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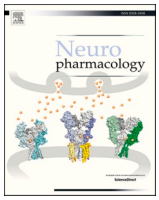

Impacts of xylazine on fentanyl demand, body weight, and acute withdrawal in rats: A comparison to lofexidine

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ABSTRACT

The opioid use landscape has recently shifted to include xylazine, a veterinary anesthetic, as an adulterant in the fentanyl supply. The health impacts of xylazine as an emerging fentanyl adulterant has raised alarm regarding xylazine as a public health threat, warranting research on the impacts of xylazine on fentanyl's behavioral effects. No prior studies have evaluated the effects of xylazine on fentanyl consumption at various unit doses, fentanyl demand, or withdrawal as compared to the Food and Drug Administration-approved opioid withdrawal medication, lofexidine (Lucemyra®). This is important because lofexidine and xylazine are both adrenergic α2a (A2aR) agonists, however, lofexidine is not a noted fentanyl adulterant. Here we evaluated xylazine and lofexidine combined with self-administered fentanyl doses in male and female rats and evaluated fentanyl demand, body weight, and acute withdrawal. Consumption of fentanyl alone increased at various unit doses compared to saline. Xylazine but not lofexidine shifted fentanyl consumption downward at a number of unit doses, however, both lofexidine and xylazine suppressed fentanyl demand intensity as compared to a fentanyl alone control group. Further, both fentanyl + lofexidine and fentanyl + xylazine reduced behavioral signs of fentanyl withdrawal immediately following SA, but signs increased by 12 h only in the xylazine co-exposed group. Weight loss occurred throughout fentanyl SA and withdrawal regardless of group, although the xylazine group lost significantly more weight during the first 24 h of withdrawal than the other two groups. Severity of weight loss during the first 24 h of withdrawal was also correlated with severity of somatic signs of fentanyl withdrawal. Together, these results suggest that body weight loss may be an important indicator of withdrawal severity during acute withdrawal from the xylazine/fentanyl combination, warranting further translational evaluation.

1. Introduction

In the rapidly changing landscape of the opioid epidemic, xylazine is being increasingly detected in the illicit opioid supply that has consistently contained fentanyl since 2016 (Friedman et al., 2022; Pesce et al., 2023). When used as a veterinary anesthetic, xylazine is administered in combination with other compounds such as ketamine in order to reduce the amount of ketamine required to achieve an adequate plane of anesthesia and provide muscle relaxation and sedation (Olson and McCabe, 1986; Wellington et al., 2013). In humans, co-exposure to xylazine, an adrenergic α2a receptor (A2aR) agonist, with fentanyl has been associated with increased overdose deaths (Johnson et al., 2021) and necrotic non-injection site flesh wounds (Soderquist et al., 2023). Importantly, a large majority of individuals with xylazine exposure state that they do not intentionally seek it out, and also state that xylazine makes withdrawal from other substances worse (Spadaro et al., 2023). Despite this clinically important public health crisis, there is not much known regarding the behavioral or biological impacts of xylazine when

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it is combined with fentanyl. In 2023, one study showed that exposure to the xylazine/fentanyl combination prolonged brain hypoxia in mice, and it was concluded that xylazine may attenuate the brain's compensatory mechanisms to counteract brain hypoxia (Choi et al., 2023). Another recent study showed that xylazine increased fentanyl lethality in rodents, and that the μ opioid antagonist naloxone, but not the A2aR antagonist yohimbine, prevented this enhancement of lethality (Acosta-Mares et al., 2023). In our recent rat study, we found that xylazine and fentanyl co-self-administration (SA) dose-dependently suppressed intake in both sexes and induced a unique withdrawal syndrome in females that was not altered by acute naloxone treatment (Khatri et al., 2023). Together, these results suggest that there may be important unique biological and behavioral consequences when xylazine and fentanyl are used in combination.

Management of opioid withdrawal with A2aR agonists has been repeatedly shown to alleviate symptoms of withdrawal during the acute phase, including anxiety, chills, nausea, sweating, diarrhea, and craving (Gowing et al., 2016; Gripshover and Kosten, 2022). Notably, xylazine exists in a family of A2aR agonist compounds that also includes lofexidine (Lucemyra®) and clonidine. Clonidine has been approved as an antihypertensive medication since the 1960s and used off-label to treat opioid withdrawal for decades (Kleber et al., 1980; Washton and Resnick, 1981). Lofexidine is FDA-approved for the treatment of clinical opioid withdrawal symptoms (Hermes et al., 2019). Several studies have shown that individuals treated with lofexidine during abrupt opioid withdrawal remain opioid-free for a longer period of time than patients treated with clonidine (Gripshover and Kosten, 2022). Given that lofexidine shows efficacy in the treatment of OUD but is not a noted adulterant in the opioid drug supply, understanding the differences between this compound and xylazine is important.

To our knowledge, there is no information on how intravenous lofexidine may impact opioid SA. One prior study showed that lofexidine potentiated cocaine's reinforcing effects when it was administered intramuscularly by reducing cocaine SA without impacting food SA (Kohut et al., 2013). This study further showed that when intravenously self-administered, lofexidine induced a leftward shift in the cocaine SA dose-effect curve. The translational conclusion of this paper was that lofexidine may potentiate risk for cocaine use in individuals who use multiple drugs of dependence. Since that study, there have been no studies evaluating lofexidine's effects on opioid dose-response curves, leaving a large gap in the rigor of prior research.

There is a dearth of information regarding the impacts of xylazine on fentanyl as well as the lack of understanding of the differences between xylazine and lofexidine, and limited studies leveraging basic science to understand these relationships (Gipson and Strickland, 2023). Thus, the purpose of the current project was to extend the findings of our prior study (Khatri et al., 2023) and determine how adulteration of the intravenously self-administered fentanyl infusion with xylazine impacts the fentanyl dose-response curve, fentanyl demand, and acute withdrawal syndrome in rats, and how these effects compare to the intravenous adulteration of fentanyl with lofexidine during SA. We transformed the dose-response data into demand curves, as this analysis has translational value and is utilized in both clinical (Petry and Bickel, 1998) and preclinical (Fragale et al., 2019) opioid research. We hypothesized that fentanyl would function as a reinforcer when self-administered intravenously, and that xylazine would suppress fentanyl consumption and thus induce a leftward and/or downward shift in the fentanyl dose-response curve. This hypothesis was based on clinical reports that xylazine may extend the "high" of fentanyl (Friedman et al., 2022) which could result in decreased fentanyl intake, similar to xylazine's ability to decrease the necessary ketamine dose during anesthesia. Similarly, we hypothesized that xylazine would decrease intensity of demand. Given the noted clinical differences in how lofexidine and xylazine are utilized, we hypothesized that the impacts on the fentanyl dose-response curve and fentanyl demand would be specific to xylazine and that lofexidine would not induce a leftward shift or alter demand parameters compared to control. Preclinically, there is also evidence that fentanyl exposure is associated with weight loss (Koek, 2014), and opioid withdrawal is associated with decreased feeding behavior (Schoenbaum et al., 1989). Although there are no clinical reports involving xylazine and weight change patterns or appetite, in the veterinary literature there is evidence that feeding behavior significantly decreases in the first 24 h following xylazine injections in deer (Simpson et al., 1983; Warren et al., 1984). Clinically, there is a signal that body weight index may impact fentanyl clearance, with higher body mass index (BMI) scores being associated with slower fentanyl clearance (Luba et al., 2023). In the current study, rats were food restricted, and were exposed to fentanyl with or without xylazine during SA. Given that both xylazine and opioids are associated with body weight loss, we hypothesized that rats would demonstrate greater weight loss following exposure to the fentanyl + xylazine combination as compared to the fentanyl alone control condition. There is also clinical evidence that lofexidine decreases caloric intake during withdrawal from cannabis (Haney et al., 2008). Thus, we hypothesized that fentanyl + lofexidine would also be associated with greater withdrawal-associated weight loss as compared to the fentanyl alone control condition. Finally, given that lofexidine is FDA-approved to treat opioid withdrawal, we hypothesized that it would suppress acute somatic signs of fentanyl withdrawal, which would be exacerbated by xylazine when compared to fentanyl alone.

