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AN EXPERIMENTAL THERAPY FOR OPIOID WITHDRAWAL SYNDROME

by

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ABSTRACT

AN EXPERIMENTAL THERAPY FOR OPIOID WITHDRAWAL SYNDROME

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The ongoing opioid crisis in the United States needs alternative therapeutics. To explore the role of the 5-HT_{2A} serotonin receptor in opioid physical dependence and withdrawal syndrome, morphine dependent rats were treated with pimavanserin, a highly selective 5-HT_{2A} inverse agonist in current medical use. In experiment 1, rats were rendered morphine-dependent after seven days of continuous infusion at 0.6 mg/kg/hr. On the seventh day, morphine infusion ceased, and a day later, rats were injected with either 0.3 or 1.0 mg/kg pimavanserin or saline. A non-morphine dependent saline-infused control group received only saline. One hour post injection, rats were observed under blind conditions for somatically expressed behavioral withdrawal signs utilizing a validated observation checklist. Compared to morphine dependent/saline-injected rats, the non-dependent rats and both morphine-dependent pimavanserin dose groups exhibited significantly reduced withdrawal signs, $p < .001$, based on Tukey's HSD test for non-independent pairwise comparisons. The higher pimavanserin dose (1.0 mg/kg)

fully reversed the effect of morphine infusion on withdrawal signs, while the lower dose (0.3 mg/kg) largely reversed it. In experiment 2, utilizing only non-dependent/saline-infused rats, pimavanserin showed no significant effect on overall withdrawal signs. Given pimavanserin's high selectivity for the 5-HT_{2A} serotonin receptor, these findings indicate that the activity of this receptor plays a role in opioid physical dependence. These results suggest the need for further research on pimavanserin as a novel therapeutic for managing the aversive withdrawal symptoms associated with opioid withdrawal syndrome.

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CHAPTER I: INTRODUCTION

Opioid Crisis

The severity of the opioid crisis in the United States has reached unprecedented levels. According to the Centers for Disease Control and Prevention (CDC), in 2021, there was an average of 220 daily deaths due to opioid overdoses, a tenfold increase from 1999 (CDC, 2022). The age group most affected by this tragedy is 18 to 44 years old (*National Vital Statistics System - Mortality*). This life stage contains formative years often focused on pursuing education, building careers, and forming families. The social consequences of opioid deaths are profound, including family trauma, domestic violence, and stigma (Chapman et al., 2021). Moreover, the economic impact of opioid dependency is substantial, with the United States Joint Economic Committee estimating a \$1.5 trillion spent on the opioid crisis in 2020 (United States Joint Committee, 2020). These costs encompass health care expenses, crime, lost productivity, and loss of life.

Various factors contribute to the crisis, including overprescription, influence from pharmaceutical companies, insufficient regulation, and an increased use of both older opiates such as heroin and newer synthetics such as fentanyl (Americas, 2023). However, arguably the biggest contributor to this ongoing public health crisis is the ease with which individuals can become addicted.

Opioid drugs

There is a large family of opioid drugs chemically modified from morphine (such as heroin) or synthesized with related molecular structures (such as fentanyl). Morphine is considered the prototypic opiate drug and is the most frequently used in laboratory studies of opiate effects (Opioids, 2020).

In clinical settings, opioids serve a crucial role in the management of debilitating pain associated with cancer, surgery recovery and palliative care. These drugs have a wide variety of immediate actions that are useful in medicine, including relief of pain and painful emotions, sedation, relief from respiratory congestion and from dysentery. In addition, opioids are a vital component in anesthesia for their sedative effects (Ferry, 2022). Although opiates were critical in advancing medicine, there are severe, negative consequences of their unsupervised use. The use of opioids while under the care of a physician can provide a substantial increase in quality of life, nonetheless, opioid use comes at a considerable risk due to their side effects and addictive properties.

Opioid Withdrawal Syndrome

The Diagnostic and Statistical Manual, 5th edition, defines opioid use disorder as “a problematic pattern of opioid use that leads to clinically significant distress or impairment” (American Psychiatric Association, 2013). A serious result of opioid use disorder is Opioid Withdrawal Syndrome (OWS), the combination of negative symptoms a user experiences when attempting to cease drug use (Shah, 2023).

The initial appeal of opioids stems from their positive euphoric reinforcing effects. As neural circuits adapt to the flood of drugs in the nervous system, more of the drug is necessary to maintain its effects known as tolerance. Physical dependence develops from repeated use, whereby individuals experience unpleasant withdrawal symptoms even after periods of abstaining from the drug. Physical dependence introduces negative reinforcement aspects, providing relief by avoiding aversive symptoms. Consequently, the threat of opioid withdrawal syndrome becomes a compelling motivation for persisting drug use (Koob, 2020). Understanding the neurobiological elements involved in physical dependence and opioid withdrawal syndrome is crucial in developing alternative therapies.

Mechanism of opioid action in the CNS

The term “opioid” refers to a broad category of drugs, including natural substances like morphine, synthetic like fentanyl, and semi-synthetic like oxycodone, all classified as narcotics. The body also produces its own natural pain-relieving neuropeptides known as endogenous opioids, including endorphins, enkephalins and dynorphins. These neuropeptides are synthesized in the brain and are activated in times of stress, exercise, or pain. They primarily exert their physiological effects through opioid receptors: mu, delta, and kappa. Functionally, they are receptor agonists that bind to and activate opioid receptors (i.e., mu receptor). However, opioids such as morphine are also receptor agonists at the mu receptor, mimicking a similar response (Moal & Koob, 2007). Morphine’s primary mechanism of actions occur through mu opioid receptors, and the elimination of morphine effects at mu receptors in knockout mice confirms this pharmacological evidence (Childers, 1997).

Neuron communication

Neurons generate electrical signals, known as action potentials. Electrical activity allows information to be transmitted down the length of the neuron. When the action potential reaches the axon terminal of the presynaptic neuron, it triggers the release of neurotransmitters from synaptic vesicles into the synaptic cleft. These neurotransmitters cross the synaptic cleft and bind to receptors on the postsynaptic neuron. If the postsynaptic electrical charge rises above a threshold, the signal propagates to the next neuron.

Presynaptic neurons release excitatory neurotransmitters (e.g., Glutamate) that result in a depolarization in the postsynaptic neuron, continuing the signal to the postsynaptic neuron. In contrast, presynaptic neurons that release inhibitory

neurotransmitters (e.g., GABA) result in a hyper-polarization in the postsynaptic neuron, interfering with the propagation of the signal.

Presynaptic and Postsynaptic Inhibition

Opioids stop signal transmission between neurons by inhibition. Presynaptic inhibition occurs when opioid drugs bind to opioid receptors on the presynaptic neuron preventing the release of neurotransmitters. Therefore, in the event of an action potential, the lack of neurotransmitter release prevents the propagation of the signal.

Postsynaptic Inhibition occurs when opioid drugs bind to opioid receptors on the postsynaptic neuron, preventing depolarization as well as the formation of an action potential.

