Classically Conditioning Morphine Response Profile to Reduce Behavioral Dependence Withdrawal in Male Sprague-Dawley Rats

By

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Allegheny College

Department of Neuroscience

Department of Psychology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Rodney Clark

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Abstract

The opioid epidemic continues to worsen across the United States. As more individuals continue to fall victim to substance use disorders, it is becoming even more paramount that we identify possible therapies to help those struggling towards rehabilitation. This study aimed to determine whether utilizing both contextual and discrete cues through classical conditioning could be a viable method to reduce withdrawal symptoms both during and after rehabilitation. Twice daily 10 mg/kg subcutaneous morphine administration were given to male Sprague-Dawley rats until behavioral tolerance thresholds were met as measured by responding for water in operant chambers. Withdrawal symptoms were measured based on changes in completed lever response sets, grooming behaviors, defecation amount, and weight — compared to baseline measures prior to drug exposure. Three groups were maintained for this study: a no-cue control which received no exposure to the conditioned stimuli (CS) during withdrawal, a context-cue-only experimental group which was only exposed to the contextual CS of the injection room, and an all-cue experimental group which was exposed to both the contextual CS and received a saline injection functioning as the discrete CS. Statistical analysis indicated a significant difference in grooming behaviors between the three cue groups ($p < 0.001$), across the five days of withdrawal ($p = 0.008$), as well as an interaction between them ($p = 0.014$). Significant differences were also found across the days of the withdrawal phase in completed response sets, defecation, and weight; with weight also having a significant difference between the three cue groups. These three measures, by the end of the 5 days, returned close to their baseline values. These changes across the days indicated a reduction of withdrawal across the five days, suggesting a longer tolerance phase was necessary. These results indicate separate levels of success in conditioning procedures reducing symptoms between the forms of withdrawal being measured. Such conclusions call for more withdrawal symptoms to be explored in future studies.

Introduction

Opioid Prescriptions and Risks

Overprescription of opioids in the United States has become a growing issue in recent years, and the overall effect has become widespread. In comparison to global healthcare systems, the US prescribes more than 50 times the amount of opioids than the rest of the world combined (Tick et al., 2018). Much of this high level of prescription came about through drastic shifts in care to address patient's pain. Prior to the 1990s, most doctors did not address acute pain symptoms in hospitals. With a shift occurring due to push back from the American Pain Society arguing that pain should be treated as another vital sign, a new way to assess patient pain and how to address it accordingly was generated (Max et al, 1995). A secondary reason for the overprescription of opioids in the US paradoxically comes about from limitations of opioid prescription policies. Due to its schedule II level, patients must present a handwritten prescription from their physician. Because of this, most physicians will prescribe opioids in excess (even when they may not be required at all) to prevent the patient from having to return to the physician's office/hospital in order to obtain a refill (Theinsen et al., 2018).

A major concern from this excessive prescription is that even a single prescription of opioids has been shown to lead to chronic use of opioids (Alam et al., 2012; Brummett et al., 2017; Clarke et al., 2014; Goesling et al., 2016). One study indicated that of first-time opioid users, 8.2% continued daily use of their prescribed opioids 6 months after their total knee replacement surgery (Goesling et al., 2016). A second study observed that from a group of 391,139 first time opioid user patients following short-stay surgery, 7.7% of those patients maintained their prescription 1 year following the surgery (Alam et al., 2012). The results from these two studies indicate just how hazardous the overprescription of opioids following any form of surgery could be to the development of a dependence on the drug.

The fallout from this overprescription is what has resulted in the opioid epidemic that we see in the United States today. Recent studies have indicated that approximately 6% of the United States population between the ages of 15-64 have abused opioids, with that number likely being even higher due to social desirability bias. When comparing this to the rest of the world's population reporting less than 1% of people in that age range having abused opioids (Theinsen et al., 2018), the magnitude of this epidemic becomes quite apparent. Furthering this concern, in the United States, opioids have led to a greater number of overdoses than any other drug since 2017, with those numbers only continuing to rise above the other drugs of abuse (Ahmad et al., 2022).

The overprescription of opioids is certainly a key factor in the development of the epidemic. A study in 2013 found that of more than 3 million patients, 25% showed at least one sign of potential misuse by the end of their 47-day supply and at least 5% of those showed at least two (Liu et al., 2013). Furthermore, another study found that 80% of nonmedical opioid use in high schoolers (which made up 12.9% of the participants) had obtained their opioids from previous prescriptions (McCabe et al., 2012). The results from these studies emphasize the impact that the overprescription problem has on the opioid epidemic. It should be noted, however, that this is not the only factor. A full meta-analysis found further impact from demographic data such as gender, education, culture, and even religion (Stoicea et al., 2019). The evidence for a multifactorial issue only strengthens the complexity of opioid use disorders and the present epidemic, a pattern that is further seen in the variety of treatment techniques.

For immediate treatment of an individual who has overdosed, naloxone (an opioid receptor antagonist) can be applied which will counter the effects of the opioid. Naloxone can increase an individual's respiratory rate, even if the overdosed person has stopped breathing entirely in some cases (CDC, 2022). However, opioid antagonists are not only utilized in emergencies to reverse an overdose. Naltrexone (another majorly opioid antagonist) is used as a pharmacotherapy generally taken orally. Naltrexone has been found to be successful when taken as prescribed, however, it depends on the patient to commit to their treatment plan – among other factors. This issue is emphasized by studies that have indicated a lack of success when it comes to the implementation of Naltrexone. It was found that when compared to benzodiazepines, buprenorphine, or even a placebo, there was no significant difference in the abstinence from the drug of abuse between participants during and following treatment (Minozzi et al., 2011). Methadone – a far less potent opioid that acts on the same receptors as morphine and other analgesic opioids – is another pharmacotherapy option that exists for those seeking reprieve from their opioid use disorder. Generally, this drug is utilized as an outpatient treatment option (though the longer the patient stays in the treatment facility, the more successful the treatment tends to be) in an attempt to provide an alternative to the normal opioid of abuse when living their day-to-day lives. Most studies indicate that a higher dose of methadone results in decreased use of the abused drug, with very few reporting successes with low doses (Ward et al., 1994). The requirement for a higher dose of methadone in order to reduce use of the abused drug is obviously a paradoxical issue, one that could be seen as quite problematic for a long-term treatment option.

In addition to the pharmacotherapies available, there are also several group therapy options, as well as cognitive therapies. Both can be applied in addition to the pharmacotherapies mentioned previously (NIDA, 2020). Though notably, many of these therapies do not fully help those struggling in the long term – with a relapse rate of 40-60% for substance use disorders such as opioids and alcohol (NIDA, 2023). One potential reason for this is that often the cues associated with the substance use disorder are not focused on during treatment. A problem that is emphasized by the fact that many times, even after years of sobriety, being exposed to the same environment in which drug use was commonplace will result in a relapse of drug use (O'Brien et al., 1992). These issues require further investigation into new therapies or methodologies to help those struggling through opioid use disorders. But in order to discover these new therapies, we must first understand how opioids affect the body to begin with, which will be explored in the next section.

Morphine Pharmacology

When considering why opioids are prescribed for pain, as well as understanding their propensity to lead to substance abuse disorders, one must examine the mechanisms behind how these drugs act on the body. An opioid's overall physiological effects depend on what receptor it has the greatest affinity for binding to. There are three main opioid receptors: MOR (μ), KOR (κ), and DOR (δ), each with their own main physiological effects, among other side effects. μ receptors when activated tend to result in feelings of euphoria mainly through dopaminergic activity, κ results in feelings of dysphoria through gamma-aminobutyric acid (GABA)ergic activity, and δ leads to a mix of these two responses (Dhaliwal & Gupta, 2021). Opioids used to treat pain related symptoms generally have greater affinity for μ receptors due to the euphoric effects as described previously, however, they also tend to carry high levels of abuse liability (Negus & Freeman, 2018). For this reason, this section will mainly discuss the functionality of μ opioid receptors and what leads to their activation and aftereffects of that activation.

The μ opioid receptors are G-protein coupled receptors (GPCRs), meaning that when a ligand – such as an endorphin, or morphine – binds to it, the G-protein is activated leading to a biochemical cascade. The α-GTP both inhibits adenylyl cycles which reduces cAMP levels, as well as interacts with ion channels to overall reduce the amount of neurotransmitter released

(Pathan & Williams, 2012). This biochemical cascade event produces the analgesic effects of opioids, as well as other side effects produced from μ receptors and the other types of opioid receptors, such as a reduced respiration rate, euphoria, rigid muscles, and constipation just to name a few (Rosenblum et al., 2008).

Medically, morphine is generally prescribed for its analgesic properties. Unfortunately, prolonged use of morphine eventually develops tolerance, diminishing its effectiveness in long-term patients. In such cases, patients must be given an increased dose to overcome the aforementioned tolerance to the drug. This increases the exposure to morphine's reinforcing properties and ultimately puts them at a higher risk of developing withdrawal symptoms when morphine use is stopped (Kumar et al., 2001; LeResche et al., 2015). These reinforcing properties are in part caused by the relaying circuitry of the Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAc).

The VTA contains a high concentration of MORs which when activated, modulates DA projections from the VTA to the GABAergic medium spiny neurons of the NAc. This increased concentration of DA in these regions is preferentially upregulated through the increased production of tyrosine hydroxylase (TH) – which is used in the synthesis of DA. Chronic use of morphine causes an increased activation rate of these neurons due to long-term potentiation (LTP), which when combined with the increased production of DA due to TH, creates something of a positive feedback loop (Kim et al., 2016). Generally, this process can occur with any reinforcing drug or behavior. However, due to the potency of morphine and other (μ) opioid agonists, the process begins to require that level of activation in order to experience the reinforcing (euphoric) effects; which reduces the reinforcing properties of other agents. This, in essence, describes one aspect of the development of an opioid use disorder.

However, before any of this process can begin, the drug must first enter the system. Opioids with an affinity for the μ receptors have a variety of methods to do this. Most opioids prescribed to hospitalized patients receive the drug via IV – though occasionally subcutaneous or other enteral routes are used instead – whereas most out-patient prescriptions are taken orally (Kestenbaum et al., 2014). Whatever the route of administration, the overall effect of the drug is exceptionally similar, with little difference in the pharmacodynamics of the drug in the system (Hasselström & Säwe, 1993). Interestingly, the analgesic effects of morphine are not directly related to its concentration in the brain following administration. It has been found in rat models that the analgesic effects remain well after the concentration of morphine has been reduced through excretion (Dahlström et al., 1978). This is likely due to the biochemical cascade described above causing longer lasting effects due to changes in neurochemical concentrations. A result of this elongated reaction to the receptor being activated is that the effects of the drug can remain even after most of the drug has dissipated from the system.

The resulting effects from morphine administration include, but are not limited to analgesia, constipation, reduce respiratory/cardiovascular rate, and sedation (KuKanich & Wiese, 2015). Due to tolerance, as an individual continues to use the drug at the same dosage, these effects are reduced, generally requiring a greater amount of the drug to be administered in order for the desired effects to be felt (Martin et al., 1963). The development of tolerance and much of the basis of the present study is based on classical conditioning surrounding drug use, which will be explored in the next section.

Classically Conditioning the Effects of Morphine

Classical conditioning is the process in which two stimuli are repeatedly temporally paired. The exposure of one of these stimuli – the unconditioned stimulus (UCS) – naturally results in an unconditioned response (UCR). Through repetitive pairings the second, neutral stimulus (NS) will elicit a similar response to the UCS, at which point the NS is considered a conditioned stimulus (CS). This process was accidentally discovered by the Russian physiologist, Ivan Pavlov, while studying canine digestion physiology. In his study, Pavlov was measuring salivation levels as a dog digested food. However, an unexpected outcome resulted from a bell that would ring as an experimenter entered the room to give the dog its food. As time went on, the dog would begin salivating when the bell sounded, whether the experimenter had food with them or not. In this situation, the bell had become a CS with the salivation becoming a conditioned response (CR) resulting from the bell ringing (Pavlov, 1906). Classical conditioning is considered the basis of associative learning in organisms, however, as time has gone on further studies have indicated similar implications within the realm of drug effects.