2. Methods

2.1. Subjects

51 Long Evans rats (26 male, 25 female) were purchased from Charles River. Male rats were 225–250g and female rats were 200–225g upon initiation of experimentation and were individually housed on a 12-h reverse light cycle with ad libitum access to food and water prior to experimental procedures. Animals were handled daily and 2 animals were excluded from the dose-response and withdrawal phases due to catheter patency failure or death during surgery, for a total of 49 rats finishing the dose-response phase. All animal use practices were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky (UK; Protocol #2020–3438).

2.2. Study drugs

Xylazine (injectable solution) was purchased from Covetrus (Portland, ME). For xylazine dilution, 1.5 mL of stock solution (100 mg/mL) was added to the fentanyl stock solution and then further diluted by 10 and adjusted for body weight dose. Fentanyl HCl was gifted from the National Institute on Drug Abuse (NIDA, Bethesda, MD) through the NIDA Drug Supply Program or purchased from Cayman Chemicals (Ann Arbor, Michigan). Lofexidine HCl was also purchased from Cayman Chemicals.

2.3. Surgery

Jugular vein catheter surgery was conducted in all rats. Rats were anesthetized with intramuscular (IM) ketamine (80–100 mg/kg) and xylazine (8 mg/kg), and aseptic surgical techniques were utilized. Rats received a 7-day recovery period prior to SA procedures. Rats were given cefazolin (100 mg/kg; i. v.), heparin (10 usp, i. v.), and meloxicam (1 mg/kg, i. m.) on the day of surgery and once daily for three days during the post-surgical recovery period. Heparin was administered throughout the post-surgical recovery period and throughout SA to maintain catheter patency. Catheter patency was checked throughout SA experiments using Brevital (3 mg/kg i. v.), which induces and maintains anesthesia for 2–5 min. Please see our prior studies for additional surgical details (Khatri et al., 2023; Maher et al., 2022).

2.4. Operant conditioning chambers

28 sound-attenuated Med Associates chambers with ventilation fans were used for this study. One active and one inactive lever was presented at the beginning of each session. Above each lever was a stimulus light, and each chamber contained a house light. Infusion pumps were located outside of each chamber. Experimental events were recorded by MED-PC software on a computer in the experimental room (previously described in (Maher et al., 2022; Maher et al., 2021)).

2.5. Food training

All rats were food restricted to 85% of free feed body weight and then underwent one 15-h overnight food training session prior to initiation of fentanyl SA. In this session, one active lever press (fixed ratio (FR)-1) resulted in the delivery of one chow pellet (45 mg; BioServ, Flemington, NJ). Food pellets were also delivered non-contingently every 9 min. Operant chambers were illuminated with a houselight during the overnight session to simulate the reverse light cycle, and water was provided ad libitum during the session. Both active and inactive lever presses were recorded, and acquisition criterion were set at a 2:1 active:inactive lever press ratio during the session. If this was not reached, an additional overnight session was conducted.

2.6. SA procedures

Acquisition. Following intravenous jugular vein catheter surgery (see experimental timeline in Fig. 1A), rats were food restricted to 85% of their free feed weight (established prior to initiation of experimental procedures) throughout experimentation and food trained prior to SA procedures (see Maher et al., 2022). During SA sessions, upon an active lever press (FR-1), lights above both levers were illuminated and a tone (2900 Hz) was presented simultaneously with drug infusion. Infusions were followed by a 20-s dark timeout period, during which active lever responses were recorded but produced no consequences. An inactive lever was present at all times but produced no consequences when pressed. Responding of 66.67% or higher on the active lever for 2 consecutive sessions was considered meeting acquisition criteria. Rats then underwent 10 2-h sessions of fentanyl SA, whereby 3.2 μg/kg/infusion fentanyl (0.1 mL/infusion; (Hammerslag et al., 2020; Seaman and Collins, 2021) was delivered across 5.9 s following one response on the active lever (FR-1).

Fentanyl Dose-Response. Following 10 sessions of fentanyl SA, rats underwent a dose-response phase in which rats completed 3 sessions each of 6 doses of fentanyl (0, 0.1, 0.32, 1.0, 3.2, and 10 μg/kg/infusion) in randomized order. Saline sessions (the 0 μg/kg/infusion fentanyl dose which either included just saline, saline + xylazine, or saline + lofexidine) was randomly interpolated with the other fentanyl doses. Rats were split into thirds (half male, half female per condition) and randomly assigned based on similar consumption in the acquisition phase as in our prior publications (Maher et al., 2022, 2023) to receive either lofexidine (0.1 mg/kg/infusion; Kohut et al., 2013), xylazine (0.15 mg/kg/infusion; Khatri et al., 2023), or vehicle (a fentanyl control group). The lofexidine dose was selected because it was previously