Ascending and Descending Pain Pathways

Our bodies have two primary pain-related pathways. The ascending pathway transmits pain signals from the periphery (e.g., a tack in the hand) through various nuclei to the brain. The descending pathway blocks the ascending pathway, effectively discontinuing the perception of pain. Habitual use of opioids leads to physiological modifications in neural pain pathways in ways that lead to addiction (Kosten & George, 2002).

A validated model for testing physical dependence

Malin et al. (1992) introduced a rat model of nicotine physical dependence and withdrawal. Its validation has been reviewed (Malin, et al 2001) previously. Nicotine physical dependence is closely related to opiate physical dependence, since nicotine releases enkephalins and endorphins, which stimulate opiate receptors (Lichtenstein et al., 2019). Therefore, the withdrawal signs are remarkably similar to opiate dependence.

The 5-HT_{2A} receptor and pimavanserin

The drug pimavanserin is a highly selective inverse agonist of the 5HT_{2A} serotonin receptor. An inverse agonist drug reduces the activity of an intrinsically active receptor below its basal level and blocks the effects of an agonist drug (e.g. opioids) at the receptor (Howland, 2016). Inverse agonists shut down the effects of a receptor on its cell, regardless of whether or how much it is stimulated by ligands (transmitters, hormones, or drugs) binding to it. Some receptors affect the constitutive or intrinsic activity of their cells in the absence of transmitters or hormones binding (Berg & Clarke, 2018). Such inverse agonists shut down or reduce both intrinsic and stimulated activity.

Pimavanserin dose-dependently reduced physical withdrawal signs of nicotine dependence (Malin et al., 2019). Extensive evidence suggests relationships between nicotine and opioid physical dependence (Malin, et al. 2016). For example, the opioid antagonist naloxone can precipitate nicotine withdrawal syndrome (Malin, Anderson & Goyarzu, 2016). There are also known interactions between the 5-HT_{2A} receptor and opioid mechanisms. Modulation of 5-HT_{2A} receptors affects the sensitivity of opioid receptors and pharmacological actions (Pang, et. Al 2016) These relationships are further described in the discussion section of this thesis. Due to the close relationship between nicotine and opioid physical dependence the reasonable next step is to test pimavanserin effects on opioid withdrawal symptoms.

Aims

The purpose of this experimental study is to explore the role of the 5-HT_{2A} receptor as a possible contributor to opioid physical dependence. The current study attempts to determine whether the inverse agonist pimavanserin can reduce or eliminate somatically expressed withdrawal signs associated with opioid withdrawal syndrome.

The number of somatically expressed withdrawal signs served as the operational definition of physical dependence.

Hypotheses

Hypothesis 1: Rats dependent on morphine will show a reduction in somatically expressed behavioral withdrawal signs following the cessation of morphine infusion when injected with the 5-HT_{2A} serotonin inverse agonist Pimavanserin.

Hypothesis 2: The effect of Pimavanserin will vary with dosage, with a higher dose providing greater relief from somatically expressed behavioral withdrawal signs.

Hypothesis 3: Administering pimavanserin to rats not dependent on morphine will have a much less effect on behavioral withdrawal signs.

CHAPTER II: MATERIALS AND METHODS

Protocols

All experimental protocols were approved by the University of Houston Clear Lake Animal Care and Use Committee and carried out in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council, 2011). Prior to participation, all researchers and volunteers in the rodent laboratory completed mandatory training and successfully passed exams administered by the Collaborative Institutional Training Initiative (CITI Program).

Procedures

All rats were received at the University of Houston Clear Lake animal research facility and identified by numerically marking each tail. The assigned animal ID did not indicate the specific research treatment intended for that rat. To uphold the reliability and validity of the results, strict double-blind procedures were enforced, ensuring that neither researchers nor observers were aware of the treatment administered to each rat. The rats were randomly divided into cohorts to facilitate the identification of surgical and experimental procedures. To ensure surgical skill proficiency, all surgical researchers in the rodent animal laboratory underwent comprehensive training in both rodent handling and surgical skills. All activities were supervised by the Principal Investigator.

A previous study utilizing a rodent model of morphine withdrawal served as reference in the planning of experimental design, statistical analysis, data collection, surgery, management of controlled substances, drug infusion rates and checklist of somatically expressed withdrawal symptoms in a rodent (Malin et al., 2019). The number of somatically expressed withdrawal signs served as the operational definition of physical dependence.

Subjects

Fifty-three male Sprague-Dawley rats were chosen due to previous studies validating this strain in a rat model of morphine physical dependence (Malin et al., 2019). Rats were acquired from Envigo. This strain is commonly used in studies investigating the impact of drugs in the nervous system. All rats were housed in a climate-controlled room with corn cob bedding, unrestricted access to food and water. Rats were maintained on a 12-hour dark (red light exposure) and 12-hour light (white light exposure) daily cycle with all experimental testing occurring during the white light phase. Following observations, to prevent any subsequent suffering, rats were humanely euthanized using 10 ml Isoflurane for 20 minutes.

Materials

Rat housing, consisted of single (24.2 x 14 x 10 cm) and double (48.3 x 27.9 x 20.3 cm) sizes, constructed with transparent plastic walls and floor, with a mesh top. Inside, each housing unit contained corn cob bedding, a water bottle and food feeder. For somatically expressed behavioral observations, the double sized plastic container, without mesh top was utilized. A stopwatch was operated for consistent observation monitoring time (i.e., 20 minutes).

Surgical tools required for implant and explant surgeries included an electrical hair clipper, curved scissors, round spatula, wound clipper with clips, forceps, alcohol pads, surgical pads, surgical blades, surgical gloves, sterile cotton tip applicators, 1 ml syringes, 26-gauge single injection needle, fifty-three Alzet 2ML osmotic minipumps and 70-90% ethanol spray for equipment sterilization. Surgical tools used between surgeries were sterilized using a bead sterilizer. Post-surgery, all surgical tools were sterilized in an autoclave. All surgical procedures were conducted under a fume hood to facilitate ventilation from isoflurane anesthesia.

Medications

Isoflurane, obtained from Millipore Sigma in St. Louis, MO was administered to rats as an inhalant for surgical anesthesia and post experiment euthanasia. Topical lidocaine analgesia, sourced from a local drug store, was applied twice daily to the wound site as part of post-surgical care. Morphine sulfate obtained from Sigma Aldrich was placed into the mini pumps for the morphine-dependent rat cohorts. Pimavanserin was provided by Acadia Pharmaceutical Corporation, San Diego, CA.