In 1975, Ader & Cohen were conducting a study on taste aversion through conditioning procedures between saccharine flavored water and cyclophosphamide. Cyclophosphamide is a drug which causes nausea in its users, which was its main effect property being studied at the time. However, cyclophosphamide also functions as an immunosuppressant, so while the animal subjects were consistently being exposed to both cyclophosphamide and saccharine flavored water, the water became a CS for the immunosuppressant effect of the cyclophosphamide. This resulted in the rats having a severely decreased immune response. The weakened immune system made them exceptionally vulnerable to diseases, resulting in many of the rats dying unexpectedly (Ader & Cohen, 1975). The unanticipated results of this study indicate that drug effects (UCR) could be classically conditioned to a CS.

While Ader & Cohen made this discovery with cyclophosphamide, opposite results have also occurred from some drug related CSs. There are cases in which a CS, rather than generating a CR, will instead cause a conditioned compensatory response (CCR). This results from shifts in the organism's physiology as a result of exposure to the CS. A pertinent example of this comes from substance abuse disorders. Individuals who have maintained a consistent or similar environment for drug self-administration can experience withdrawal symptoms and relapse after exposure to those environmental stimuli due to CCR (Siegel, 1999). The resulting withdrawal symptoms from this CCR are considered drug-opposite effects. These symptoms and the resulting relapse occur even after years of abstinence from the drug & not existing in that environment (O'Brien et al., 1992). One resulting effect of the CCR can be observed when someone with an opioid use disorder takes something close to their normal dose in an unfamiliar environment. In this case, the CCR does not occur due to a lack of environmental CS, and so the body was not prepared for that significant of a drug intake. In some cases, this phenomenon can result in the individual overdosing (Siegel & Ellsworth, 1986).

Notably, these CCRs are a result of environmental (or contextual) cues. An interesting phenomenon has been observed in some studies indicate that when a conditioned *discrete* stimulus is applied to an individual that has been exposed to those environmental stimuli, they can experience what is considered a drug-like effect instead. Case studies of individuals who have experience this are considered by some to be "needle junkies" as injecting themselves with an inert or less potent substance in the same fashion they would the normal drug of abuse in the same environment, leads to similar physiological/psychological effects to if they had taken the drug itself (O'Brien et al., 1992).

One particular study that aimed to investigate the effect of these contextual and discrete stimuli was conducted in 1983 through a series of experiments, in which rats developed a conditioned morphine tolerance via subcutaneous injections, one group within a distinctive injection environment and a second group in a non-distinct environment. Tolerance was based on responsiveness to a shock test; a test that uses a jump/flinch apparatus attached to a shock grid floor to measure the level of response following exposure to a shock. As the subjects became more tolerant to the drug, they would also become more reactive to the shock test. The initial finding emphasized contextual mediated tolerance – a proposed concept in which tolerance to a drug is dependent on the environment in which it is used (Siegel, 1975). This particular study found that those rats who only were given injections in the distinctive environment developed tolerance faster than the non-distinct group. Furthermore, when all groups were injected within the distinctive environment, the groups who were consistently injected within that environment showed a greater level of response to the shock test than those who had only just experienced the distinctive environment. This indicated that the contextual environment helped mediate the development and maintenance of tolerance (Tiffany et al., 1983).

A second experiment within this study explored the effect of discrete stimuli within the basis of classically conditioning morphine effects. For this, similar parameters for the initial study were used; in which rats were injected within either a distinct environment or indistinct until tolerance had been reached, then morphine injections were replaced with injections of saline. Following these injections, the same shock test was investigated and surprisingly, there were no significant differences observed between any of the groups on responsiveness to the shock test. This would indicate that discrete stimuli (saline injection) do not successfully generate a conditioned response similar to that of morphine as the drug-like effect of morphine was not replicated through exposure to the CS (Tiffany et al., 1983).

One issue with the conclusion drawn by Tiffany’s study is that it focused solely on the analgesic effects of morphine and observed them via shock tests. However, as emphasized by

Ader & Cohen’s study, drugs have a plethora of possible effects with a wide response profile. For that reason, investigating only one of the possible effects of a drug is a substantial shortcoming of the study, and further investigation should be conducted to investigate the greater response profile of morphine and whether it can be replicated through Pavlovian conditioning.

The Present Study

The present study sought to address the shortcomings resulting from Tiffany’s study, and further investigate the level to which morphine’s response profile can be classically conditioned in rats. Further, this study investigated the amount to which withdrawal symptoms are observed through the same classical conditioning interventions.

Utilizing operant conditioning techniques, rats were trained on a lever pressing task as a way to observe tolerance development and subsequent withdrawal symptoms. Making use of behavioral tolerance theory – in which the disruption caused by drug use is diminished after repeated exposure to a substance (Krasnegor, 1978; Cappell & LeBlanc, 1981) – this study seeks to observe shifts in response rates based on level of exposure to various conditioned stimuli (both contextual and discrete). The purpose of this was to observe the more diverse response profile of morphine, rather than investigate a singular physiological effect such as its analgesic properties. This technique makes it possible to not only actively observe the onset of tolerance, but also investigate shifts in behavioral measures during withdrawal as well.

While investigating shifts in behavioral measures, other forms of physiological withdrawal were also observed during this study. The three forms of withdrawal being observed included grooming behaviors, defecation, and changes in body weight. Prior studies have indicated that grooming behaviors take a sharp increase following naloxone induced withdrawal in rat populations. Along with this, due to morphine’s constipating effect, during withdrawal, there is a greater tendency to observe defecation and urination (KuKanich et al., 2015). The final symptom of withdrawal will be passively observed during the already required daily weight collection. Studies have indicated that at the onset of withdrawal, there is an initial phase of a sharp decline in body mass, followed by a quick increase; even above the original mass (Mishra et al., 2017).

A multitude of studies observing the development of tolerance and withdrawal of morphine in rats indicated the use of 10 mg/kg of morphine twice per day for at least 10 days via subcutaneous injections was effective at developing a tolerance to the drug in rats. Additionally, in order to induce a state of withdrawal, an intraperitoneal injection of 2 mg/kg of naloxone was used in previous studies (Craft et al., 1999; Maguire et al., 2016; Mishra et al., 2017). Such parameters remaining consistent throughout other studies provide a clear starting point for developing an onset of morphine tolerance and initiating withdrawal symptoms. Despite other studies observing withdrawal utilizing naloxone as mentioned previously, this study only observed the natural onset of withdrawal rather than a drug induced condition. Not only will this allow for a more applicable methodology to be utilized in potential future clinical trials, it also ensures that discrete cues only center around morphine injections and no other.

It should also be noted, that the majority of previous studies focus solely on male rats. It is likely that based on the fact that female rats experience less of an effect from morphine than male rats, that such dosages may need to be different for female rats in order to develop a tolerance or dependence (Loyd et al., 2008; Shansky & Murphy, 2021). For this reason, this study will only utilize male rats. Future research should investigate whether this is replicable in female rats.

The goal of this study was to determine whether the development and maintenance of conditioned stimuli from the environment during consistent subcutaneous morphine administration could successfully reduce behavioral withdrawal symptoms experienced by male Sprague-Dawley rats when performing a learned motivated behavior after such morphine injections are transitioned into saline injections. Based on past information collected from both pre-clinical and clinical trials, it is anticipated that environmental cues (auditory, olfactory, and visual) provided with a substitute injection (saline) can replicate the response profile of morphine and therefore reduce the behavioral withdrawal symptoms in the rats. Furthermore, previous research indicates that the rats that receive conditioned stimuli with no contingent saline injections following their presentation will experience drug opposite effects (CCR), resulting in worsened withdrawal symptoms.

Based on previous studies, it was anticipated that during the withdrawal phase of the study, the rats exposed to both environmental and discrete cues would score most similarly in all categories of measure – completed response sets, grooming behavior, weight, and defecation – to their baseline data. Whereas rats exposed only to environmental cues would perform extremely differently in all categories of measure, with completed response sets, grooming behavior, and defecation predicted to be much higher during the withdrawal phase, and weight reduced. The rats exposed to no purposeful CS were predicted to perform somewhere between those two groups in all measures.

Methods

Subjects

Twelve Sprague-Dawley male rats approximately 10 weeks in age, housed in hanging wire cages in groups of 4, were used in this study. Each cage contained two food troughs and one water bottle. Rats were put on a 12-hour water deprivation prior to each operant session in order to introduce a motivating operation (MO). Rats were weighed each day prior to their nighttime dose of morphine (their morning dose used the previous night's mass). The colony room was maintained on a 8.5:14.5-hour dark/light schedule (Lights on 7:30 AM-11:00 PM). Rats were put in operant boxes at 8 PM. There were three groups in this study: The No-Cue-Control Group (n = 4), the All-Cue-Experimental Group (n = 4), and the Context-Cue-Only-Experimental Group (n = 4).

Materials

Based on prior methods utilized in developing a morphine tolerance in male rats: twice daily (8 AM & 8 PM) subcutaneous morphine injections (10 mg/kg) (Mishra et al., 2017) were given to each rat during the tolerance phase. Saline was given in a volume of 1 ml/kg of body weight via subcutaneous injection into the same injection site. Experimenter-administered morphine and saline were injected via a 1cc syringe with a 25-gauge needle with a 10 mg/ml solution. White noise was presented during each morphine injection for the entirety of the time the injections took, along with an additional ten minutes. The white noise used was played from a phone via the YouTube app, the video was called: “White Noise Black Screen | Sleep, Study, Focus | 10 Hours”. The video was played at around 58 decibels. Transportation cages to the experimental room were plastic tubs with wood chip bedding and a wire lid.

Apparatus

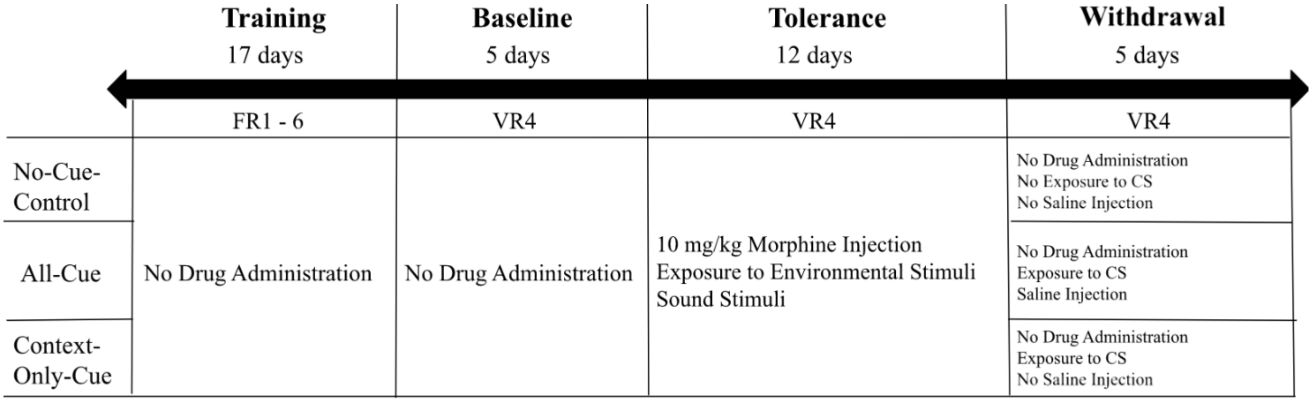
A standard Lafayette Instruments co. operant conditioning chamber (model #80001) served as the apparatus. The chamber was equipped with two levers and two stimulus lights. Scheduling of all experimental events and data collection of lever responses was accomplished through a Lafayette operant conditioning console (model #81335). Due to malfunctioning within some of the boxes, the left lever was used as the response lever for every trial.

Design

The independent variables of this study centered around different types of exposure to conditioned stimuli during the period of withdrawal from morphine. All groups received morphine injections while being exposed to contextual cues (white-noise, bright room, same experimenter), as well as discrete cues (injection). Following the development of behavioral tolerance to morphine (as described below), all rats were taken off the drug. At which point, the No-Cue-Control Group was not exposed to any purposefully conditioned stimuli prior to being placed in the operant chamber. The All-Cue-Experimental Group was exposed to both the contextual cues, as well as the discrete cue via a saline injection into the same injection site, prior to being placed in the operant chamber. The Context-Cue-Only-Experimental Group was exposed to the context cues, however, they did not receive a saline injection prior to being placed in the operant chamber.

This experiment measured the number of response sets completed during operant sessions under a Variable Ratio 4 (VR4) schedule – that is, the average number of responses it takes to receive a reinforcer (water) will be 4. Between-group measures were done to compare the deviation of the average number of completed response sets from each experimental group during the withdrawal phase of the study in comparison to each rat's baseline. This analysis observed how the level of behavioral dysregulation of the learned behavior changed based on exposure to different levels of CSs as a way to infer a change in withdrawal symptoms. Further analysis observed specific withdrawal symptom differences between the groups, namely grooming behavior, defecation behavior, and changes in the animal’s mass.