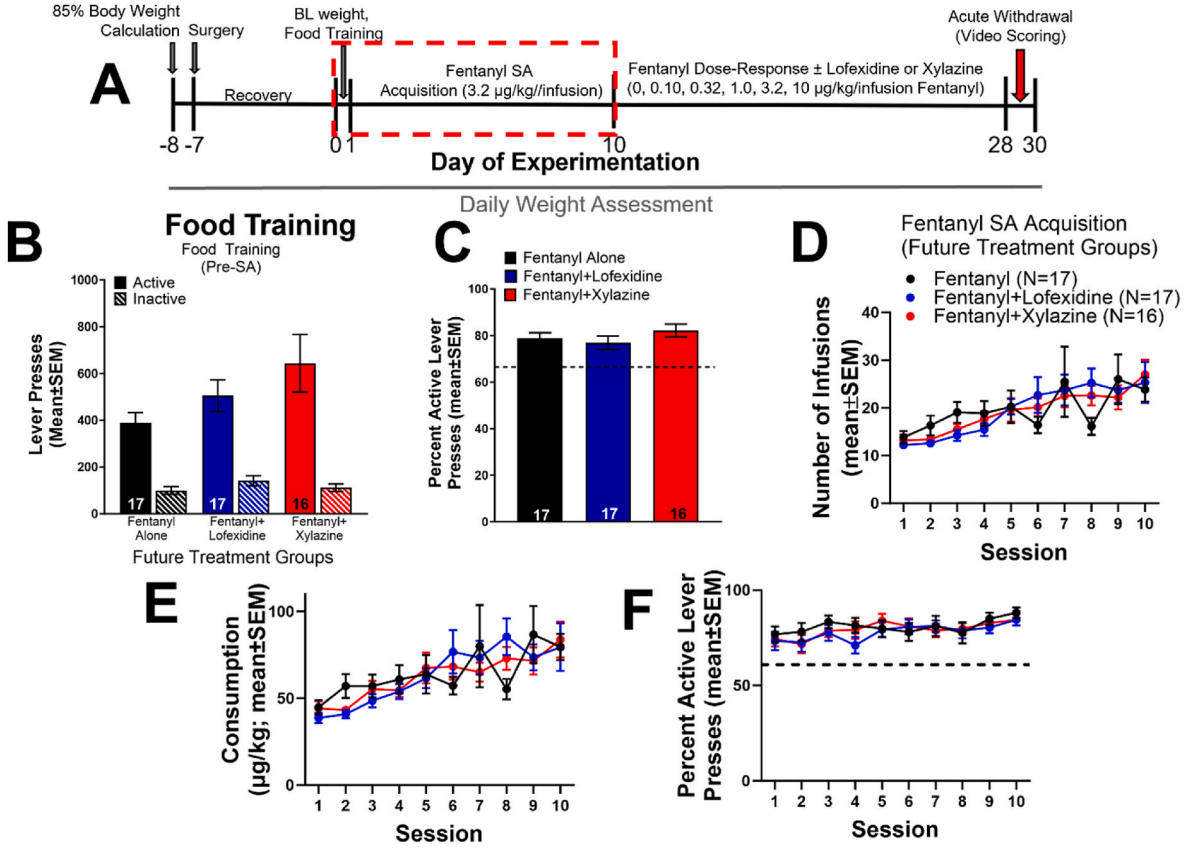


Fig. 1. Fentanyl SA During Acquisition. (A) Experimental timeline; red box indicates the phase of the experiment. **(B)** The number of active and inactive lever presses from future treatment groups (to ensure no differences in baseline food training experience). There was only a significant main effect of lever (*$p < 0.05$), with no differences in lever pressing between future groups. **(C)** Active lever discrimination did not differ as a function of sex or future group. Horizontal dotted line in C = 66.67% active lever press discrimination. **(D)** The number of fentanyl infusions across the first 10 sessions of fentanyl SA did not differ between future fentanyl alone, fentanyl + lofexidine, and fentanyl + xylazine groups ($p > 0.05$). **(E)** Consumption (infusions x dose) as a function of treatment group. **(F)** There were no future group differences in active lever press discrimination during fentanyl acquisition, and all rats reached the 2:1 active:inactive lever press criterion (dotted line at 66.67% active lever press discrimination). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

found to shift the cocaine dose-response curve leftward when added to an intravenous cocaine infusion during SA (Kohut et al., 2013), and the xylazine dose was selected because it is the middle dose tested in Khatri et al. (2023), where we found that it suppresses fentanyl consumption during SA.

2.7. Somatic signs of acute withdrawal

Following the dose-response phase, a subset of rats (N = 32; withdrawal was not scored following the initial cohort of rats and thus 19 rats were not included in withdrawal analyses) underwent observational testing for somatic signs of spontaneous withdrawal consistent with our prior publications (see (Gipson et al., 2020; Khatri et al., 2023) at 0, 12, 24, and 48 h post SA. The 0 h timepoint represents baseline behavioral signs after SA prior to drug discontinuation. The justification for evaluation of this timepoint is to determine how somatic signs evolve from baseline during the acute withdrawal phase (Dunn et al., 2019; Gipson et al., 2020). Rats were placed in a clear Plexiglas chamber (9.6 × 9.6 x 14.6in; L x W x H) and recorded for 10 min after 5 min of habituation. Trained scorers were blinded to group and timepoint and scored withdrawal signs from the video recordings using a standardized scoring protocol (Gipson et al., 2020). Sixteen signs were evaluated, including digging, jumping, rearing, grooming, diarrhea, piloerection, genital licks, teeth chatters, chewing, ptosis, escape attempts, eye blinks, foot licks, writhing, head shakes, and body shakes.

2.8. Statistical analysis

Food training lever press data, drug infusions, and lever press discrimination (active, inactive) across sessions and withdrawal signs were analyzed using linear mixed effects (LME) modeling (JMP software from SAS). Tukey's HSD was used as post-hoc pairwise comparisons on nominal variables where appropriate ($\alpha = 0.05$). Lever discrimination was assessed as the ratio of active/active + inactive lever presses (including infusion-earning and timeout active lever presses, as well as all inactive lever presses). Data from the acquisition phase was analyzed via LME to remove any baseline differences in fentanyl consumption prior to group assignment during the dose-response phase. Data for the fentanyl dose response were averaged across the last 2 sessions at each dose and analyzed using LME. A quadratic trend analysis was also conducted on data from the dose response phase to test for the presence of ascending and descending dose response curve limbs. Subject was treated as a random factor and session was treated as a continuous factor in all relevant analyses. Model fits were conducted to determine the best model fit for the datasets where appropriate, and the models with the lowest Akaike information criterion (AICc) were selected. The total number of infusions per animal during acquisition was recorded per session and compared across groups using LME.

2.9. Demand analyses

To determine fentanyl demand, infusion data from the fentanyl dose response curves were transformed to determine consumption (calculated as number of infusions earned x dose delivered) and demand curves were calculated according to the exponential demand equation (Hursh and Silberberg, 2008):

$$log\ Q = log\ Q_0 + k(e^{-\alpha \bullet Q_0 \bullet C} - 1)$$

where Q represents consumption, Q_0 represents the estimated maximum consumption at a zero-unit price point, α represents the rate of change of consumption, C is unit price, and k is a constant. Demand curves were fit to the data via nonlinear mixed effects (NLME) modeling in *R* using the 'nlme' package (Hofford et al., 2016; Powell et al., 2020). The global constant k had a best-fit value of 1.32 for all experiments. Graphing was performed in Prism 10.1 (Graphpad Software, San Diego, CA). Data are represented as means ± standard error of the mean (SEM) where appropriate.

2.10. Body weight analysis

Baseline body weight was initially evaluated prior to experimentation, while rats were on free feed. 85% of the 100% free feed weight was calculated and set as the target food restriction weight. Body weight was measured daily, and the percent change from the pre-experimental baseline was calculated. Weight was also examined by calculating Area Under the Curve (AUC) for the changes in body weight, which were used in separate analyses of variance (ANOVAs) to compare groups for each of the acquisition, dose-response, and withdrawal phases. Only animals that were tested for withdrawal (N = 32) were included in the body weight analysis because weights were examined across all phases of experimentation and compared separately during the acquisition, dose-response, and withdrawal phases.