Pre-Operative Care

For the induction of inhalant anesthesia, rats were placed in an induction chamber connected to a respirator and vaporizer system that mixed isoflurane with oxygen. The induction concentration of isoflurane was 3-5% mixed with 100% oxygen at a flow rate of 0.5-1.0 liters per minute. Following the loss of postural control, but maintaining a steady breathing rate, the animal was removed from the induction chamber and inhalation anesthesia was continuously applied through a nose cone mask. To test adequate concentration of isoflurane, a flexor withdrawal response (i.e., a pinch to the foot pad) was tested. If absent, the concentration of isoflurane was reduced to 1-2.5% and the oxygen flow rate reduced to 0.4-0.8 liters per minute. If present, the concentration of isoflurane was increased. A steady state of anesthesia was determined adequate based on a steady breathing rate and absence of withdrawal response from the foot pad pinch. Anesthesia levels were monitored throughout each procedure and levels adjusted as necessary.

Pump Implantation

To maintain a constant delivery of morphine, the rats were surgically implanted with a 2ML Alzet osmotic minipump. While under anesthesia, the rat's skin was shaved over the regions to undergo incision, from back of the neck down the back. The shaved

area was scrubbed with Betadine. A $\frac{3}{4}$ inch incision was made to the scapular region (i.e., between the shoulder blades). The incision was subcutaneously injected with 0.25 ml bupivacaine local analgesic (0.025%). A blunt spatula was used to create a large pocket under the skin of the subjects intrascapular region. Then, the osmotic minipump filled with morphine or saline solutions was inserted into the large pocket. The incision edges were pressed together and sealed with sterile wound clips. Both antibiotic and lidocaine ointments were applied topically to the incision site. The surgical process was performed in approximately 5 minutes per rat.

Pump Explantation

To initiate spontaneous withdrawal, the drug-infusing osmotic minipump must be removed. While under anesthesia, a second incision (about $\frac{1}{2}$ inch) was made just below the pump. The pump was gently squeezed out, the incision closed with wound clips, 0.25 ml bupivacaine subcutaneously injected, and antibiotic ointment applied. This surgery was performed in approximately 3 minutes per rat.

Injections

A 26-gauge single injection needle was employed at the scruff of the rodent's neck for all subcutaneous injections, including the administration of the experimental treatments: pimavanserin or saline.

Post Operative Care

Immediately following surgery, a warming pad was placed under the rat's cage to help maintain body temperature. Researchers closely monitored rats, noting attitude, gait, and posture on a rodent surgery log sheet (Appendix B). Following recovery from anesthesia, rats were returned to their home cage in a single unit housing. Post-surgery, animals were monitored daily, receiving topical lidocaine two times a day to minimize pain and distress. A daily post-surgery log sheet (Appendix C) was used to monitor all

conditions including pain or distress, inactivity, bleeding, abnormal gait, loss of righting response or any miscellaneous signs of illness. No problems occurred and there was never a need to contact the research veterinarian.

Surgical Sterility

Surgeries were performed under sterile conditions inside an exhaust vent hood to prevent contamination. Surgical sites were scrubbed with betadine. Sterile surgical gloves were used and changed between each surgery. Sterile surgical pads, alcohol pads, sterile cotton tip applicators were all changed between each surgery. To maintain sterility of surgical tools between surgeries, tools were placed inside a bead sterilizer for 15 seconds, cooled for 30 seconds and rinsed with sterile saline in a stainless-steel bowl. Following each cohort surgery, all surgical tools were sterilized once again by use of an autoclave

Experiment Timelines

The overall timeline remained largely consistent with both experiment 1 and 2 (Figure 1). During pre-experiment days (3 days) rats received gentle handling to encourage habituation and reduce any initial stress. The surgical timeline unfolded as follows: On day 1, the implant surgery of an Alzet 2ML osmotic mini pump was performed subcutaneously. In experiment 1 pumps were filled with morphine sulfate dissolved in saline or with saline alone. In experiment 2, all pumps were filled with saline alone. Subsequently, days 1-7 involved post-surgery care and continuous subcutaneous infusion from the minipumps flowed. On day 7, minipump explant surgery. On day 8, 23 hours after minipump removal, subcutaneous injections were initiated. The timer for the 20-minute observation period began one hour post subcutaneous injection. Both implant and explant surgeries were performed under the vent hood using isoflurane anesthesia.

Experiment 1: Effect of pimavanserin on spontaneous morphine withdrawal

Subjects

The study subjects consisted of 37 male Sprague-Dawley rats with an average weight of 286.2 ± 33.7 grams ($M \pm SD$).

Infusions

While under isoflurane anesthesia, the rats were surgically implanted subcutaneously with an Alzet 2ML1 osmotic minipump. The subcutaneous placement of the minipumps facilitated continuous and controlled infusion of the morphine solutions. These minipumps were specifically designed to provide a continuous infusion of medications over a period of seven days. On the seventh day, the minipumps were extracted while the rats were under isoflurane anesthesia.

Injections

Twenty-three hours following minipump removal and one hour prior to the behavioral observation, rats received subcutaneous injections using a 1 ml sterile syringe with 26-gauge needle. The rat was injected with either 1.0 mg/kg pimavanserin dissolved in saline, 0.3 mg/kg pimavanserin in saline, or saline alone. The injection volume in milliliters equaled the rat's weight in kilograms. For example, a 250-gram rat would be administered 0.25 ml. Dosage was determined by varying the concentration of pimavanserin.

Treatment Groups

This study included two control and two experimental groups. The control groups are listed as follows: a positive control group for morphine withdrawal ($n = 9$), which received morphine infusion and saline injections, and a negative control group to assess the absence of morphine withdrawal ($n = 10$), which received saline alone. The two experimental treatment groups are listed as follows: a group ($n = 9$) that received

morphine infusion and 0.3 mg/kg/hr. pimavanserin injections, and another group (n = 9) that received morphine infusion and 1.0 mg/kg/hr. pimavanserin injections.

Behavioral Observation

Observations began one hour post injection, to allow for development of pharmacokinetic effects. The animals and solutions were assigned codes, and the observation of rats were conducted under blind conditions (where experimenters are unaware of which rats had which treatment). The rat was placed within a transparent plastic container measuring 48.3 x 27.9 x 20.3 cm. Utilizing a standard checklist of rodent opiate withdrawal signs, observations continued for a duration of 20 minutes (Appendix A). The behaviors were monitored for number of occurrences during the 20-minute observation period. An animal's overall withdrawal score equaled the total number of occurrences across all categories. Somatically expressed behavioral signs recorded included wet-dog shakes, abdominal writhes, and a miscellaneous category consisting of a range of less frequent behaviors such as cheek tremors, teeth chattering, vacuous chewing, ptosis, hindfoot scratching, hindfoot foot-licks, and spontaneous seminal ejaculations.