Procedure



	Training 17 days	**Baseline** 5 days	**Tolerance** 12 days	**Withdrawal** 5 days
	FR1 - 6	VR4	VR4	VR4
No-Cue-Control	No Drug Administration	No Drug Administration	10 mg/kg Morphine Injection Exposure to Environmental Stimuli Sound Stimuli	No Drug Administration No Exposure to CS No Saline Injection
All-Cue				No Drug Administration Exposure to CS Saline Injection
Context-Only-Cue				No Drug Administration Exposure to CS No Saline Injection

Fig. 1. A basic overview of the experimental timeline.

During the training phase of the study, each rat was weighed prior to each operant session. Operant sessions during this phase of the study began at 45 minutes in length and then shortened to 30 minutes by day 14. During this phase, rats were put on a 12-hour water deprivation schedule beginning at 8 AM and ending upon return to home cages after their operant session. Rats were auto-shaped in the chambers to press the lever with at least 15g of force. Initially, each rat was put on a Fixed Ratio 1 (FR1) schedule – meaning that one press of the correct lever resulted in a water droplet being delivered to a collection tray. Following this condition, rats were elevated to an FR2 schedule – meaning two presses of the active lever were required to receive one drop of water. Following this, rats were moved to an FR3 schedule, and so on until each rat was responding to an FR6 schedule. After each rat had time to perform the task at the FR6 level, the Baseline phase of the experiment began.

During the baseline phase of the study, rats were placed on a VR4 schedule. Each rat was weighed prior to each operant session. Operant sessions lasted for 30 minutes. Responses were recorded during this time, as well as the amount of defecation and grooming behaviors to be compared later. Grooming behaviors (i.e. self-licking, nibbling fingers, “wet dog” shakes, and rubbing face) were recorded for a total of 4 minutes per rat, with 2 minutes with each rat at the beginning of the operant session and for 2 minutes at the end, each day the rat that began the grooming behavior observations was shifted to account for any shifts in behavior throughout an operant session. The defecation amount was recorded at the conclusion of each session via counting in the collection trays at the base of each chamber. Baseline measures were concluded after 5 days of data collection.

Following the baseline phase of the study, the tolerance phase began. During this time, each rat remained on a VR4 schedule within the operant chambers and operant sessions lasted 30 minutes. The same recording of grooming behaviors, responses, and defecation amounts were conducted. However, after each rat was weighed, all groups were taken to a separate injection room with bright lights and white noise playing for contextual cues. While in this room, each rat received a subcutaneous injection of morphine. After the last rat’s morphine injection, the rats remained in the room with the light on and white noise playing for 10 minutes. This process was repeated both morning (8AM) and night (8PM). Following the PM injection period, rats began their operant session. The order of injection changed by group and by rat each day to account of any shifts in the metabolism of the drug across the period of injections. This phase of the study ended after each rat returned to within 20% of its baseline response rate (10% margin above or below baseline); indicating that each rat had become behaviorally tolerant to the morphine injections. In the case of this study, this phase lasted a total of 12 days.

Following the tolerance phase of the study, the withdrawal phase began. During this period, each group received different levels of cues. The All-Cue-Experimental Group were brought into the injection room with the same light and white noise being played, however, they were given an injection of saline rather than morphine. Following this injection, the group stayed in the injection room for the same 10-minute time period after the last injection. Following this time period, the group was again put in the operant chambers under a VR4 schedule for 30 minutes with the same grooming, defecation, and response recording procedures in place. The Context-Only-Experimental Group were brought into the same injection room with the same light and white noise; however, they did not receive any injection. They were exposed to these conditions for a 10-minute time period. Following this time period, the group was again put in the operant chambers under a VR4 schedule for 30 minutes with the same grooming, defecation, and response recording procedures in place. The No-Cue-Control Group was not exposed to the injection room or any of its related cues and instead was brought straight to the operant chamber room and put in the operant chambers under a VR4 schedule for 30 minutes with the same grooming, defecation, and response recording procedures in place. This phase of the study lasted for 5 days.

At the conclusion of the withdrawal phase, the rats were euthanized, and a statistical analysis was conducted based on the data that was collected.

Statistical Analysis

Statistical analysis was done through a between-group 3X6 Mixed Factorial ANOVA. The first independent variable (IV) had three levels, which consisted of the three levels of exposure to CSs during the Withdrawal Phase: No-Cue-Control, Combined-Cue-Experimental, and Context-Cue-Only-Experimental. The other IV had five levels, one measure for each day of the Withdrawal Phase. The dependent variable (DV) being observed was the average number of completed response sets of each rat from each group during the Baseline Testing Phase, subtracted from the average number of completed response sets of each rat from each group for each day of the Withdrawal Phase. This form of test was run three more times, with the variation in the dependent variable, namely: grooming behavior, weight, and defecation. Each variable had its measurements from each day of withdrawal subtracted by the average score from the baseline phase. The significance level of these tests were set at $\alpha > 0.05$ and post hoc tests were performed where appropriate.

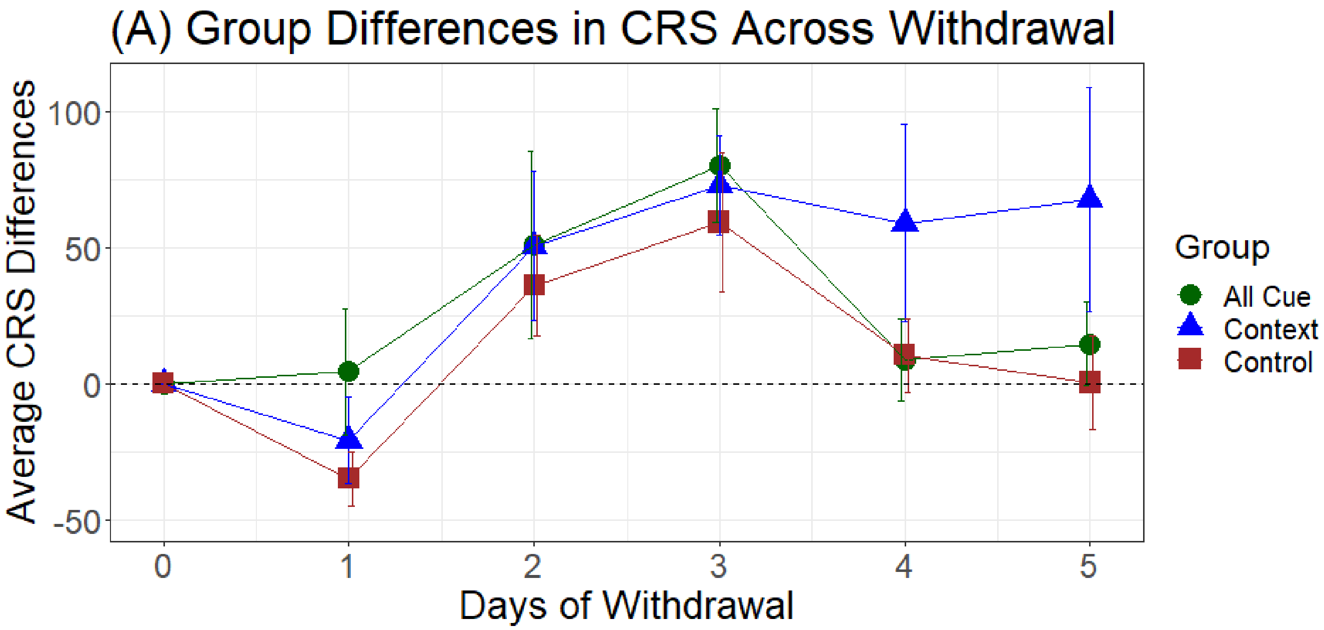
Any significant interactions were followed up on using a one-way ANOVA, in which the independent variable was again the three levels of exposure to CSs during the Withdrawal Phase: No-Cue-Control, Combined-Cue-Experimental, and Context-Cue-Only-Experimental, each day of withdrawal being held as a constant. Once again, the significance level of these tests was set at $\alpha > 0.05$ and post hoc tests were performed where appropriate. All statistical analyses were conducted in JASP, and graphs were constructed in R.

Results

Observed Behaviors

Completed Response Sets

A 3X6 factorial ANOVA – with cue groups as the between-subjects factor and day as the within-subject factor – was performed for changes in completed response sets between the baseline average and the days of the withdrawal period (Figure 2.A). A significant main effect was found across the days of withdrawal [$F(5,54) = 6.261$; $p < 0.001$]. No significant difference was found between cue groups during the withdrawal phase [$F(2,54) = 2.203$; $p = 0.120$]. No significant interaction was found between the days of the withdrawal period and the groups [$F(10,54) = 0.676$; $p = 0.741$]. Post hoc analysis (Table 1) showed significant differences between the baseline average and day 3 ($p = 0.002$), day 1 and day 2 ($p = 0.009$), and day 1 and day 3 ($p < 0.001$).



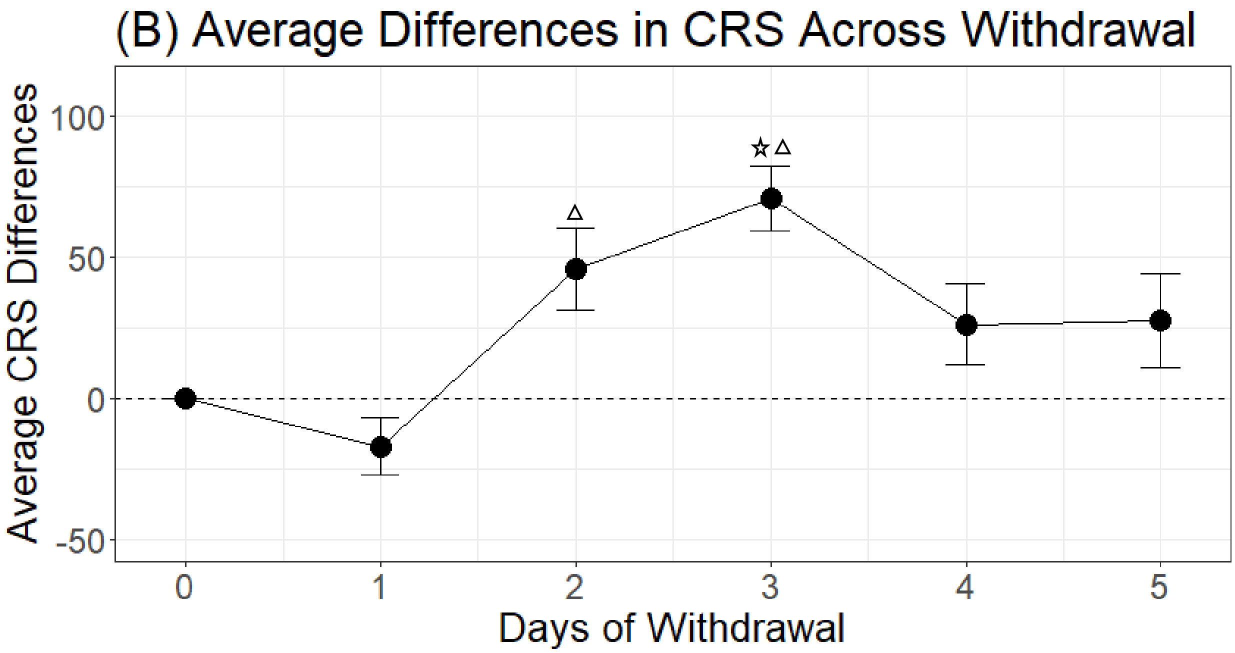
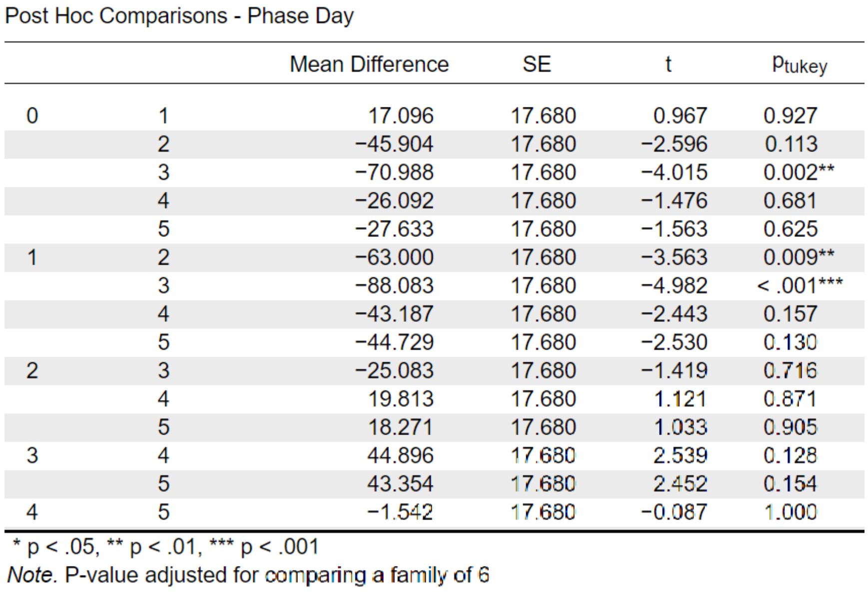