3. Results

3.1. Food training

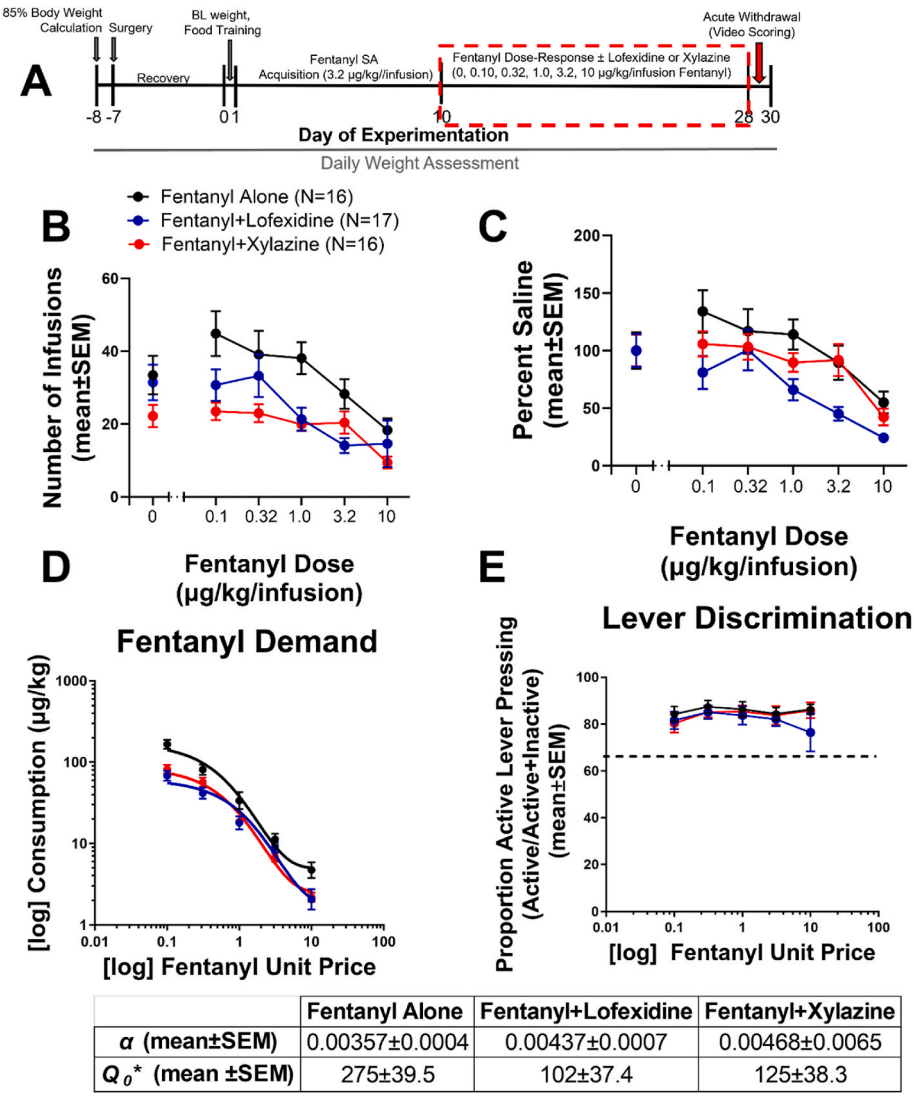
All rats underwent one 15h food training session. LME was conducted separately on pellets earned, lever pressing to the active and inactive levers, and the proportion of active lever pressing (active/active + inactive). LME analysis on number of pellets earned revealed no main effect of future assigned group, sex, or sex × group interaction (p's > 0.05). These results indicate that there was no difference in the number of pellets earned between future groups during food training. LME on active and inactive lever pressing reveled only a main effect of lever ($F_{1,44} = 73.41$; $p < 0.05$; Fig. 1B), with no other main effects of interactions (p's > 0.05). Tukey's post-hoc analysis of lever revealed that pressing was higher on the active lever as compared to the inactive lever. Next, LME conducted on lever discrimination revealed no main effects of group or sex, or group × sex interaction (p's > 0.05; Fig. 1C). These results indicate that all groups met the lever discrimination criterion (2:1 active:inactive lever press ratio, or 66.67% active lever pressing) regardless of future group assignment.

3.2. Fentanyl SA acquisition

LME was conducted on the number of infusions earned during fentanyl SA acquisition, which revealed a significant main effect of session (Fig. 1D; $F_{1,43} = 17.69$; $p < 0.05$), but no other significant main effects or interactions (p's > 0.05). Results indicate no differences in baseline fentanyl infusions prior to group assignment, and baseline infusions did not differ as a function of biological sex at the 3.2 μg/kg/infusion training dose. Data are also presented as consumption (infusions x dose; Fig. 1E). LME conducted on lever discrimination revealed only a main effect of session ($F_{1,43} = 13.43$; $p < 0.05$; Fig. 1F), with no other significant main effects or interactions (p's > 0.05). These results revealed no differences prior to group assignment in lever discrimination; however, active lever discrimination appeared to improve across sessions.

3.3. Impact of lofexidine and xylazine on fentanyl SA across doses

Next, LME analysis was conducted on infusions from the within-subject fentanyl dose-response curve with or without lofexidine or xylazine combined with fentanyl (see Fig. 2A for an experimental timeline). The model that excluded sex (delta AICc = 47) and treated dose as a nominal factor (delta AICc = 42) was the best fit. There was a significant main effect of group (Fig. 2B; $F_{2,46.38} = 7.23$; $p < 0.05$) and a main effect of dose ($F_{5,212.8}$) = 18.64; $p < 0.05$), but no significant group × dose interaction ($p > 0.05$). Given the significant main effect of group, Tukey's HSD was conducted on the nominal group variable and significant differences between the fentanyl + xylazine and fentanyl +



	Fentanyl Alone	Fentanyl+Lofexidine	Fentanyl+Xylazine
α (mean±SEM)	0.00357±0.0004	0.00437±0.0007	0.00468±0.0065
Q_0* (mean ±SEM)	275±39.5	102±37.4	125±38.3

Fig. 2. The Impacts of Lofexidine and Xylazine on Consumption of Fentanyl SA at Various Unit Doses. (A) Experimental timeline; red box indicates the phase of the experiment. **(B)** Consumption of fentanyl during intravenous SA differed as a function of unit dose. Both xylazine and lofexidine decreased fentanyl consumption across fentanyl doses as compared to the fentanyl alone control group when plotted as number of infusions (*$p < 0.05$; main effect of group) as well as **(C)** when plotted as percent of the respective group saline condition. **(D)** Both xylazine and lofexidine decreased demand intensity (Q_0) but did not impact fentanyl demand elasticity (α). Q_0 and α values are represented in the table as mean ± SEM. *$p < 0.05$. **(E)** Lever discrimination did not differ as a function of fentanyl unit price during the dose-response phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lofexidine and control groups were found (though the xylazine and lofexidine groups did not differ from each other significantly). Data from the three groups were further analyzed via quadratic trend analysis, which revealed a significant quadratic trend for the fentanyl alone group ($F_{1,211.3} = 18.97$; $p < 0.05$), but not the fentanyl + lofexidine, or the fentanyl + xylazine groups ($p > 0.05$). We hypothesized that saline intake in the fentanyl alone group would be lower than the 0.1 µg/kg/infusion fentanyl dose. Thus, a paired *t*-test was run on these two dataponts in the fentanyl alone control group, which confirmed this hypothesis ($t_{12} = 2.35$, $p < 0.05$). LME was then conducted on data transformed to percent of the corresponding group saline condition (0 µg/kg/infusion fentanyl dose). Model fits revealed that the model that included sex and treated dose as a nominal variable was the best fit (delta AICc = 55) and revealed a significant main effect of group (Fig. 2B; $F_{2,43.63} = 3.65$; $p < 0.05$) and dose ($F_{5,198} = 17.85$; $p < 0.05$), with no other significant main effects or interactions (p's > 0.05). Tukey's HSD was conducted on the nominal group variable, which revealed the fentanyl + lofexidine but not fentanyl + xylazine group significantly differed from fentanyl control group. Tukey's HSD on the nominal dose variable revealed that the 10 µg/kg/infusion dose was significantly lower than all other doses, and the 3.2 µg/kg/infusion dose was significantly different from 0, 0.1, and 0.32 µg/kg/infusion doses. Together, these data suggest that fentanyl functioned as a reinforcer, and both lofexidine and xylazine suppressed fentanyl consumption across a range of fentanyl doses.