Statistical Analysis

One-way analyses of variance (ANOVA) analyzed overall somatically expressed behavioral signs, wet-dog shakes, abdominal writhes, and miscellaneous signs as a function of dose. Subsequently, post hoc comparisons were conducted between pairs of treatment groups using Tukey's Honest Significant (HSD) test. To assess the dose-dependent effect of pimavanserin, a linear trend analysis evaluated the relationship between the total signs and dosage. The effect sizes of pimavanserin were calculated using Cohen's d. Percentage reduction of those signs attributable to morphine infusion was computed by the formula:

morphine-infused/saline-injected mean – morphine-infused/pimavanserin-injected mean

morphine-infused/saline-injected mean – saline-infused/saline-injected mean

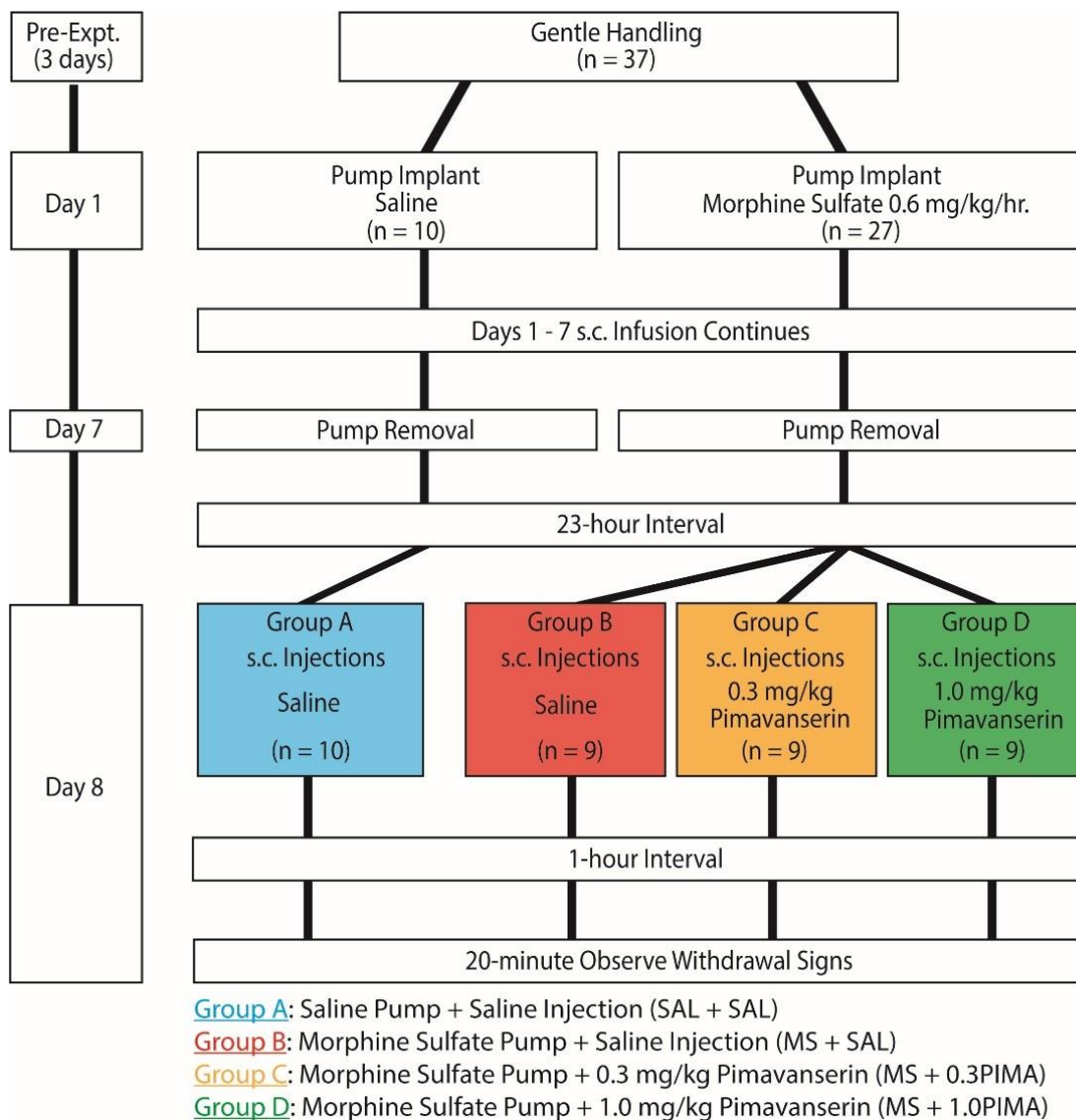


Figure 1:

Timeline and Group Procedures in Experiment 1

Group A: Negative control group for morphine dependence

Group B: Positive control group for morphine dependence

Group C: Morphine dependent/lower dose (0.3 mg/kg) pimavanserin treated

Group D: Morphine dependent/higher dose (1.0 mg/kg) pimavanserin treated

Experiment 2: Effects of pimavanserin on non-dependent rats

To assess somatically expressed behavioral withdrawal effects of pimavanserin, if any, on non-morphine dependent rats, a second experiment was necessary.

Subjects

The study subjects consisted of 17 male Sprague-Dawley rats that had never been exposed to morphine with an average weight of 278.9 ± 13.1 grams ($M \pm SD$).

Infusions

While under isoflurane anesthesia, the rats were surgically implanted subcutaneously with an Alzet 2ML osmotic minipump. These minipumps were filled with saline solution to provide a continuous infusion of saline over a period of seven days. On the seventh day, the minipumps were extracted while the rats were under isoflurane anesthesia.

Injections

Twenty-three hours of the removal of the infusion minipumps, these rats were subcutaneously administered either a dose of 1.0 mg./kg. /hr. of pimavanserin in saline, which was determined as the most effective dose in Experiment 1 ($n = 8$), or an injection of saline alone ($n = 8$). These sample sizes basically match those employed in Experiment 1. As in experiment 1, the subcutaneous injections were administered one hour prior to conducting a 20-minute blind observation to assess somatically expressed behavioral signs typical in opioid withdrawal syndrome.

Treatment Groups

This study included two experimental groups. A 1.0 mg./kg. /hr. of pimavanserin in saline treated group ($n = 8$) and a saline-only control group ($n = 8$).

Behavioral Observation

The animals and solutions were assigned codes to ensure experimenter blind conditions and the rats were observed within a transparent plastic container measuring 48.3 x 27.9 x 20.3 cm for a duration of 20 minutes. Experimenters recorded all somatically expressed behavioral signs that were recorded in Experiment 1, including wet-dog shakes, abdominal writhes, and a miscellaneous group of less frequent behaviors such as cheek tremors, teeth chattering, vacuous chewing, ptosis, hindfoot scratching, hindfoot foot-licks, and spontaneous seminal ejaculations.

Statistical Analysis

Independent samples t-tests compared the two treatment groups for overall somatically expressed behavioral signs, wet-dog shakes, abdominal writhes, and miscellaneous signs.

CHAPTER III: DATA ANALYSIS & RESULTS

Experiment 1: Effect of pimavanserin on spontaneous morphine withdrawal

Overall withdrawal signs

Overall withdrawal signs (Figure 2) were analyzed by a one-way analysis of variance (ANOVA), yielding a statistically significant treatment effect of pimavanserin, ($F_{3,33} = 11.40, p < .001$). Subsequent post-hoc comparisons (i.e., Tukey's HSD Test) revealed the positive control group, consisting of morphine-dependent rats receiving saline injections, exhibited significantly more withdrawal signs compared to the negative control group receiving saline only ($p < .001$). Moreover, this positive control group exhibited more withdrawal signs than the 1.0 mg/kg. /hr. pimavanserin group ($p < .001$) and the 0.3 mg./kg. /hr. pimavanserin group ($p < .001$). No other significant post-hoc differences were observed.