Figure 2. Error bars in all graphs represent standard error of the mean. A dashed line was added at 0 to represent baseline data. **(A)** Changes in completed response sets (CRS) across the days of the withdrawal phase compared to the average completed response sets during baseline. The baseline is shown through day 0 of phase day. Averages across the days began around the baseline and generally increased. **(B)** Shows the collapsed data of each cue group across the withdrawal phase. △ day 1 $p < 0.05$, ☆baseline (day 0) $p < 0.05$



Post Hoc Comparisons - Phase Day

		Mean Difference	SE	t	p_{tukey}
0	1	17.096	17.680	0.967	0.927
	2	−45.904	17.680	−2.596	0.113
	3	−70.988	17.680	−4.015	0.002**
	4	−26.092	17.680	−1.476	0.681
	5	−27.633	17.680	−1.563	0.625
1	2	−63.000	17.680	−3.563	0.009**
	3	−88.083	17.680	−4.982	< .001***
	4	−43.187	17.680	−2.443	0.157
	5	−44.729	17.680	−2.530	0.130
2	3	−25.083	17.680	−1.419	0.716
	4	19.813	17.680	1.121	0.871
	5	18.271	17.680	1.033	0.905
3	4	44.896	17.680	2.539	0.128
	5	43.354	17.680	2.452	0.154
4	5	−1.542	17.680	−0.087	1.000

* $p < .05$, ** $p < .01$, *** $p < .001$

Note. P-value adjusted for comparing a family of 6

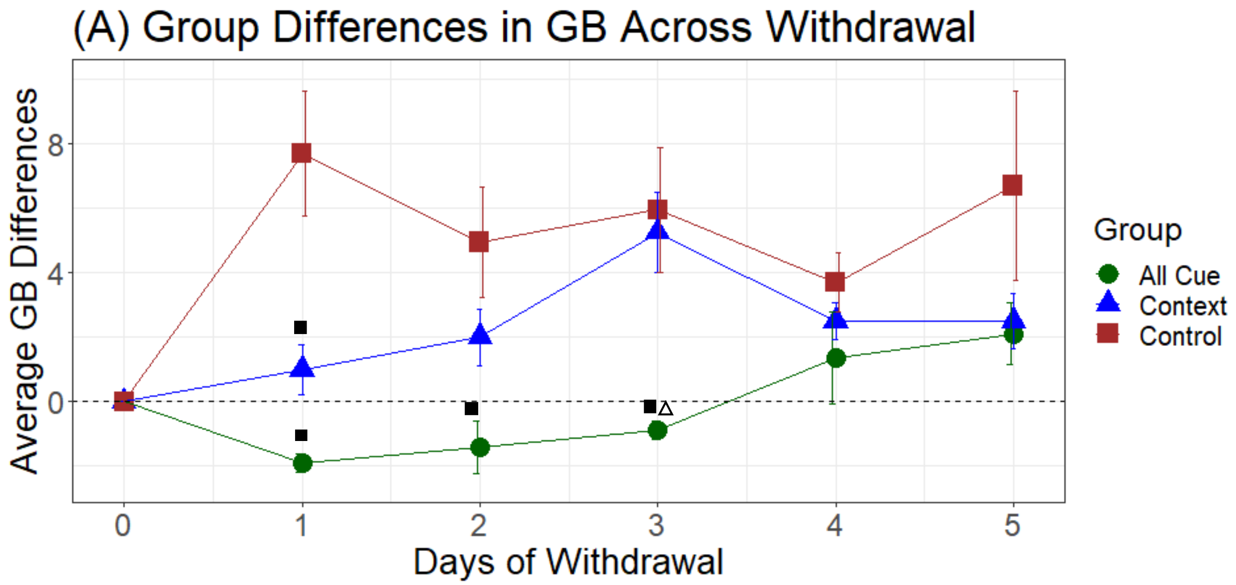
Table 1. Post hoc analysis of completed response sets across the five days of withdrawal.

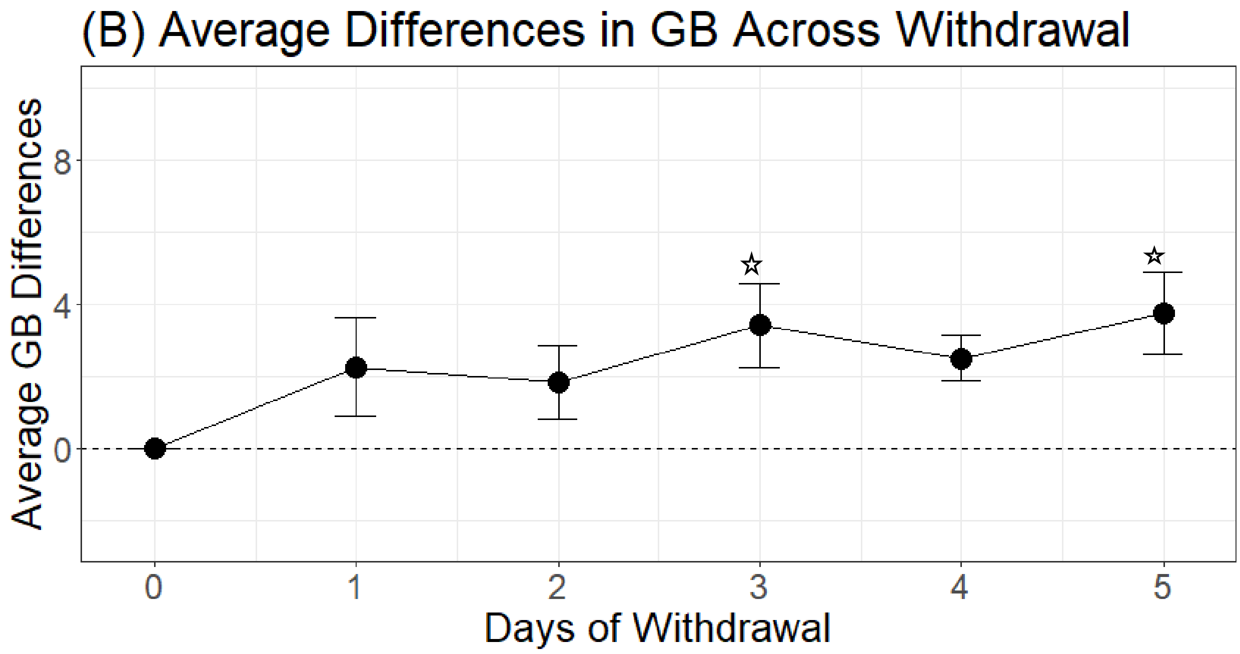
Grooming Behaviors

A 3X6 factorial ANOVA – with cue groups as the between-subjects factor and day as the within-subject factor – was performed for changes in grooming behaviors between the baseline average and the days of the withdrawal period (Figure 3.A). A significant main effect was found between the cue groups during the withdrawal phase [$F(2,54) = 23.919$; $p < 0.001$]. A significant main effect was also found across the days of the withdrawal phase [$F(5,54) = 3.489$; $p = 0.008$]. A significant interaction was found between the days of the withdrawal period and the groups [$F(10,54) = 2.527$; $p = 0.014$].

Post hoc analysis (Table 2) showed significant differences between the baseline average and day 3 ($p = 0.016$), baseline average and day 5 ($p = 0.006$). Post hoc analysis (Table 3) showed significant differences between the all-cue group and the context group ($p = 0.006$), the all-cue group and the control group ($p < 0.001$), and the context group and the control group ($p = 0.002$).

To explore the interaction between groups and days during the withdrawal phase in concern to grooming behavior: each day was held constant, comparing across cue groups. A significant main effect was found on day 1 [$F(2,9) = 16.259$; $p = 0.001$]. Post hoc analysis of day 1 showed a significant difference between the all-cue group and the control group ($p < 0.001$), and the context group and the control group ($p = 0.009$). A significant main effect was found on day 2 [$F(2,9) = 6.933$; $p = 0.015$]. Post hoc analysis of day 2 showed a significant difference between the all-cue group and the control group ($p = 0.012$). A significant main effect was found on day 3 [$F(2,9) = 7.822$; $p = 0.011$]. Post hoc analysis of day 3 showed a significant difference between the all-cue group and the control group ($p = 0.014$), and the all-cue group and the context group (*p* = 0.025). No significant main effect was found on day 4 [*F*(2,9) = 1.267; *p* = 0.327]. No significant main effect was found on day 5 [*F*(2,9) = 1.905; *p* = 0.204].





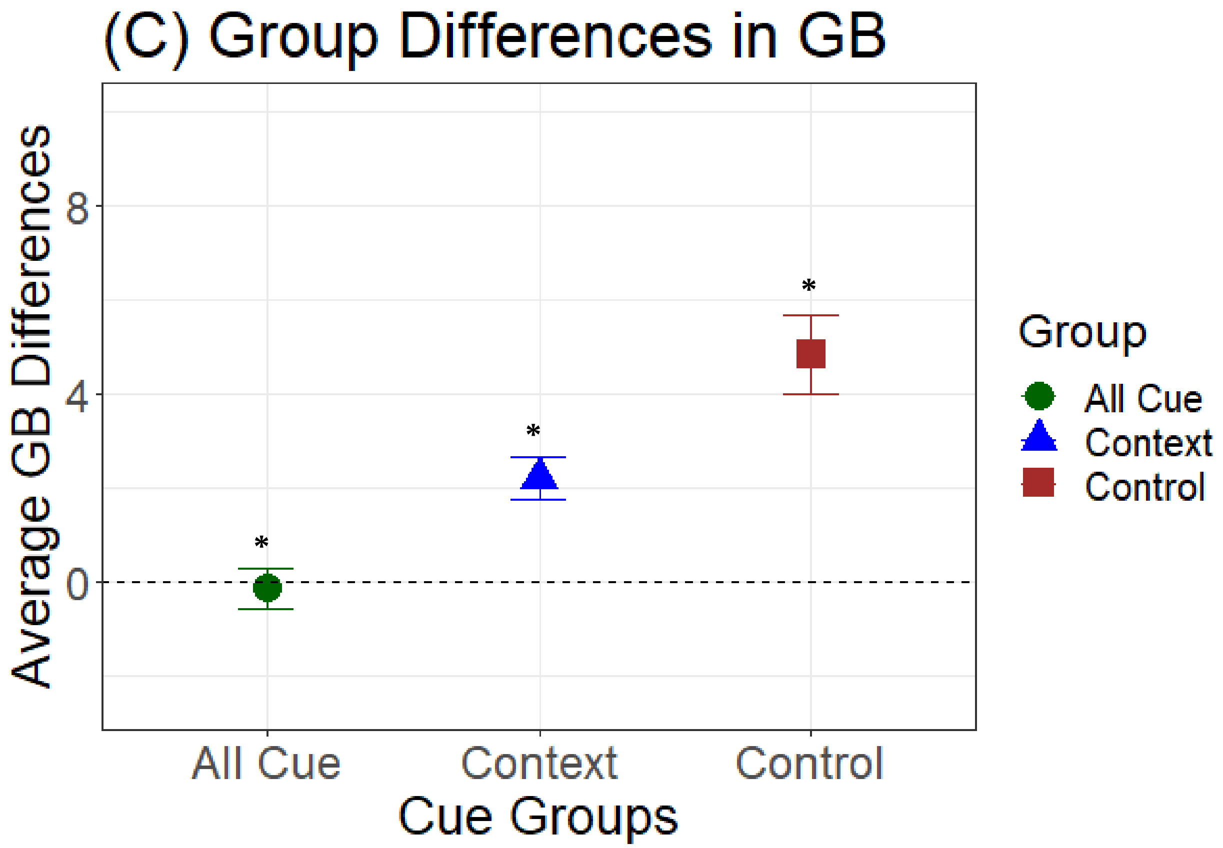
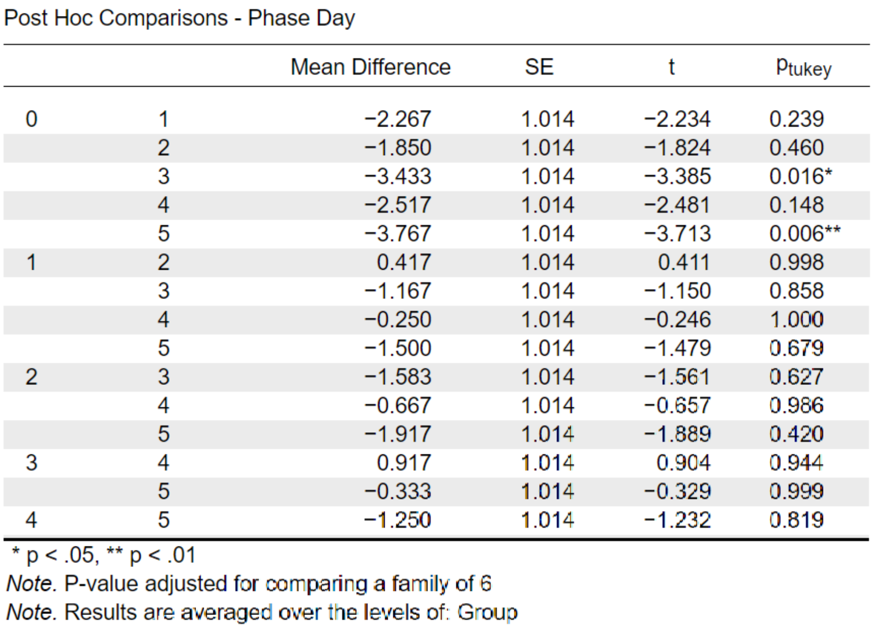