Next, consumption from the dose-response curves were transformed into demand curves (Fig. 2B). The best model fit included sex and group in the model (AICc delta = 28, relative to the next best model fit, which included only group as a variable). NLME revealed a significant main effect of group on demand intensity (Q_0; $F_{2,169} = 4.16$, $p < 0.05$), with contrasts indicating significance between the fentanyl alone control group and both the fentanyl + lofexidine and fentanyl + xylazine groups but not between the fentanyl + lofexidine and fentanyl + xylazine groups themselves. There was no main effect of group on demand elasticity (α; $F_{2,169} = 1.88$, $p > 0.05$), and there was also no main effect of sex on Q_0 or α (p's > 0.05). The global best fit k value was 1.61.

Active lever press discrimination data from the last 2 sessions of each dose was averaged and analyzed via LME, which revealed no main

effects or interactions (p's > 0.05), and responding on the active lever remained consistently above 66.67% regardless of unit price (Fig. 2C). These results indicate that although fentanyl + xylazine and fentanyl + lofexidine suppressed demand intensity, alteration of the fentanyl infusions with these drugs did not impair active lever discrimination at any unit price (dose) tested.

3.4. Fentanyl SA induces loss of body weight

Rats were weighed daily throughout the experimental timeline (Figs. 1A, 2A and 4A) from acquisition, dose-response, and withdrawal phases. Overall, weight decreased across the experiment (Fig. 3A), but there were no differences in body weight change across the three phases of experimentation as a function of group, as shown by the area under the curve (AUC) graphs, which were compared via individual ANOVAs (p's > 0.05; Fig. 3B). We hypothesized that body weight would decrease significantly more in the fentanyl + xylazine group as compared to the fentanyl control group during the first 24 h of withdrawal, thus weight change from 0 h (immediately following the last SA session) to 24 h was calculated and analyzed via LME. The model that excluded sex was the best model fit (delta AICc = 6). A significant main effect of group was found ($F_{2,27} = 6.49$, $p < 0.05$; Fig. 4B), and Tukey's HSD post-hoc analysis revealed that the fentanyl + xylazine group demonstrated significantly greater weight loss during the first 24 h of withdrawal compared to the fentanyl alone control group (there were no other significant differences between groups). Together, these data demonstrate that chronic exposure to fentanyl with xylazine may induce more weight loss during the acute withdrawal syndrome than the other studied conditions.

3.5. Differences in the emergence of fentanyl withdrawal signs between lofexidine and xylazine following SA

A subset of rats from each group (12 from fentanyl alone, 9 from fentanyl + lofexidine, and 11 from fentanyl + xylazine) was evaluated for somatic signs of withdrawal across the acute withdrawal phase (including 0, 12, 24, and 48 h following SA; see Supplemental Material (S1), Table 1 for mean and standard deviation of each individual sign). Model fits were conducted to determine the lowest AICc values. The best model fit was one that treated timepoint as a nominal variable (delta AICc = 74) and included sex as a nominal variable (delta AICc = 10). LME revealed a significant main effect of timepoint (Fig. 4C; $F_{3,76.39} = 23.53$; $p < 0.05$) and a group × timepoint interaction ($F_{6,76.37} = 2.29$; $p < 0.05$), but no main effect of group ($p > 0.05$). Tukey's HSD post-hoc analyses conducted on the nominal variable to timepoint revealed significantly lower withdrawal signs in the fentanyl + xylazine group at the 0 h timepoint as compared to the 12, 24, and 48 h timepoints, as well as the fentanyl + xylazine 0 h timepoint being significantly lower compared to the 24 and 48 h timepoints of the fentanyl alone control group. There was also significance of the fentanyl + lofexidine 0 h timepoint compared to the 24 and 48 h fentanyl + lofexidine group timepoints. The fentanyl + lofexidine group was also significantly different at the 0 h timepoint compared to the fentanyl control group at 24 and 48 h. Thus, there was a difference in the rate at which withdrawal signs in the different groups rebounded to the fentanyl control group levels, with the fentanyl + xylazine group increasing from 0 h by the 12 h timepoint, but the fentanyl + lofexidine group remaining lower than fentanyl control and fentanyl + lofexidine 0 h until the 24 h timepoint. These results may reflect the withdrawal suppression ability of lofexidine, which has been shown to be clinically efficacious (Hermes et al.,

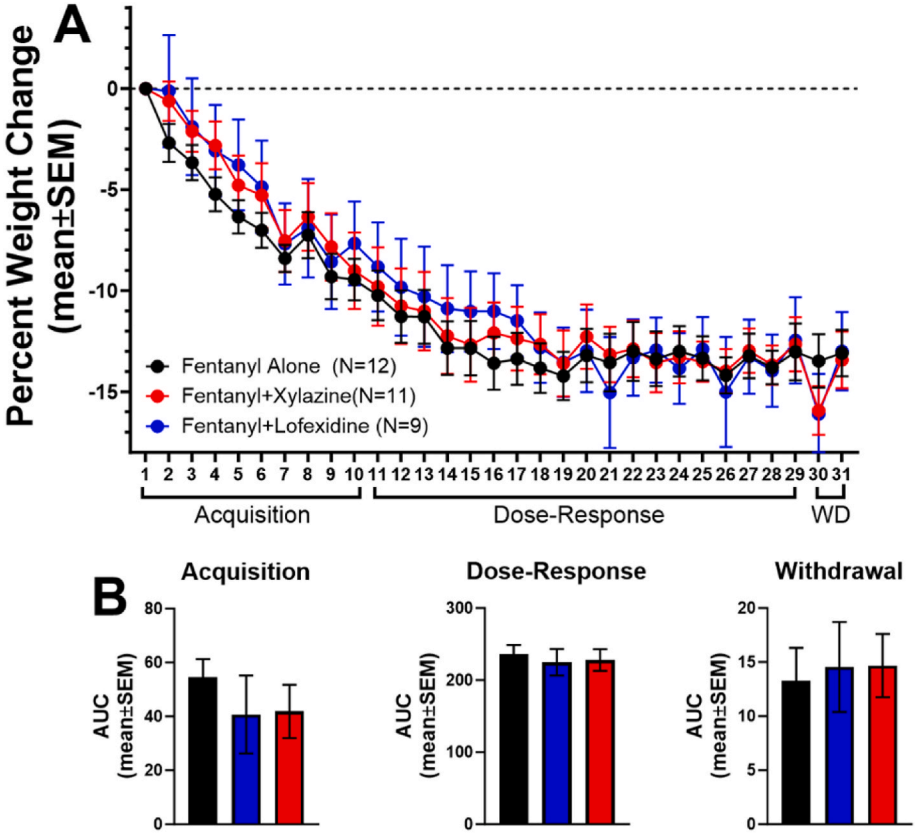


Fig. 3. Fentanyl SA and Withdrawal Induces Body Weight Loss. (A) Daily weight measurements of rats throughout experimentation indicated body weight loss compared to baseline (pre-SA) in all three treatment groups (fentanyl alone, fentanyl + lofexidine, fentanyl + xylazine). **(B)** Area under the curve (AUC) analysis indicated no group differences in the rate of weight loss during the acquisition, dose-response, and withdrawal phases (WD = withdrawal; Fentanyl alone control group = 6 male, 6 female, fentanyl + lofexidine = 5 male, 4 female; fentanyl + xylazine = 5 male, 6 female).