Trend analysis among the three morphine-dependent groups revealed a statistically significant decreasing linear trend of overall withdrawal signs in relation to pimavanserin dose, ($F_{1,24} = 25.56, p < .001$). The effect directly attributed to morphine dependence (calculated as the difference between the mean of the morphine-infused/saline-injected group and the mean of the saline-infused/saline-injected group) amounted to 31.81 signs, reflecting a 122.45% increase from the negative control group. The 0.3 mg./kg. /hr. dose of pimavanserin led to an 88.71% reduction in the signs associated with dependence, while the 1.0 mg./kg. /hr. dose resulted in a 103.34% reduction. Per Cohen's *d* for mean differences between the positive control group and both the 0.3 mg./kg. /hr. and 1.0 mg./kg. /hr. pimavanserin groups were calculated as 1.79 and 2.47, respectively, both of which fall within the category of large effect sizes.

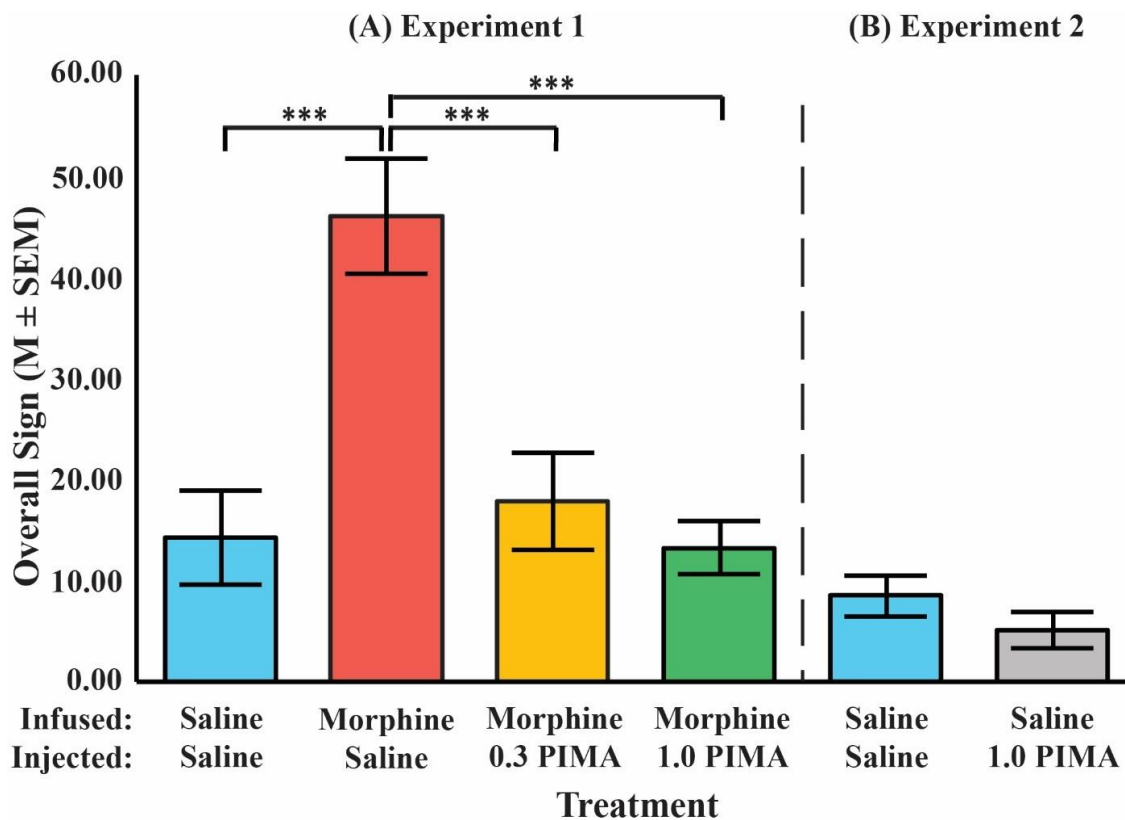


Figure 2:

Experiment 1 and 2 One-way ANOVA of Overall Signs ($M \pm SEM$)

The treatment effect in experiment 1 was highly significant, $p < .001$. *** $p < .001$ vs. all other groups in experiment 1 (Tukey's HSD test). *** $p < .001$ vs. all other groups. The top line of the legend indicated 7 days chronic morphine or saline infusion. The bottom line specifies saline or pimavanserin (PIMA) injection on the day following termination of infusion. Data are group means $\pm SEM$, standard error of mean.

Individual Signs

Wet dog shakes (Figure 3) were the predominant withdrawal sign in the positive control group (morphine-infused/saline-injected). One-way ANOVA of the four treatment groups revealed a highly significant effect of treatment, ($F_{3,33} = 21.46$, $p < 0.001$). There was a significant decreasing linear trend of wet dog shakes as a function of pimavanserin dose, $p < .001$.

The effect of actual morphine dependence, indicated by the difference between the morphine-infused/saline-injected group mean and the saline-infused/saline-injected group mean, was 13.87 signs or a 177.82% increase from non-dependent control rats. The 0.3 mg/kg pimavanserin dose reduced these signs related to dependence by 109.81%, while the 1.0 mg/kg dose reduced them by 136.94%. Effect sizes (Cohen's d) comparing morphine-infused/saline to the 0.3 and 1.0 mg/kg pimavanserin groups were 2.76 (large) and 4.10 (large), respectively.