Figure 3. Error bars in all graphs represent standard error of the mean. A dashed line was added at 0 to represent baseline data. **(A)** Changes in grooming behavior (GB) across the days of the withdrawal phase compared to the average grooming behavior during baseline. The baseline is shown through day 0 of phase day. ■Control Group $p < 0.05$, △Context Cue Group $p < 0.05$ **(B)** Shows the collapsed data of each cue group across the 5 days of the withdrawal phase. ☆baseline (day 0) $p < 0.05$ **(C)** Shows the collapsed data of each day into each cue group. All groups showed significant differences from each other (*) $p < 0.05$.



Post Hoc Comparisons - Phase Day

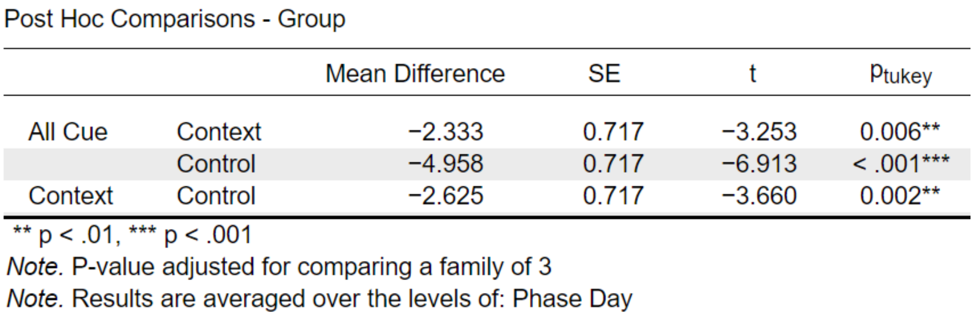
		Mean Difference	SE	t	p_{tukey}
0	1	−2.267	1.014	−2.234	0.239
	2	−1.850	1.014	−1.824	0.460
	3	−3.433	1.014	−3.385	0.016*
	4	−2.517	1.014	−2.481	0.148
	5	−3.767	1.014	−3.713	0.006**
1	2	0.417	1.014	0.411	0.998
	3	−1.167	1.014	−1.150	0.858
	4	−0.250	1.014	−0.246	1.000
	5	−1.500	1.014	−1.479	0.679
2	3	−1.583	1.014	−1.561	0.627
	4	−0.667	1.014	−0.657	0.986
	5	−1.917	1.014	−1.889	0.420
3	4	0.917	1.014	0.904	0.944
	5	−0.333	1.014	−0.329	0.999
4	5	−1.250	1.014	−1.232	0.819

* p < .05, ** p < .01

Note. P-value adjusted for comparing a family of 6

Note. Results are averaged over the levels of: Group

Table 2. Post hoc analysis of grooming behaviors across the five days of withdrawal.



Post Hoc Comparisons - Group

		Mean Difference	SE	t	p_{tukey}
All Cue	Context	−2.333	0.717	−3.253	0.006**
	Control	−4.958	0.717	−6.913	< .001***
Context	Control	−2.625	0.717	−3.660	0.002**

** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 3

Note. Results are averaged over the levels of: Phase Day

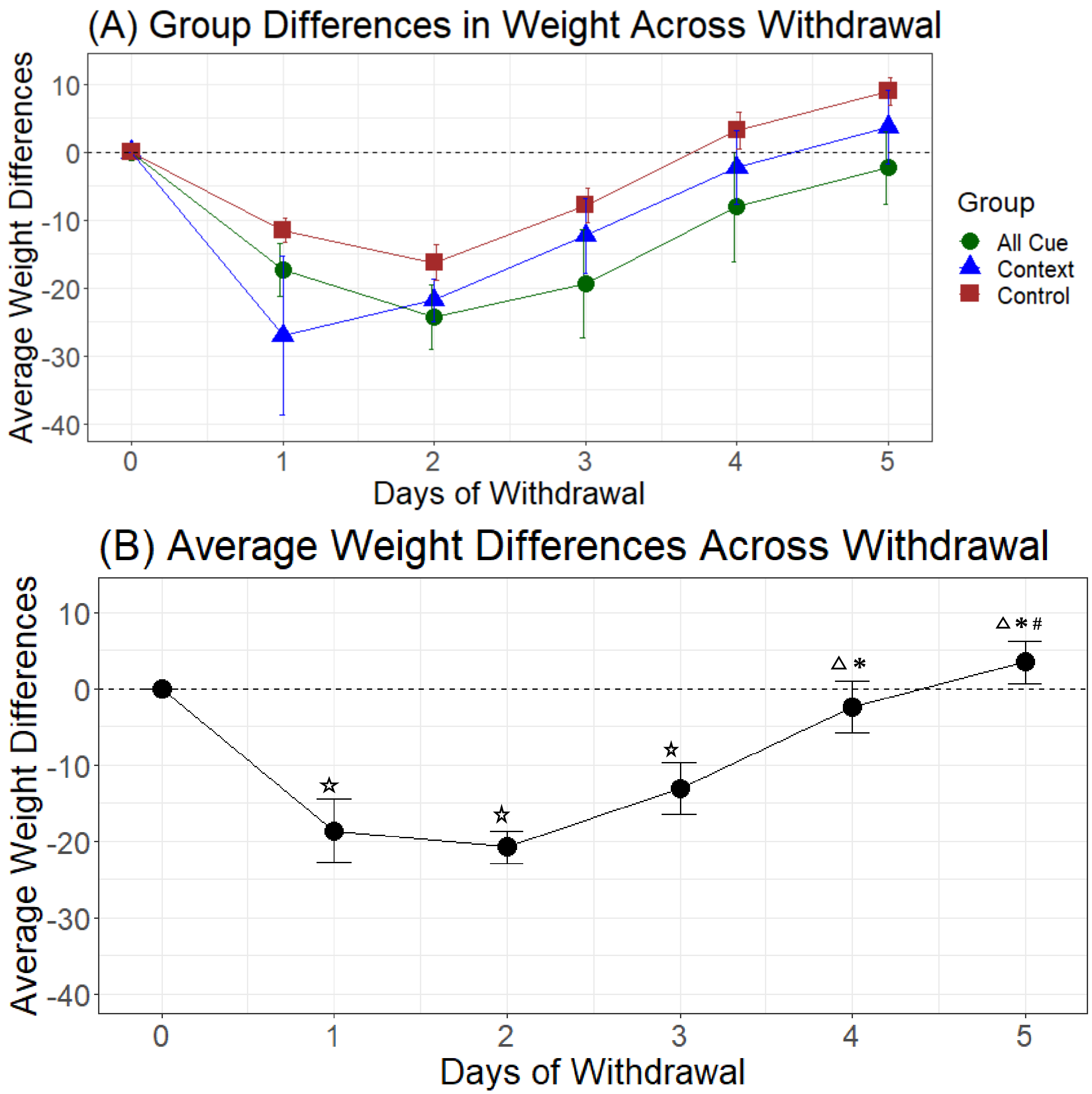
Table 3. Post hoc analysis of grooming behaviors between the three cue groups.

Physiological Measures

Weight

A 3X6 factorial ANOVA – with cue groups as the between-subjects factor and day as the within-subject factor – was performed for changes in rats' weight between the baseline average and the days of the withdrawal period (Figure 2.A). A significant main effect was found across the days of withdrawal [$F(5,54) = 12.430$; $p < 0.001$]. A significant main effect was found between cue groups during the withdrawal phase [$F(2,54) = 4.110$; $p = 0.022$]. No significant interaction was found between the days of the withdrawal period and the groups [$F(10,54) = 0.564$; $p = 0.836$].

Post hoc analysis (Table 4) showed significant differences between the baseline average and day 1 ($p < 0.001$), the baseline average and day 2 ($p < 0.001$), the baseline average and day 3 ($p = 0.028$), day 1 and day 4 ($p = 0.003$), day 1 and day 5 ($p < 0.001$), day 2 and day 4 ($p < 0.001$), day 2 and day 5 ($p < 0.001$), and day 3 and day 5 ($p = 0.002$). Post hoc analysis (Table 5) showed significant differences between the all-cue group and the control group ($p = 0.022$).