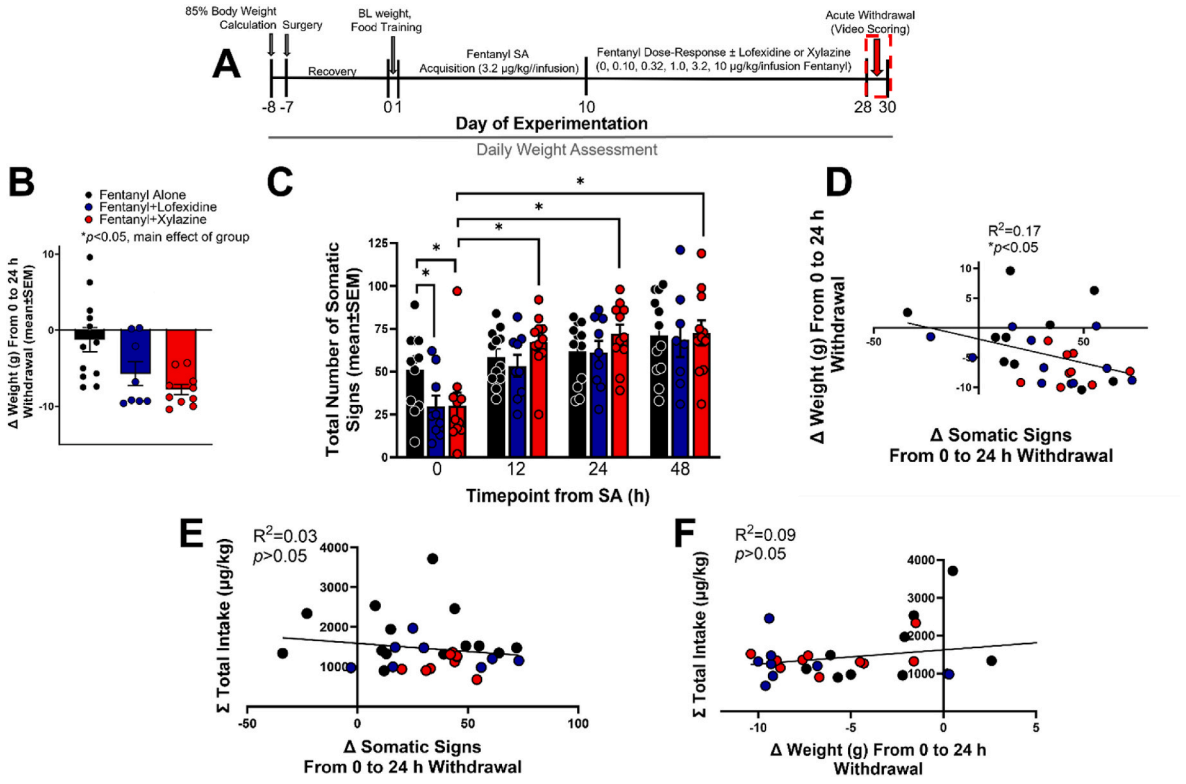


Fig. 4. Xylazine and Lofexidine Shift the Trajectory of Fentanyl Withdrawal Symptomatology. (A) Experimental timeline; red box indicates the phase of the experiment. **(B)** Xylazine induced significant weight loss when self-administered with fentanyl during SA as compared to a fentanyl alone control group (*$p < 0.05$; main effect of group). Body weight was not significantly different between the fentanyl + lofexidine and fentanyl control group. **(C)** Somatic signs of fentanyl withdrawal were significantly suppressed in the fentanyl + lofexidine and fentanyl + xylazine groups immediately following SA (0 h timepoint) but rebounded by 12 h in the fentanyl + xylazine group. In contrast, rats in the fentanyl + lofexidine group demonstrated no difference in withdrawal signs between the 0 and 12 h timepoints but showed significantly higher signs by 24 h (asterisks denote significance at $p < 0.05$). **(D)** Severity of somatic signs of withdrawal at 24 h is significantly correlated with severity of weight loss (*$p < 0.05$), but neither **(E)** the change in somatic signs of withdrawal from 0 to 24 h or **(F)** the change in body weight from 0 to 24 h were significantly correlated with the total amount of drug consumed (in µg/kg) across experimentation. Fentanyl alone control group = 6 male, 6 female, fentanyl + lofexidine = 5 male, 4 female; fentanyl + xylazine = 5 male, 6 female. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2019). The fentanyl alone control group did not significantly differ in total somatic signs across the acute timepoints. To determine if the patterns of somatic signs of withdrawal were related to weight loss during the acute withdrawal phase, we correlated the change in body weight from 0 to 24 h withdrawal to the change in somatic signs from these 2 timepoints. Interestingly, change in weight was significantly correlated with the change in somatic signs of withdrawal, indicating that the more weight a rat lost during the first 24 h of withdrawal from SA, the more somatic signs of withdrawal were observed (Fig. 4D; $R^2 = 0.17, p < 0.05$). Next, correlations were conducted on total consumption of fentanyl with or without xylazine or lofexidine during SA and either the change in somatic signs from 0 to 24 h or the change in body weight from 0 to 24 h. No significant correlations were found between the summation of consumption and the change in somatic signs (Fig. 4E; $R^2 = 0.03, p > 0.05$) or the change in body weight (Fig. 4F; $R^2 = 0.09, p > 0.05$). Together, these results indicate specificity of the relationship between withdrawal severity and loss of body weight and suggest that body weight may be an important biological indicator of severity of the withdrawal experience.

4. Discussion

Here we show that consumption of fentanyl during SA was dose-dependent, consistent with other literature showing that consumption of fentanyl changes when unit dose is manipulated (Mavrikaki et al., 2017). In line with our hypothesis, we also show that the combination of fentanyl with xylazine decreased fentanyl consumption when tested at multiple unit doses. Interestingly, lofexidine also decreased fentanyl SA at both higher and lower fentanyl doses. When data were transformed as percent of respective group saline conditions, results support that xylazine generally suppressed responding for both saline and fentanyl and could be indicative of some overall response/behavioral suppression, an effect not induced by lofexidine. Observation of the animals indicated that both lofexidine and xylazine induced sedation when these compounds were added to the fentanyl infusion. However, only xylazine (and not lofexidine) significantly reduced saline intake, suggesting that lofexidine alone does not inhibit locomotor activity or induce sedation at the dose tested here. It is possible that these effects are dose-dependent, and additional studies with different xylazine and lofexidine doses are warranted to evaluate this possibility. Demand curves demonstrated that both xylazine and lofexidine significantly decreased Q_0 compared to control, indicating that both of these adrenergic α2a agonists specifically suppress maximum fentanyl consumption at zero unit price. Importantly, this parameter provides an estimated amount of fentanyl an animal would take if the commodity was free, demonstrating the intensity of demand. That all rats showed systematically decreased fentanyl consumption as unit price increased supports that all groups were equally sensitive to changes in fentanyl unit price when it was manipulated by decreasing the dose (i.e., there were no group differences in demand elasticity).