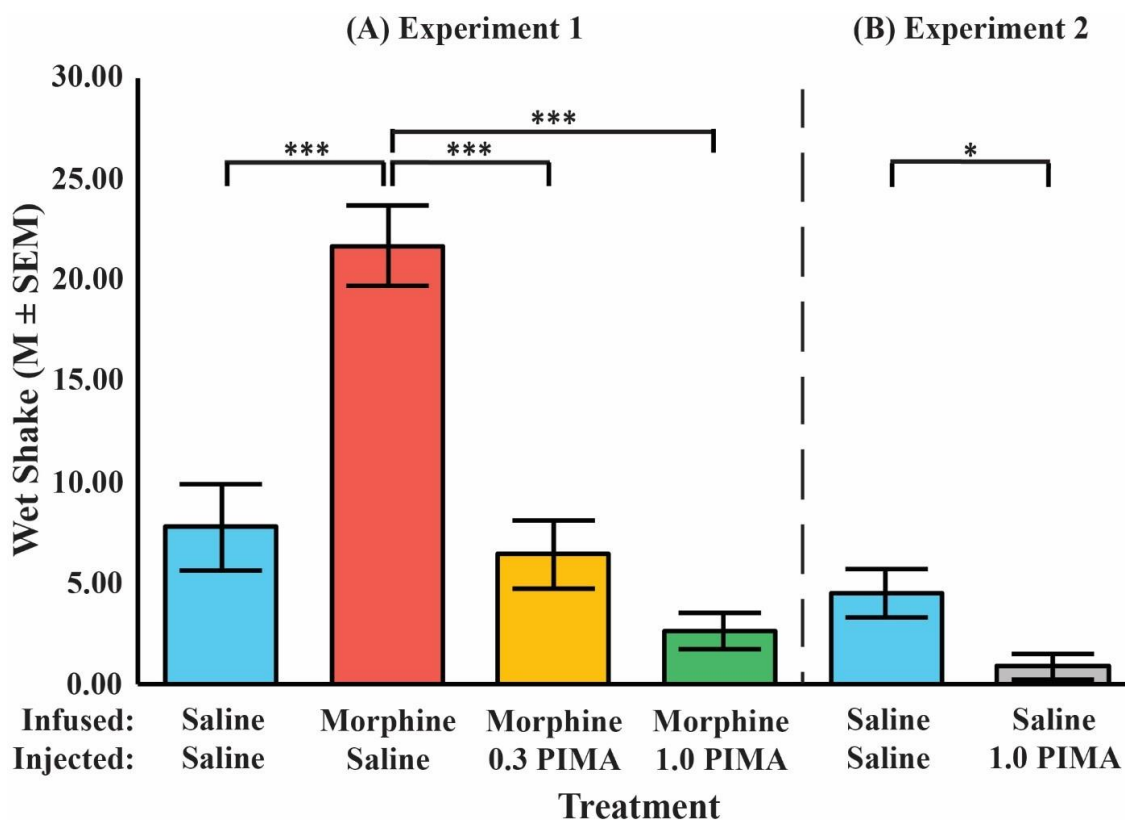


Figure 3:

Experiment 1 and 2 Wet Dog Shakes (M ± SEM)

One-way ANOVA: the treatment effects in experiment 1 was highly significant, $p < .001$

*** $p < .001$ vs. all other groups in experiment 1. *** $p < .001$ vs. all other groups

* $p < .05$ versus saline-injected group in experiment 2. The top line of the legend indicates 7 days chronic morphine or saline infusion. The bottom line specifies saline or pimavanserin (PIMA) injection on the day following Data are group means ± SEM, standard error of mean.

Abdominal writhes (Figure 4) represented the second most common withdrawal sign in the positive control group undergoing morphine withdrawal. One-way ANOVA analysis of writhes revealed a significant treatment effect, ($F_{3,33} = 7.16$, $p < .001$).

Tukey's HSD Test indicated that the morphine-infused/saline-injected group exhibited significantly more writhes than the negative control group (saline-infused/saline-injected), $p = 0.002$, the 0.3 mg/kg pimavanserin group, $p = 0.005$, and the morphine dependent 1.0 mg/kg pimavanserin group, $p = 0.003$.

The effect attributed to actual morphine dependence, calculated as the morphine-infused/saline-injected group mean minus the saline-infused/saline-injected group mean, was 2.34 signs. The 0.3 mg/kg dose of pimavanserin reduced these signs associated with dependence by 94.49%, while the 1.0 mg/kg dose reduced them by 99.57%. Effect sizes (Cohen's *d*) comparing the morphine-infused/saline positive control group to the 0.3 and 1.0 mg/kg pimavanserin groups were 1.21 (large) and 1.28 (large), respectively.

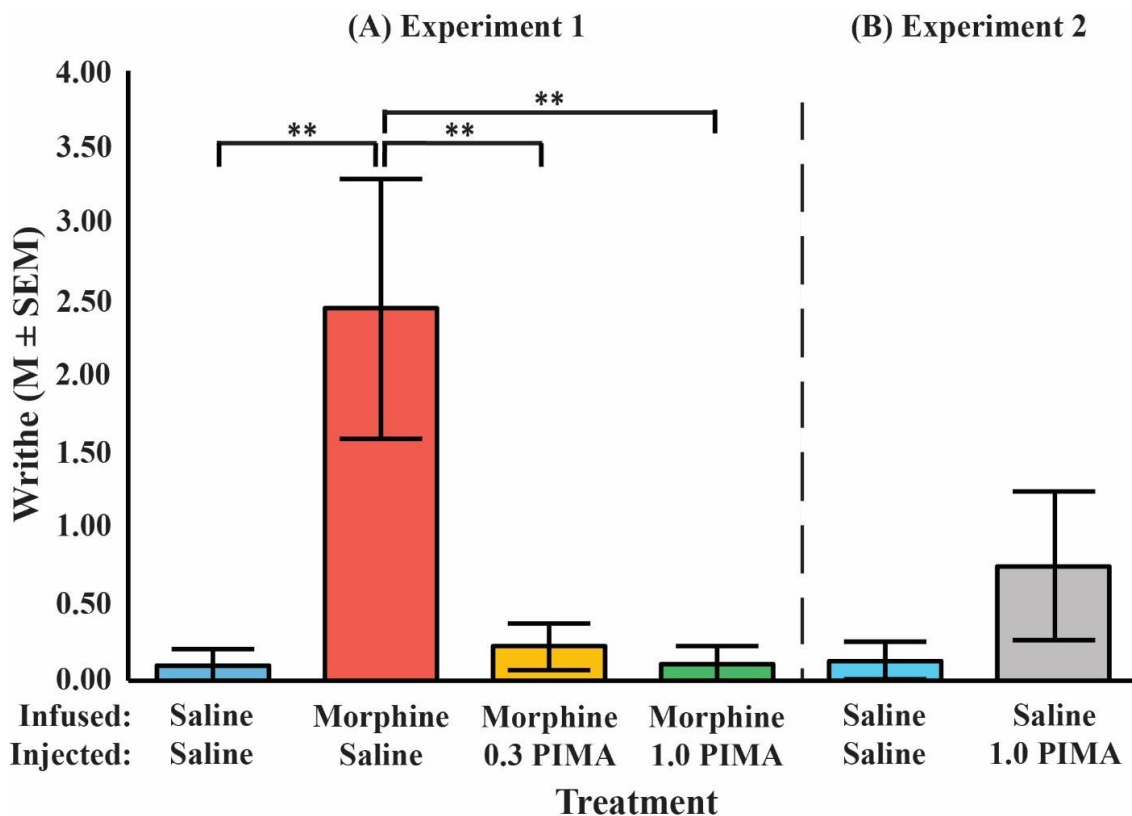


Figure 4:

Experiment 1 and 2 Writhe ($M \pm SEM$)

One-way ANOVA: the treatment effect was highly significant in Experiment 1, $p = .001$.

** $p < 0.01$ vs. all other groups in Experiment. The top line of the legend indicated 7 days chronic morphine or saline infusion. The bottom line specifies saline or pimavanserin (PIMA) injection on the day following termination of infusion. Data are group means $\pm SEM$, standard error of the mean.