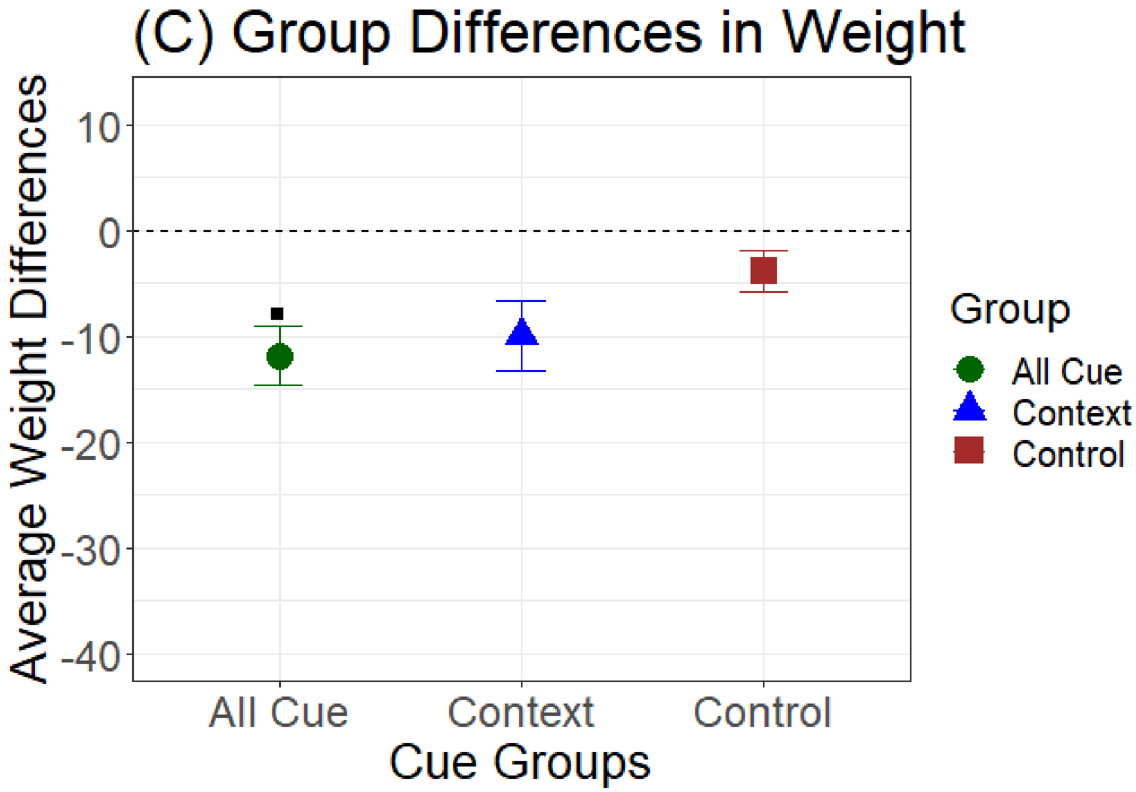
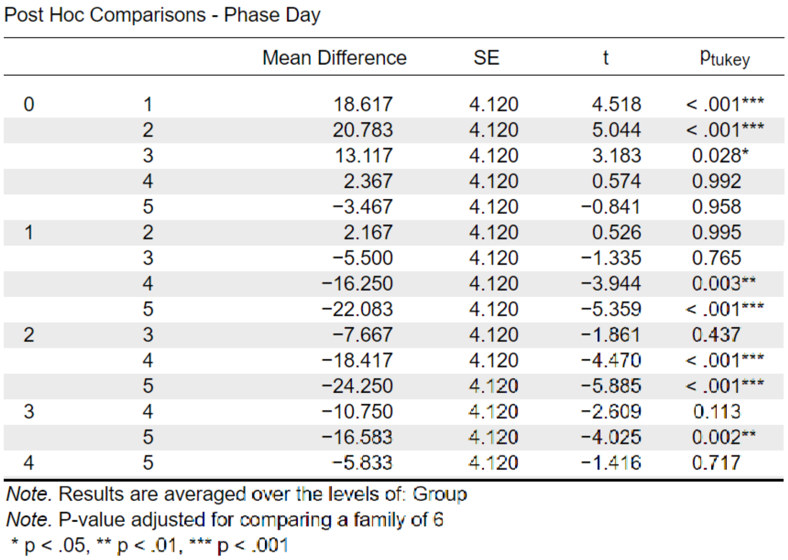


Figure 4. Error bars in all graphs represent standard error of the mean. A dashed line was added at 0 to represent baseline data. **(A)** Changes in weight across the days of the withdrawal phase compared to the average weight during baseline. The baseline is shown through day 0 of phase day. **(B)** Shows the collapsed data of each cue group across the 5 days of the withdrawal phase.☆ baseline (day 0) $p < 0.05$, △ day 1 $p < 0.05$, * day 2 $p < 0.05$, # day 3 $p < 0.05$
(C) Shows the collapsed data of each day into each cue group. ■ Control group $p < 0.05$.

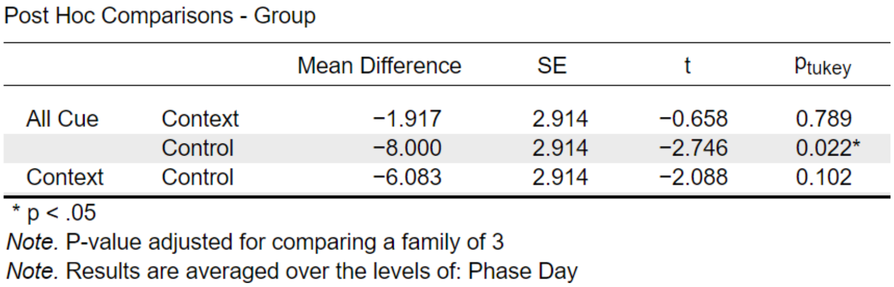


Post Hoc Comparisons - Phase Day

		Mean Difference	SE	t	p_{tukey}
0	1	18.617	4.120	4.518	< .001***
	2	20.783	4.120	5.044	< .001***
	3	13.117	4.120	3.183	0.028*
	4	2.367	4.120	0.574	0.992
	5	−3.467	4.120	−0.841	0.958
1	2	2.167	4.120	0.526	0.995
	3	−5.500	4.120	−1.335	0.765
	4	−16.250	4.120	−3.944	0.003**
	5	−22.083	4.120	−5.359	< .001***
2	3	−7.667	4.120	−1.861	0.437
	4	−18.417	4.120	−4.470	< .001***
	5	−24.250	4.120	−5.885	< .001***
3	4	−10.750	4.120	−2.609	0.113
	5	−16.583	4.120	−4.025	0.002**
4	5	−5.833	4.120	−1.416	0.717

Note. Results are averaged over the levels of: Group
Note. P-value adjusted for comparing a family of 6
* p < .05, ** p < .01, *** p < .001

Table 4. Post hoc analysis of weight across the five days of withdrawal.



Post Hoc Comparisons - Group

		Mean Difference	SE	t	p_{tukey}
All Cue	Context	−1.917	2.914	−0.658	0.789
	Control	−8.000	2.914	−2.746	0.022*
Context	Control	−6.083	2.914	−2.088	0.102

* $p < .05$

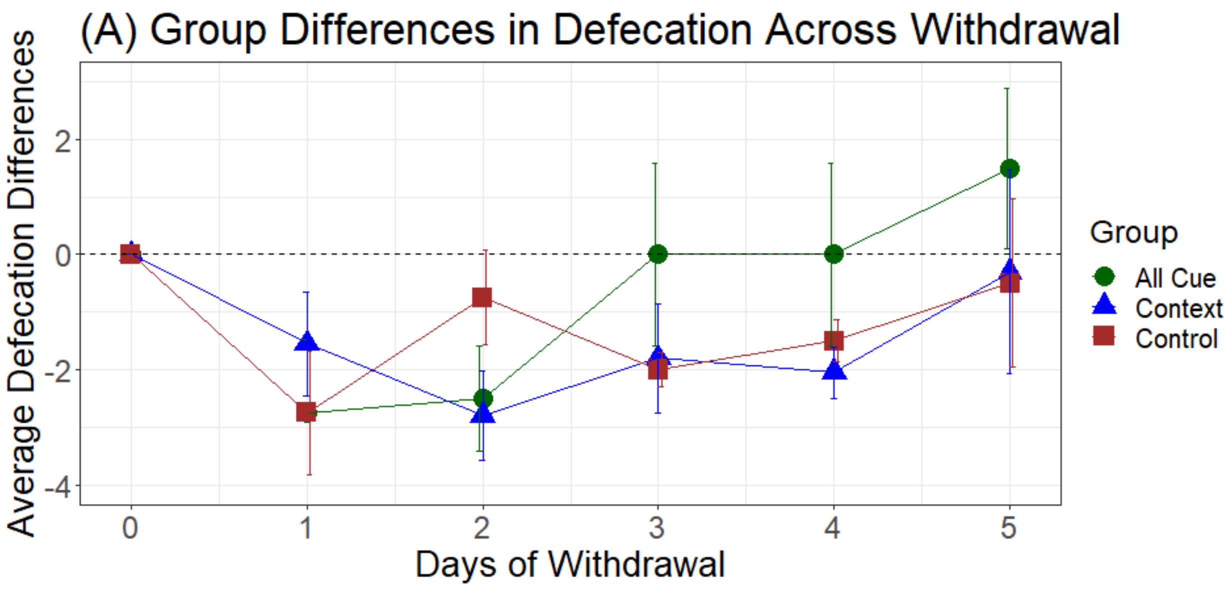
Note. P-value adjusted for comparing a family of 3

Note. Results are averaged over the levels of: Phase Day

Table 5. Post hoc analysis of weight between the three cue groups.

Defecation

A 3X6 factorial ANOVA – with cue groups as the between-subjects factor and day as the within-subject factor – was performed for changes in defecation amounts between the baseline average and the days of the withdrawal period (Figure 5.A). A significant main effect was found across the days of withdrawal [$F(5,54) = 3.037$; $p = 0.017$]. No significant difference was found between cue groups during the withdrawal phase [$F(2,54) = 0.975$; $p = 0.384$]. No significant interaction was found between the days of the withdrawal period and the groups [$F(10,54) = 0.786$; $p = 0.642$]. Post hoc analysis (Table 6) showed a significant difference between day 1 and day 5 ($p = 0.039$).



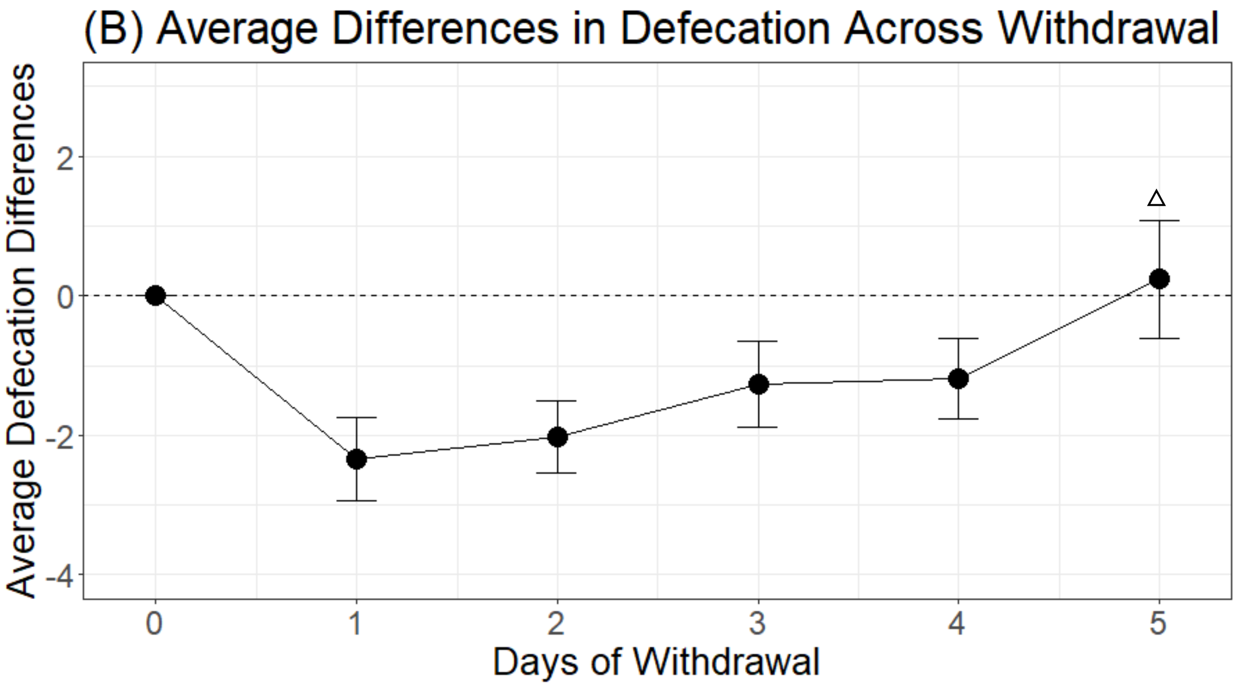
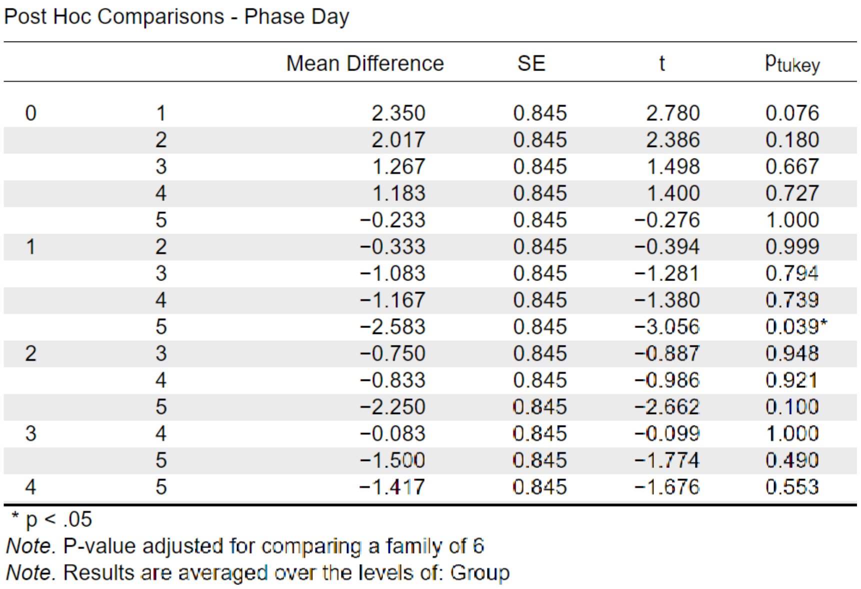


Figure 5. Error bars in all graphs represent standard error of the mean. A dashed line was added at 0 to represent baseline data. **(A)** Changes in defecation amount across the days of the withdrawal phase compared to the average defecation amount during baseline. The baseline is shown through day 0 of phase day. Averages across the days began around the baseline and generally increased. **(B)** Shows the collapsed data of each cue group across the withdrawal phase. △ day 1 $p < 0.05$.