Here we show that co-exposure to fentanyl with xylazine and with lofexidine during SA suppressed the total number of acute behavioral signs of withdrawal at 0 h (immediately following SA) as compared to fentanyl alone, but the signs rebounded in the fentanyl + xylazine group

within 12 h and were maintained through the 24 h and 48 h timepoints. Interestingly, there was a prolonged suppression of somatic signs in the fentanyl + lofexidine group, with signs significantly rebounding by the 24 h timepoint but remaining lower at the 12 h timepoint. These results demonstrate that withdrawal suppression may last longer following lofexidine versus xylazine co-exposure. Given that lofexidine suppresses opioid withdrawal at the clinical level, the current results with lofexidine are consistent with this clinical effect although the ability of lofexidine to suppress acute withdrawal appears to diminish after the first 12 h of lofexidine exposure. In humans, the half-life of lofexidine is 11 h (Al-Ghananeem, 2009). Although we could not find data on the half-life of lofexidine in rats, the rebound of somatic signs of withdrawal by the 12 h timepoint found in the current study is consistent with the pharmacokinetics of this compound in humans. With xylazine, our prior study found that the half-life of intravenous xylazine in rats was 0.838 h (Khatri et al., 2023), consistent with our current data showing a rebound of somatic signs of withdrawal within 12 h after xylazine exposure. Together, these results suggest that there are important differences between the two A2aR agonists in duration of withdrawal sign suppression, warranting clinical studies evaluating lofexidine in the context of withdrawal from fentanyl and xylazine.

Consistent with prior reports that weight loss corresponds with chronic opioid exposure (Mitzelfelt et al., 2011) and withdrawal (Koek, 2014) in rodents, our data show that all rats demonstrated weight loss throughout experimentation, with no group differences in this effect. When the weights were analyzed from the end of SA to the first 24 h of withdrawal, there was a significant group difference whereby rats in the fentanyl + xylazine group demonstrated more severe weight loss at this acute withdrawal timepoint. Further, weight loss severity between the 0 and 24 h timepoints was correlated with severity of somatic signs of withdrawal, indicating that weight loss magnitude may be associated with withdrawal severity within the first 24 h. Clinically, there are no reports evaluating body weight loss during fentanyl + xylazine withdrawal, however, there appear to be meaningful interactions between BMI, fentanyl clearance, and withdrawal severity (Luba et al., 2023). Together, these studies and our current data warrant further evaluations of the relationships between body weight, fentanyl + xylazine SA, and withdrawal.

4.1. The fentanyl dose-response curve: prior research and defining fentanyl as a reinforcer

Our results show that fentanyl consumption decreased as unit dose increased, reflective of a descending limb of a typical inverted-U dose response curve (Mavrikaki et al., 2017; Townsend et al., 2019). In one prior study, peak fentanyl intake was found at a 1.0 μg/kg/infusion fentanyl dose, with lower (0.32) and higher doses (3.2, 10) reflecting ascending and descending limbs of an inverted U-shaped curve (Townsend et al., 2019). Importantly, a quadratic trend was evident in the fentanyl alone group, supporting that an ascending limb was present when the 0 μg/kg/infusion fentanyl dose was included in the analysis. Contrary to our hypothesis that an ascending limb would be evident at the 0.1 and 0.32 μg/kg/infusion doses, our data show increased intake at these lower doses. These results are consistent with another study in mice showing increased fentanyl infusions at a 0.18 μg/kg/infusion dose, with subsequent decreases in infusions as dose was increased (to 0.56, 1.8, 5.6, 18, and 56 μg/kg/infusion doses; (Leonardo et al., 2023). Further, a study evaluating heroin dose-response both within a session and between sessions demonstrated no ascending limb of the dose response curve; thus, it is possible that even lower doses than the lowest dose tested in our present study (0.1 μg/kg/infusion) as well as in the prior cited studies are needed to achieve an ascending limb. It is also possible that the disparate results between our study and Townsend et al. (2019) are due to procedural differences. Although the training dose was the same between studies (3.2 μg/kg/infusion), the prior study utilized a FR-5 schedule of reinforcement, and saline was substituted for fentanyl every other session (giving animals extended training with the saline substitution methodology). These methods contrast with ours as we utilized a FR-1 and only implemented one saline substitution randomly for 3 days.

The parameters defining drugs as reinforcers have long been established to determine if responding is due to response-contingent delivery of the drug and not due to other factors. Procedures to establish this include presentation of a second (inactive or "dummy") lever to ensure that responding is specific to the lever associated with drug delivery, reversal of the active lever and corresponding reversal of response rates, comparison of rate of responding compared to vehicle, inclusion of intermittent access schedules, and orderly dose-response relationships (Meisch, 1987). Although we did not incorporate all of these procedures, several of them were included as procedural parameters in the current study. These included an inactive (dummy) lever, saline substitution, and evaluation of a dose-response relationship. We posit that we show evidence that fentanyl functioned as a reinforcer in the current study, as the proportion of lever pressing on the active lever remained high across experimentation, including at very low unit doses of fentanyl. We further show that when saline was substituted for fentanyl, consumption of fentanyl decreased compared to active fentanyl doses providing evidence of an ascending limb of the dose-response curve. Finally, we show that when the fentanyl dose was increased, consumption decreased in an orderly fashion, indicating that consumption of fentanyl was sensitive to dose manipulation. In further support, when we included the 0 μg/kg/infusion dose (saline) in the quadratic trend analysis, a significant quadratic trend was found, indicating that responding did decrease when fentanyl was omitted from the infusion. We further show a decrease in number of infusions in the fentanyl alone condition when saline was substituted for fentanyl. Together, our results indicate that fentanyl functioned as a reinforcer in the current study.

4.2. Xylazine and the fentanyl dose-response curve

Here we show no quadratic trend when xylazine was added to the saline fentanyl infusions, and xylazine suppressed both saline and fentanyl consumption at various tested unit doses. In 2022, the Drug Enforcement Administration (DEA) issued a joint intelligence report with the Department of Justice claiming that xylazine may "attract customers looking for a longer high since xylazine is described as having many of the same effects for users as opioids, but with a longer-lasting effect than fentanyl alone" (DEA, 2022). This genesis of this statement likely came from epidemiological reports claiming that individuals seek xylazine because it "prolong [s] the duration of fentanyl injections, in particular, solving "the problem" of the "short legs" of the otherwise euphoric effects of illicitly manufactured fentanyl" (Friedman et al., 2022). While our current data may support the possibility that xylazine enhances the reinforcing efficacy of fentanyl and thus decreases intake, we cannot rule out other factors such as general response suppression in the current dataset. This is important because xylazine also suppressed saline intake, indicating that procedures that allow measurement of response rate cannot disentangle reinforcing efficacy from response rate. Thus, additional research is warranted to evaluate xylazine and fentanyl using rate-independent outcome measures, which is translationally important to determine if xylazine is extending the "high" of fentanyl, or if other factors are involved.

4.3. Lofexidine and the fentanyl dose-response curve

Lofexidine significantly lowered fentanyl consumption when evaluated at various fentanyl doses, and suppressed fentanyl demand intensity. These results are in line with findings from one study evaluating lofexidine's effects on the cocaine dose-response curve (Kohut et al., 2013). Specifically, that study found that lofexidine shifted the cocaine dose-response curve leftward, and interpreted findings as lofexidine potentiating cocaine's reinforcing effects. Importantly, the design of our

study does not allow for conclusions to be made regarding the impacts of lofexidine on fentanyl's reinforcing efficacy, as response suppression may have been the cause of the decreased fentanyl consumption found. It is also important to limit translational interpretation of our findings regarding reinforcing efficacy, as lofexidine is an FDA-approved medication for opioid withdrawal and is not approved to decrease opioid intake.

4.4. Body weight and feeding behavior during opioid exposure and xylazine

One surprising finding of the current study is that fentanyl SA induced weight loss across experimentation and that xylazine co-exposure with fentanyl exacerbated body weight loss during acute withdrawal as compared to the fentanyl alone control condition. In the clinical literature, there is evidence that body weight and opioid use are related (Luba et al., 2023). For example, one study showed that BMI indicating overweight (>25) or obese (>30) was associated with increased odds of prescription opioid use (Stokes et al., 2019). Given that fentanyl is highly lipophilic, it may be possible that absorption and excretion patterns of fentanyl may vary by body weight and fat content (Bird et al., 2023).