Scattered occurrences of a miscellaneous group of less frequent signs were also noted (Figure 5). Subjecting the aggregate number of these signs displayed by each rat to one-way ANOVA revealed a significant effect of treatment, $F_{(3,33)} = 7.13$, $p = .001$. Tukey's HSD Test indicated that the negative control group for morphine withdrawal exhibited significantly fewer of these signs than the positive control group, $p = .022$. Although no other significant differences were found, the mean difference of miscellaneous signs from the positive control group to the 1.0 mg/kg pimavanserin group approached significance, $p = .085$. The 0.3 mg/kg pimavanserin group differed non-significantly from the positive controls, $p = .119$.

In the morphine-dependent/saline injected group, 14.11 mean miscellaneous signs were attributable to morphine infusion, reflecting a 176.64% increase from miscellaneous signs in the saline-infused/saline-injected group. This effect was reduced by 77.18% with 0.3 mg/kg pimavanserin and 84.12% by the 1.0 mg/kg dose. The effect sizes (Cohen's d) comparing morphine-infused/saline vs. morphine-infused/1.0 mg/kg pimavanserin was 1.48 (large).

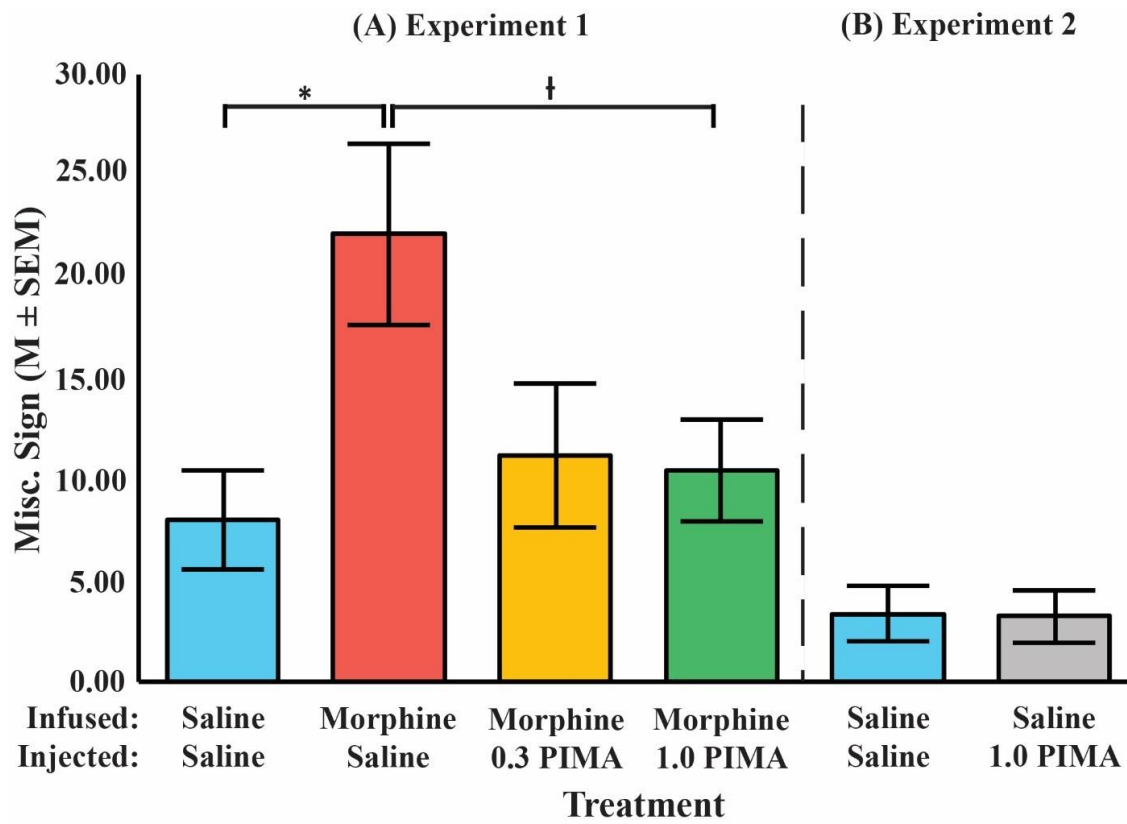


Figure 5:

Experiment 1 and 2 Miscellaneous Less frequent signs ($M \pm SEM$)

One -way ANOVA: The effect of treatment was significant in Experiment 1, $p = .001$.

* $p = .022$, $t p = .085$, Tukey's HSD test. The top line of the legend indicated 7 days chronic morphine or saline infusion. The bottom line specifies saline or pimavanserin (PIMA) injection on the day following termination of infusion. Data are group means $\pm$ SEM, standard error of the mean.

Experiment 2: Effect of pimavanserin on non-morphine dependent rats

As hypothesized, non-dependent rats exhibited far fewer behavioral withdrawal signs compared to the morphine-dependent rats in Experiment 1. The analysis depicted in Figure 2B did not reveal statistically significant pimavanserin treatment effect on overall signs, cumulated across all categories, $t(14) = 1.06$, $p = .308$. This contrasts with a significant, $p < .001$ reduction of those signs observed by 1.0 mg/kg subcutaneous pimavanserin in morphine-dependent rats in Experiment 1.

However, there were indications suggesting an effect on the relative frequency of withdrawal sign categories in the saline-infused rats. Pimavanserin significantly reduced wet shakes, $t(14) = 2.70$, $p = .023$ as illustrated in Figure 3B. Additionally, the pimavanserin group exhibited fewer miscellaneous signs, although this difference was not significant, $t(14) = .066$, $p = .948$ as reflected in Figure 5B. Conversely, the pimavanserin group had more writhes. However, this difference was also not significant, $t(14) = 1.23$, $p = .238$, as presented in Figure 4B.

CHAPTER IV:

DISCUSSION

Effects on spontaneous withdrawal

The continuous infusion of morphine led to a significant increase in spontaneous withdrawal signs, evident through marked differences between the morphine-infused/saline-injected group and the saline-infused/saline-injected group. These differences were not only statistically significant but also accompanied by large effect sizes. Pimavanserin effectively alleviated the impact of morphine infusion, as supported by several measures: statistically significant differences from the morphine-infused/saline-injected group, large effect sizes, considerable percentage reductions in withdrawal measures attributable to morphine exposures and a significant linear trend of overall signs as a function of pimavanserin dose.

Pimavanserin mechanism of action

In an earlier experiment on pimavanserin effects on anxiety measures in a model of Post-Traumatic Stress Disorder, subcutaneous pimavanserin injections at 0.1, 0.3 and 1.0 mg/kg raised plasma concentrations in a nearly linear fashion (Malin et al., 2023). The highest dose resulted in a concentration of 18 ng/mL. A standard assay for central 5-HT_{2A} receptor activity is to record numbers of head twitches induced by the selective 5-HT_{2A} agonist DOI (i.e., 2,5-dimethoxy-4-iodoamphetamine). The ability of another compound to reduce DOI induced head twitches assesses that compound suppression of 5-HT_{2A} receptor activity. Pimavanserin prevented the DOI effect, demonstrating its suppression of the 5-HT_{2A} receptor (Malin, et.al., 2023).