Post Hoc Comparisons - Phase Day

		Mean Difference	SE	t	p_{tukey}
0	1	2.350	0.845	2.780	0.076
	2	2.017	0.845	2.386	0.180
	3	1.267	0.845	1.498	0.667
	4	1.183	0.845	1.400	0.727
	5	−0.233	0.845	−0.276	1.000
1	2	−0.333	0.845	−0.394	0.999
	3	−1.083	0.845	−1.281	0.794
	4	−1.167	0.845	−1.380	0.739
	5	−2.583	0.845	−3.056	0.039*
2	3	−0.750	0.845	−0.887	0.948
	4	−0.833	0.845	−0.986	0.921
	5	−2.250	0.845	−2.662	0.100
3	4	−0.083	0.845	−0.099	1.000
	5	−1.500	0.845	−1.774	0.490
4	5	−1.417	0.845	−1.676	0.553

* $p < .05$

Note. P-value adjusted for comparing a family of 6

Note. Results are averaged over the levels of: Group

Table 6. Post hoc analysis of defecation amounts across the five days of withdrawal.

Discussion

The purpose of this study was aimed to answer the following two questions: can the response profile of morphine be replicated in male rats through classical conditioning, and subsequently, can morphine withdrawal symptoms be reduced through classical conditioning? Results from the present study demonstrated no significant differences in completed response sets or defecation measures when comparing exposure to CS. Those rats that were exposed to both environmental CS and discrete CS (all-cue) had no significant difference from those rats that were exposed to only environmental CS (context), and neither of those groups performed significantly different from those rats that were exposed to no purposeful CS (control). These results do not support the hypothesis. Though, the all-cue group did record significantly different scores from both the context cue and control group in terms of grooming behaviors (context cue was also significantly different from the control group). With all-cue being most like the baseline measure, the hypothesis was supported in this case. Notably, however, the context group had a less substantial increase from baseline than the control group, which is counter to the hypothesis. Weight demonstrated significant differences between the all-cue group and the control group, with the control group being most similar to baseline, which again is counter to the hypothesis.

Other significant differences (discussed below) were found beyond the main hypothesis which provided fascinating insight into the progression of morphine withdrawal in male Sprague-Dawley rats. These trends also help to suggest potential unforeseen shortcomings of the study, which may have led to the inconsistent results discussed above.

Observed Behaviors

Completed Response Sets

Based on the completed response sets within the operant chambers on a VR4 schedule, there was no significant main effect of cue exposure on performance. However, there was a significant main effect of day, in which rats responded significantly greater during days 2 and 3 of the withdrawal phase than both their baseline averages as well as their day 1 average. The post hoc analysis also indicated that there was no significant difference between the average baseline data and days 4 and 5, indicating that rats across the groups seemed to return to relatively normal response rates by the end of the withdrawal phase.

During the drug injection period, due to morphine's property as a depressant: responses in the operant chambers were significantly reduced — especially on the first few days of the tolerance period where responses remained at zero. So, the *increased* level of responding – particularly on days 2 and 3 of withdrawal, indicates that a drug-opposite effect occurred during the withdrawal phase of the study. This suggests that all groups experienced a form of behavioral dysregulation (withdrawal). The subsequent return to baseline averages following the spike of behavior indicates a subsiding of withdrawal behavior on days 4 and 5. Similar trends were seen in the other measures of withdrawal. These results are consistent with the findings from Tiffany's study which found no significant differences between cue exposure and responsiveness to shock tests. It should be noted, however, that the other behavioral based test in the present study *did* show an impact on types of cue and resulting withdrawal symptoms.

Grooming Behaviors

Overall, rats in the all-cue group experienced fewer withdrawal symptoms based on grooming behavior as the all-cue group behaved similarly to their baseline grooming behaviors.

Whereas both the context cue and control groups groomed more than their baseline averages, which were both significantly different from the all-cue groups. However, in this case, the control group groomed significantly more than both the context group and all-cue group, which indicates that those rats were experiencing worsened withdrawal symptoms (Pinelli & Trivulzio, 1997).

There were also significant differences across the days of the withdrawal period when collapsed between groups. Post hoc analysis indicated that there were significant differences between the baseline averages and days 3 and 5, with a fairly regular upward trend across all of the days of withdrawal. This would indicate that withdrawal symptoms – in terms of grooming behaviors – worsened across the days of the withdrawal period.

A significant interaction was also observed between groups and across days. By holding each day as a constant, post hoc analysis was conducted, which found significant differences on day 1 between all of the groups, day 2 between the all-cue group and control group, and day 3 between the all-cue group and both the context and control group, but no significant difference was found in day 4 nor day 5. This indicates that in the first three days of the withdrawal period, there was a separation between the three groups, however, by the end of the withdrawal period the groups relatively converged with each other.

This convergence by the end of the withdrawal phase could be due to the extinction of the CR over time. The speed at which the CR was extinguished should be considered as a potential pointer to needing a longer tolerance phase to increase the conditioning effect. This change could potentially result in greater differences between groups throughout the withdrawal phase in a future study.

Physiological Measures

Weight

Weight provided a physiological measure for withdrawal in this study. There were significant differences in the averages of the withdrawal phase between the control group and the all-cue group. Notably, in this measure, the control group's weight was the closest to their baseline average, whereas the all-cue and context groups were significantly lighter than their baseline averages. This was an inconsistent aspect of the results, as the significant weight loss indicates withdrawal in rats (Martin et al., 1963). This would suggest that the all-cue group was suffering from more withdrawal symptoms than the control group.

Weight also had significant differences across the days of withdrawal, with days 1, 2, and 3 being significantly less than the baseline average, as well as days 1 and 2 being significantly less than days 4 and 5, and day 3 was only significantly less than day 5. The overall trend suggested that at the start of the withdrawal phase rats were experiencing withdrawal symptoms, but as the days of withdrawal passed rats regained weight returning to close to baseline averages and therefore experiencing less withdrawal symptoms. This trend is consistent with data found from the completed response set data.

Defecation

Defecation provided a second physiological measure to consider during the withdrawal phase. While the only significant difference found across the days was between days 1 and 5 of the withdrawal phase, the overall pattern provided a very interesting trend. When compared to their baseline measures, the defecation amount was much less during the start of withdrawal; then across the days, it slowly returned to right around baseline by the final day of the withdrawal phase (notably, the p-value between the baseline measure and day 5 was 1.0, suggesting no difference).

Defecation being less across most days of withdrawal when compared to baseline is the opposite of what was anticipated for any group. Morphine causes constipation when in use, so during withdrawal, it is expected that it would cause increased defecation similar to that of a diuretic — a drug-opposite effect (Pinelli & Trivulzio, 1997). The hypothesis would have predicted then that the all-cue could have had similar rates to the baseline average, where the context and control groups would have defecated much more. The fact that all groups defecated less is odd. There are two likely reasons for these results: either some aspects of the opioid injections were unintentionally consistent with all groups and caused a CR in all groups, or rats were not maintained on the opioid injections for long enough, and so a non-withdrawal response was observed.

However, if we were to consider the overall trend, this measure matches that of the completed response set data, as well as the weight data. The first few days were significantly different from the baseline data, which was then later returned to in the later portions of the withdrawal phase. Once again suggesting that the effect of withdrawal from the drug was diminished by the end of the 5 days.

Conclusion

The results from this study produced a variety of implications. Completed response sets, weight, and defecation all followed a similar pattern; they began the withdrawal phase significantly different from the baseline, and throughout the withdrawal phase steadily returned to their relative baseline level. Those three measures allow us to see that within five days of not receiving morphine, all rats had a significant reduction in withdrawal symptoms and behaviors.

The only measure that did not follow this trend was grooming behavior, which instead showed an *increase* in withdrawal symptoms (indicated by an increased level of grooming) across the five days of withdrawal. Previous studies have indicated that some forms of morphine-based withdrawal symptoms in mice will increase across the days of withdrawal (Stevens et al., 2021). These symptoms are essentially on a delay from other withdrawal symptoms, in which they come in later, sometimes even after other symptoms have dissipated. Many of these withdrawal symptoms are focused on movement behavior – much like grooming behaviors. With this information, in conjunction with the fact that grooming behavior was the only to produce results relatively consistent to the hypothesis, perhaps only withdrawal symptoms that have delayed intense effects will be assisted by this technique.

Grooming behavior was also notable for indicating a significant difference between the groups as well. This measure partially supported the overall hypothesis, as the all-cue group maintained a relatively consistent value close to baseline; whereas the context and control groups had increased grooming behaviors from baseline during the withdrawal phase. However, the results suggested that the context group also had less withdrawal symptoms based on grooming than the control group, which does not support the hypothesis. The trend of these results was also seen in the weight measure, as there were significant differences found between groups, with all-cue and context being significantly different from the control group. As stated prior, however, while the overall order of the trend matches; the control group seemed to experience least withdrawal in terms of weight measurement than the other two groups, which is inconsistent with the grooming behaviors.

Overall, grooming behaviors provided significant evidence that the application of a discrete stimulus following contextual stimuli during withdrawal can help to reduce some forms of withdrawal. Though the inconsistencies between grooming behaviors and weight, as well as the lack of impact in completed response sets and defecation should certainly be considered evidence that not all withdrawal symptoms will be helped through this method; potentially even worsened. This study should serve as firm reasoning for the expansion of tolerance and withdrawal tests used in the study of opioids, as evidenced by the variety of results between the four tests.

Future Directions

Some of these inconsistencies may be due to the small sample size within each group. Had this study been conducted with a larger cohort, there could have been more consistent results across the different measures and likely more significant differences between groups in the other categories that did not show significance between groups. Taking this into account, this study should be furthered with greater sample sizes to better investigate the significant differences and reduce the overall impact of individual rats. It would also be beneficial to lengthen the time in which rats exist within the tolerance phase, perhaps waiting two days after the last subject reaches tolerance requirements. This would allow for more time to strengthen conditioning surrounding morphine injections and the resulting effects.

It should also be considered, as discussed previously, that not all withdrawal symptoms will be impacted the same way through cue induction. For that reason, future studies should consider expanding the number and types of withdrawal symptoms studied in order to search for potential patterns between which are helped through this technique, those that are worsened by it, and those that are unaffected. If this approach were to be continued into clinical trials, it should be used in conjunction with other current treatments to more broadly target a variety of withdrawal symptoms and assist in the return to normal life following rehabilitation. The same technique could also be explored in the use of other drug use disorders, with the same goal of identifying which symptoms could be benefitted by the use of such techniques.

This study could also be adapted into other investigations into the onset of natural withdrawal following tolerance development period. These studies could be used to better understand changes in behavior and physiology beyond any classical conditioning techniques. Such studies would be beneficial to better understand how successful future treatments are in pre-clinical trials, prior to them being adapted for human use.

References

Ader, R., & Cohen, N. (1975). Behaviorally conditioned immunosuppression. *Psychosomatic medicine, 37*(4), 333-340. DOI: 10.1097/00006842-197507000-00007

Ahmad, F. B., Rossen, L. M., & Sutton, P. (2022). Provisional drug overdose data. *Centers for Disease Control and Prevention*. https://www.cdc.gov/nchs/nvss/vsrr/drug-overdose-data.htm

Alam, A., Gomes, T., Zheng, H., Mamdani, M. M., Juurlink, D. N., & Bell, C. M. (2012). Long-term analgesic use after low-risk surgery: a retrospective cohort study. *Archives of internal medicine*, *172*(5), 425-430. DOI: 10.1001/archinternmed.2011.1827

Allahverdiyev, O., Türkmen, A. Z., Nurten, A., Şehirli, İ., & Enginar, N. (2015). Spontaneous withdrawal in intermittent morphine administration in rats and mice: effect of clonidine coadministration and sex-related differences. *Turkish journal of medical sciences, 45*(6), 1380-1389. doi:10.3906/sag-1408-137

Brummett, C. M., Waljee, J. F., Goesling, J., Moser, S., Lin, P., Englesbe, M. J., ... & Nallamothu, B. K. (2017). New persistent opioid use after minor and major surgical procedures in US adults. *JAMA surgery*, *152*(6), e170504-e170504. doi:10.1001/jamasurg.2017.0504

Cappell, H., & LeBlanc, A. E. (1981). Tolerance and physical dependence. *Research advances in alcohol and drug problems*, 159-196. DOI: 10.1007/978-1-4615-7740-9_5

Centers for Disease Control and Prevention. (2022, September 7). *Lifesaving naloxone*. Centers for Disease Control and Prevention. Retrieved December 14, 2022, from https://www.cdc.gov/stopoverdose/naloxone/index.html#:~:text=Naloxone%20quickly%20reverses%20an%20overdose,opioids%20like%20fentanyl%20are%20involved.