Mechanistically, it is unclear how A2aR agonists may induce body weight loss. However, studies have shown that A2aRs play a critical role in the physiological control of subcutaneous adipose tissue lipolysis, which is a metabolic process in adipose tissue that causes the breakdown of fat and release of free fatty acids and glycerol into the blood (John et al., 2016; Langin, 2006). Reducing fat content and increasing energy expenditure helps reduce body weight. Fentanyl increases brown adipose tissue (BAT) sympathetic nerve activity, which may be related to fentanyl-induced changes in body temperature (Cao and Morrison, 2005) and increased energy expenditure. To date, nothing is known regarding how xylazine may impact the absorption, distribution, metabolism, and excretion of fentanyl or how this may be related to withdrawal. However, there is supporting literature evaluating A2aR agonists as anti-obesity treatments, with preclinical studies showing the efficacy of compounds within this family in reducing body weight and fat content and improving glucose tolerance in high fat diet-fed mice (DeOliveira et al., 2017; Kim et al., 2022). Thus, it is possible that xylazine reduces body weight by reducing body fat content. However, additional studies to make this conclusion are needed. Our current study does not allow for these types of conclusions to be made regarding the effects seen and mechanisms of the body weight loss. These observed effects may be through A2aR agonism or through other signaling pathways. Thus, future studies are warranted to understand the mechanistic underpinnings of our observed effects on body weight.

It is also important to consider that endogenous opioids are involved in feeding behavior, including μ opioid receptors (MORs; Bodnar, 2004). In our study, although rats lost weight, clinical signs indicated that rats consistently ate all of the daily food that was provided and lost weight despite increasing the number of grams of food across experimentation. Together, it is possible that agonism of MORs by fentanyl across the experiment increased appetite and therefore increased feeding behavior in the current study (consistent with historical findings of increased appetite with chronic opioid exposure throughout the literature, see Martin et al., 1963; Thornhill et al., 1978; Thronhill et al., 1976 for examples). Indeed, the increase in feeding behavior actually sensitizes with repeated exposure to opioids, with no evidence of tolerance to this effect (Bodnar, 2004; Jalowiec et al., 1981). Further, naloxone administration decreases feeding behavior (Holtzman, 1974), demonstrating a potential link between withdrawal from opioids and reductions in appetite. While it is unclear if xylazine potentiated this effect in the current study, it is possible given the directionality of effects in the literature. Our results are also consistent with a decrease in appetite in deer during the first 24 h following xylazine exposure (Simpson et al., 1983; Warren et al., 1984). Given that there are no prior data examining feeding behavior, weight changes, and withdrawal following chronic fentanyl and xylazine exposure, our data support that additional mechanistic studies are warranted in this area.

4.5. Possible neurobiological mechanisms underlying effects of lofexidine and xylazine on fentanyl SA

Presently, nothing is known regarding the neurobiology underlying fentanyl and A2aR agonism when these compounds are concurrently present. Mechanistically, fentanyl is a potent agonist of MORs (James and Williams, 2020), and as noted above, both xylazine and lofexidine are agonists of A2aRs. Although it is not clear how MORs and A2aRs interact within the brain reward pathway when opioids and A2aR agonists are concurrently present, both receptors activate common signal transduction pathways mediated through the inhibitory G proteins (Gi/Go). A2 cell groups send projections to the ventral tegmental area (VTA; Mejias-Aponte et al., 2009), a brain region that contains dopamine cell bodies and plays a critical role in drug use (Nestler, 2005), and A2aR agonism increases the regularity of VTA dopamine (DA) cell firing (Grenhoff and Svensson, 1989) and also decreases opioid withdrawal (Katz, 1986; Uhde et al., 1980). MORs colocalize with A2aRs (Jordan et al., 2003) and communicate at the receptor level to inhibit them and reduce cellular function (Vilardaga et al., 2008). The consequence of MOR-induced suppression of A2aRs is decreased activity of intracellular signaling cascades (Jordan et al., 2003). As well, A2aR agonism (in the absence of MOR activation) increases the frequency of VTA GABAergic mini-inhibitory presynaptic currents (mIPSCs; (Cathala et al., 2002). In the presence of xylazine or lofexidine and fentanyl, however, it is not known if the intracellular consequences MOR/A2a agonism are uniquely changed, warranting future mechanistic study.

Given that lofexidine is used to treat opioid withdrawal but unlike xylazine is not utilized as an opioid adulterant, there may be important mechanistic differences in the pharmacology which may underlie these effects. For example, although lofexidine binds to serotonin $(5\text{-HT})_{1A}$ receptors (Raffa, 2019), there is no direct evidence that xylazine binds to these receptors and thus future studies are warranted to evaluate the mechanistic underpinnings of the differences between xylazine and lofexidine.

4.6. Limitations of the current study

Limitations of our study include that only one dose of xylazine and lofexidine were examined, however, other doses may impact the fentanyl dose-response curve differently. Further, the current study did not provide extended training with saline substitution for fentanyl, which may have impacted rates of saline intake. Here, we did not evaluate different fentanyl training doses, which may have impacted response rates during the dose-response phase. As noted previously, an intermittent access schedule was not incorporated into the study design (Meisch, 1987), and additional lower doses beyond 0.1 μg/kg/infusion were not evaluated here. Also noted above is the fact that all procedures utilized in the current study allow for evaluation of rate-dependent measures, and do not evaluate the impact of xylazine on fentanyl in the context of other outcomes such as choice procedures. We also did not power our study to detect sex differences in withdrawal, warranting additional studies in this area. Given that this is the first report to examine body weight loss during acute withdrawal from fentanyl + xylazine and the first to find that weight loss may be related to severity of somatic signs of withdrawal, additional studies are needed to replicate these effects. Finally, we did not directly evaluate locomotor activity in the present study. These limitations warrant future research in these areas to provide additional information regarding xylazine's impacts on fentanyl.

4.7. Translational implications and future directions

The results from this study provide novel information regarding the impacts of xylazine, a current adulterant found in the fentanyl drug supply that is associated with increased overdose deaths and other deleterious health impacts, on fentanyl's effects during SA and withdrawal and provides a direct comparison to the FDA-approved medication for opioid withdrawal that shares a primary mechanism of action, lofexidine. Our results show similarities and differences in these compounds on fentanyl demand intensity and acute withdrawal, respectively. Our results are novel in that they indicate substantial body weight loss during the first 24 h of withdrawal from fentanyl + xylazine combination that may be associated with a more severe opioid withdrawal syndrome relative to other conditions. Although replication is needed, our data may indicate a translationally important datapoint for clinical treatment settings to consider and examine. These data also support a prominent role of the adrenergic system in fentanyl effects and withdrawal and suggest that pharmacological differences in these medications confer potentially meaningful differences in clinical effect profiles. Effects observed here, including decreases in responding and withdrawal rebound, are consistent with reports from persons with fentanyl + xylazine co-exposure who indicate displeasure at the extreme sedation and more severe withdrawal syndrome following co-exposure versus fentanyl alone (Spadaro et al., 2023). Moreover, similarities in effect profiles of lofexidine, an FDA-approved withdrawal treatment, and xylazine suggests evaluations of lofexidine treatment for fentanyl + xylazine co-exposures are warranted.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109816.

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