yang, B., Wei, J., Ju, P., & Chen, J. (2019). Effects of regulating intestinal microbiota on anxiety symptoms: a systematic review. *Gen Psychiatr*, *32*(2), e100056. https://doi.org/10.1136/gpsych-2019-100056