Clarke, H., Soneji, N., Ko, D. T., Yun, L., & Wijeysundera, D. N. (2014). Rates and risk factors for prolonged opioid use after major surgery: population based cohort study. *Bmj, 348*. doi: https://doi.org/10.1136/bmj.g1251

Craft, R., Stratmann, J., Bartok, R., Walpole, T., King, S. (1999). Sex differences in development of morphine tolerance and dependence in the rat. *Psychopharmacology, 143*(1–7). https://doi.org/10.1007/s002130050911

Dahlström, B. E., Paalzow, L. K., Segre, G., & Ågren, A. J. (1978). Relation between morphine pharmacokinetics and analgesia. *Journal of pharmacokinetics and biopharmaceutics, 6*(1), 41-53. DOI: 10.1007/BF01066062

Dhaliwal, A., Gupta, M. (2021) Physiology, Opioid Receptor. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK546642/ on 2022, September 10. PMID: 31536249

Fujii, M. H., Hodges, A. C., Russell, R. L., Roensch, K., Beynnon, B., Ahern, T. P., Holoch, P., Moore, J. S., Ames, S. E., & MacLean, C. D. (2018). Post-Discharge Opioid Prescribing and Use after Common Surgical Procedure. *Journal of the American College of Surgeons*, *226*(6), 1004–1012. doi: 10.1016/j.jamcollsurg.2018.01.058

Goesling, J., Moser, S. E., Zaidi, B., Hassett, A. L., Hilliard, P., Hallstrom, B., ... & Brummett, C. M. (2016). Trends and predictors of opioid use following total knee and total hip arthroplasty. *Pain, 157*(6), 1259. DOI: 10.1097/j.pain.0000000000000516

Hasselström, J., & Säwe, J. (1993). Morphine pharmacokinetics and metabolism in humans. *Clinical pharmacokinetics*, *24*(4), 344-354. DOI: 10.2165/00003088-199324040-00007

Kestenbaum, M. G., Vilches, A. O., Messersmith, S., Connor, S. R., Fine, P. G., Murphy, B., ... & Muir, J. C. (2014). Alternative routes to oral opioid administration in palliative care: a review and clinical summary. *Pain Medicine*, *15*(7), 1129-1153. DOI: 10.1111/pme.12464

Kim, J., Ham, S., Hong, H., Moon, C., & Im, H. I. (2016). Brain Reward Circuits in Morphine Addiction. *Molecules and cells*, *39*(9), 645–653. https://doi.org/10.14348/molcells.2016.0137

Kitanaka, J., Kitanaka, N., Hall, F. S., Fujii, M., Goto, A., Kanda, Y., & Takemura, M. (2015). Memory impairment and reduced exploratory behavior in mice after administration of systemic morphine. *Journal of experimental neuroscience*, *9*, JEN-S25057. https://doi.org/10.4137/JEN.S25057

Krasnegor, N. A. (1978). Behavioral tolerance: Research and treatment implications. *Department of Health, Education and Welfare, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration, National Institute on Drug Abuse, Division of Research, 18,* (78-551). ASIN: B002YNRUYA

KuKanich, B., & Wiese, A. J. (2015). Opioids. *Veterinary Anesthesia and Analgesia: the fifth edition of Lumb and Jones*, 207-226. https://doi.org/10.1002/9781119421375.ch11

Kumar, K., Kelly, M., & Pirlot, T. (2001). Continuous intrathecal morphine treatment for chronic pain of nonmalignant etiology: long-term benefits and efficacy. *Surgical neurology*, *55*(2), 79-86. https://doi.org/10.1016/S0090-3019(01)00353-6

LeResche, L., Saunders, K., Dublin, S., Thielke, S., Merrill, J. O., Shortreed, S. M., Campbell, C., & Von Korff, M. R. (2015). Sex and age differences in global pain status among patients using opioids long term for chronic noncancer pain. *Journal of women's health*, *24*(8), 629-635. https://doi.org/10.1089/jwh.2015.5222

Liu, Y., Logan, J. E., Paulozzi, L. J., Zhang, K., & Jones, C. M. (2013). Potential misuse and inappropriate prescription practices involving opioid analgesics. *The American journal of managed care*, *19*(8), 648-658. PMID: 24304213

Loyd, D. R., Wang, X., & Murphy, A. Z. (2008). Sex differences in μ-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia. *Journal of Neuroscience*, *28*(52), 14007-14017. https://doi.org/10.1523/JNEUROSCI.4123-08.2008

Maguire D.R., Henson C., France C.P. (2016). Daily morphine administration increases impulsivity in rats responding under a 5-choice serial reaction time task. *Br J Pharmacol, 173*(8):1350-62. doi: 10.1111/bph.13434.

Martin, W. R., Wikler, A., Eades, C. G., & Pescor, F. T. (1963). Tolerance to and physical dependence on morphine in rats. *Psychopharmacologia*, *4*(4), 247-260. DOI: 10.1007/BF00408180

Max, M. B., Donovan, M., Miaskowski, C. A., Ward, S. E., Gordon, D., Bookbinder, M., ... & American Pain Society Quality of Care Committee. (1995). Quality improvement guidelines for the treatment of acute pain and cancer pain. *Jama*, *274*(23), 1874-1880. https://doi.org/10.1001/jama.1995.03530230060032

McCabe, S. E., West, B. T., Teter, C. J., & Boyd, C. J. (2012). Medical and nonmedical use of prescription opioids among high school seniors in the United States. *Archives of pediatrics & adolescent medicine*, *166*(9), 797-802. doi:10.1001/archpediatrics.2012.8

Miguez, G., Laborda, M. A., & Miller, R. R. (2014). Classical conditioning and pain: conditioned analgesia and hyperalgesia. *Acta psychologica*, *145*, 10-20. https://doi.org/10.1016/j.actpsy.2013.10.009

Minozzi, S., Amato, L., Vecchi, S., Davoli, M., Kirchmayer, U., & Verster, A. (2011). Oral naltrexone maintenance treatment for opioid dependence. *Cochrane Database of Systematic Reviews*, (2). DOI: 10.1002/14651858.CD001333.pub4

Mishra, P. R., Barik, M., & Ray, S. B. (2017). Effect of nimodipine on morphine-related withdrawal syndrome in rat model: An observational study. *Journal of Pediatric Neurosciences*, *12*(1), 7–14. https://doi.org/10.4103/1817-1745.205652

Negus, S. S., & Freeman, K. B. (2018). Abuse potential of biased mu opioid receptor agonists. *Trends in pharmacological sciences*, *39*(11), 916-919. DOI: 10.1016/j.tips.2018.08.007

NIDA. 2020, September 18. Principles of Effective Treatment. Retrieved from https://nida.nih.gov/publications/principles-drug-addiction-treatment-research-based-guide-third-edition/principles-effective-treatment on 2022, April 16

NIDA. 2023, March 9. Treatment and Recovery. Retrieved from http://nida.nih.gov/publications/drugs-brains-behavior-science-addiction/treatment-recovery on 2023, March 25

O'Brien C.P., Childress A.R., McLellan A.T., Ehrman R. (1992). Classical conditioning in drug-dependent humans. *Ann N Y Acad Sci*, *654*, 400-15. doi: 10.1111/j.1749-6632.1992.tb25984.x.

Pathan, H., & Williams, J. (2012). Basic opioid pharmacology: an update. *British journal of pain*, *6*(1), 11–16. https://doi.org/10.1177/2049463712438493

Pavlov, I.P. (1906). The scientific investigation of the psychical faculties or processes in the higher animals. Science, 24, 613–619. Pavlov, I.P. (1927).

Pinelli, A., & Trivulzio, S. (1997). Quantitative evaluation of opioid withdrawal signs in rats repeatedly treated with morphine and injected with naloxone, in the absence or presence of the antiabstinence agent clonidine. *Journal of pharmacological and toxicological methods*, *38*(3), 117-131.

Rahman, T. (2019). Over-prescription of opioids leading to addiction and alternatives. *Doctoral dissertation, Florida Atlantic University.*

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

Sannerud, C.A., & Young, A.M. (1986). Modification of morphine tolerance by behavioral variables. *The Journal of pharmacology and experimental therapeutics*. doi: 10.1901/jeab.2005.35-04

Shansky, R.M., & Murphy, A.Z. (2021). Considering sex as a biological variable will require a global shift in science culture. *Nature Neuroscience, 24*(4), 457-464. https://doi.org/10.1038/s41593-021-00806-8

Siegel, S. (1975). Evidence from rats that morphine tolerance is a learned response. *Journal of Comparative and Physiological Psychology*, *89*(5), 498–506. https://doi.org/10.1037/h0077058

Siegel, S. (1977). Morphine tolerance acquisition as an associative process. *Journal of Experimental Psychology: Animal Behavior Processes, 3*(1), 1–13. https://doi.org/10.1037/0097-7403.3.1.1

Siegel, S., & Ellsworth, D. W. (1986). Pavlovian conditioning and death from apparent overdose of medically prescribed morphine: A case report. *Bulletin of the Psychonomic Society*, *24*(4), 278-280. https://doi.org/10.3758/BF03330140

Siegel, S., Hinson, R. E., & Krank, M. D. (1978). The role of predrug signals in morphine analgesic tolerance: Support for a Pavlovian conditioning model of tolerance. *Journal of Experimental Psychology: Animal Behavior Processes, 4*(2), 188–196. https://doi.org/10.1037/0097-7403.4.2.188

Siegel, S., Hinson, E., & Krank, D. (1981). Morphine-induced attenuation of morphine tolerance. *Science*, 212, 1533-1534. https://doi.org/10.1126/science.7233244

Siegel, S. (1999). Drug anticipation and drug addiction. The 1998 H. David Archibald lecture. *Addiction, 94*(8), 1113-1124. DOI: 10.1046/j.1360-0443.1999.94811132.x

Stevens, S., & Mohan, S. (2021). Opioid withdrawal behavior in spiny mice: A novel preclinical model of neonatal opioid withdrawal syndrome (NOWS). *Heliyon, 7*(4). https://doi.org/10.1016/j.heliyon.2021.e06694

Stoicea, N., Costa, A., Periel, L., Uribe, A., Weaver, T., & Bergese, S. D. (2019). Current perspectives on the opioid crisis in the US healthcare system: A comprehensive literature review. *Medicine*, *98*(20), e15425. https://doi.org/10.1097/MD.0000000000015425

Theisen, K., Jacobs, B., Macleod, L., & Davies, B. (2018). The United States opioid epidemic: a review of the surgeon's contribution to it and health policy initiatives. *BJU international*, *122*(5), 754-759. DOI: 10.1111/bju.14446

Tick, H., Nielsen, A., Pelletier, K. R., Bonakdar, R., Simmons, S., Glick, R., ... & Zador, V. (2018). Evidence-based nonpharmacologic strategies for comprehensive pain care: the consortium pain task force white paper. *Explore*, *14*(3), 177-211. DOI: 10.1016/j.explore.2018.02.001

Tiffany, S.T., Petrie, E.C., Baker, T.B., & Dahl, J.L. (1983). Conditioned morphine tolerance in the rat: Absence of a compensatory response and cross-tolerance with stress. *Behavioral Neuroscience, 97*(3), 335–353. https://doi.org/10.1037/0735-7044.97.3.335

Ward, J., Mattick, R. P., & Hall, W. (1994). The effectiveness of methadone maintenance treatment: an overview. *Drug and alcohol review*, *13*(3), 327-336. https://psycnet.apa.org/doi/10.1080/09595239400185431