In contrast, pimavanserin had no significant effect on the same overall set of behaviors in rats never exposed to morphine. However, one specific behavior, wet shakes, was significantly reduced in a group of opiate-free rats. This behavioral sign was

particularly susceptible to pimavanserin in all three experiments. Even in the absence of opiate exposure, the experimental procedures provided some probable sources of irritation or distress. These stressors included repeated handling, two surgeries and carrying the osmotic pump under the skin for seven days. It is possible that pimavanserin may have altered certain responses to these stressors, such as wet shakes, even in opiate-free rats.

The 5-HT_{2A} receptor and opioids

The interactions between a 5-HT_{2A} receptor inverse agonist and chronic morphine effects are not unexpected. There are several known interactions between morphine and the 5-HT_{2A} receptor. Morphine exposure increases 5-HT_{2A} receptor expression in rodents (Pang et al., 2016; Mohammed et al., 2016) as well as increasing 5-HT_{2A} receptor-mediated actions (Marek et al., 2003). Conversely, activation or inactivation of 5-HT_{2A} receptors affect the sensitivity and internalization of opioid receptors as well as a wide variety of opioid actions. These include analgesia (Li et al., 2011), dopamine release (Auclair et al., 2004), locomotor activation and its sensitization (Li et al., 2011; Pang et al., 2016; Tao and Auerbach, 1995; Auclair et al., 2004), discriminative stimulus effect (Li et al., 2011), and naloxone-precipitated morphine withdrawal signs (Pang et al., 2016).

Related Studies

Two previous studies found that the 5-HT_{2A} antagonist MDL11939 (Pang et al., 2016), as well as the 5-HT_{2A} inverse agonist MDL100907 (Li et al., 2022), suppressed naloxone-precipitated morphine withdrawal syndrome in mice. The present study is consistent with those results, while extending those findings in several ways. It employed a different, 5-HT_{2A} receptor inverse agonist currently in medical use, and a different species (rat). It mitigated spontaneous withdrawal (the type most commonly occurring in

human addicts) as opposed to precipitated withdrawal. It evaluated a considerably different set of withdrawal behaviors. The two studies taken together suggest a major role 5-HT_{2A} receptor activity as contributing to opioid physical dependence, and as a potential target for modifying physical dependence and withdrawal syndrome.

CHAPTER V:

CONCLUSION

The statistically significant results of pimavanserin effects at the 5-HT_{2A} receptor provide a compelling argument for pursuing its potential therapeutic benefits. These experiments model effects on acute opioid withdrawal syndrome (e.g., spontaneous withdrawal). However, the experimental effects on chronic addiction are unknown.

Conditioned Place Aversion

One of the more difficult symptoms to treat in opioid addiction is relapse. The mu opioid receptors in the ventral tegmentum, nucleus accumbens and basolateral amygdala have been associated with the rewarding and euphoric effects of opioids (Volkow et al., 2019). Old environmental or social settings may revive dangerous memories. This can include the association of the euphoric effects of opioids with the relief of stress and anxiety (Ou et al., 2023). Future research might explore the role of the 5-HT_{2A} receptor in such conditioned effects.

Subsequent and Future Research

After the data presented, the UHCL animal behavioral research team (The Rat Pack) confirmed that 1.3 mg/kg pimavanserin significantly reduced the behavioral signs of naloxone-precipitated withdrawal in rats. The rats that underwent withdrawal under the influence of pimavanserin exhibited significantly less avoidance of the withdrawal chamber on the following day. This suggested that interference with the 5-HT_{2a} receptor reduces the aversiveness of opioid withdrawal syndrome.

Attempting to avoid the aversiveness of opioid withdrawal syndrome is one motivation for persisting in chronic drug usage (Koob, 2009). Therefore, activation of the 5-HT_{2A} receptor may contribute to keeping opioid users chronically trapped in their drug

habit. Inactivation of that receptor might merit further investigation as a strategy for treating opiate addiction.

These findings are consistent with the 5-HT_{2A} receptors involvement with other aversive phenomena. The 5-HT_{2A} serotonin receptor has been implicated in modulating emotional memory, mediating anxiety and defensive responses (Murnane, 2019). Weisstaub et al. (2006) found that transgenic deletion of that receptor in mice resulted in loss of anxious and avoidant behavior. Selective restoration of the receptor in neocortical layer 5 restored cautious or avoidant behavior. A plausible underlying mechanism for this finding has been identified. Glutamatergic pyramidal neurons in layer 5 are stimulated through 5-HT_{2A} receptors and, in turn, stimulate the amygdala, a region implicated in mediating anxiety and dysphoric emotions (Martín-Ruiz et al., 2001). Thus, this receptor should be investigated in connection with a wide range of dysphoric disorders.

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APPENDIX A: MORPHINE WITHDRAWAL CHECKLIST IN A RODENT

Experiment:	
Drug, Dose, & Administration	
Observers:	

Date:	
Start Time:	
End Time:	
Rat ID:	

somatically expressed behaviors							
	Total		Total		Total		Total
HS - Head Shake		SC - Skin Crawl Writhe		CT - Cheek Tremor (15 sec)		P - Ptosis (30 sec)	
BS - Body Shake		BC - Black Cat Writhe		S - Hind Foot Scratch		D - Diarrhea	
G - Gasp		C - Chew (15 sec)		GL - Genital Lick		BU - Backing Up (3 steps)	
W - Writhe		TE - Teeth Chatter (15 sec)		FL - Foot Lick		SEM - Seminal Ejaculation	
* Signs with times by them indicate latency time to call it again.							
** GL & SEM typically co-occur so only call one sign.							
*** C immediately followed by grooming is not counted.							

	Observational Behavior	Observational Count		Observational Behavior	Observational Count
Ex	HS				
1			26		
2			27		
3			28		
4			29		
5			30		
6			31		
7			32		
8			33		
9			34		
10			35		
11			36		
12			37		
13			38		
14			39		
15			40		
16			41		
17			42		
18			43		
19			44		
20			45		
21			46		
22			47		
23			48		
24			49		
25			50		

APPENDIX B:
RODENT SURGERY LOG

Operation:	
Protocol #:	
Investigator:	
Surgeon:	

Date:	
Start Time:	
End Time:	
Rat ID:	

Isoflurane Anesthesia	Dose:	Start Time:	End Time:
Inhalation:			
Injection:			
Supplemental:			

Additional Medications	Time given:	
Bupivacine analgesic:		
Triple antibiotic:		
Supplemental:		

APPENDIX C:
POST SURGERY OBSERVATIONS

Start Time:	
End Time:	
Rat ID:	

	YES	NO	Action Taken:
Pain or distress			
Alert (awake)			
Movement (gait) normal			
Wound Healing			
Eating			