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RESEARCH

Xylazine exacerbates fentanyl-induced respiratory depression and prevents rescue by naloxone in mice

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Abstract

Introduction Xylazine is a veterinary sedative and widespread adulterant of illicit opioids, where it is commonly detected in combination with the potent synthetic μ opioid receptor (MOR) agonist fentanyl in fatal overdose.

Methods We used whole body plethysmography to evaluate the respiratory effects of xylazine in the presence and absence of fentanyl in awake C57BL/6 mice of both sexes. We asked whether suppression of breathing by xylazine-adulterated fentanyl resisted reversal with naloxone. We identified receptor mechanisms necessary to reverse effects of xylazine-adulterated fentanyl on breathing.

Results Xylazine rapidly and dose-dependently suppressed minute ventilation, respiratory frequency, and tidal volume, producing profound respiratory depression at low doses. These effects were dependent on α-2 adrenergic receptors (α2ARs) and were blocked by the α2AR antagonist atipamezole. Xylazine, combined with a dose of fentanyl with modest respiratory effects, suppressed breathing with greater efficacy than when administered alone. A dose of naloxone sufficient to reverse fentanyl-induced respiratory depression was ineffective in reversing the respiratory suppression induced by xylazine-adulterated fentanyl. By contrast, combinations of naloxone with atipamezole rapidly and fully reversed respiratory suppression induced by xylazine-adulterated fentanyl. The same dose of atipamezole, administered alone, produced significant, but markedly delayed reversal.

Conclusion Xylazine suppresses breathing via activation of α2ARs, an effect enhanced by coadministration with the MOR agonist fentanyl. Respiratory suppression inflicted by the mixture of xylazine and fentanyl resisted reversal by naloxone but was fully reversible by subsequent coadministration of both naloxone and atipamezole.

Keywords Opioid epidemic · Overdose reversal · Fentanyl · Xylazine · Adulterant · Respiratory depression · Lethal overdose

Introduction

Xylazine, a relatively unregulated α-2 adrenergic receptor (α2AR) agonist and common veterinary sedative, is a widespread adulterant in the United States illicit opioid supply. Xylazine's sedative effects are viewed as extending the rewarding effects of opioids and ameliorating the effects of withdrawal (Edinoff et al. 2024; Friedman et al. 2022; Justice Department 2022). Consequently, the mixture of xylazine with illicit opioids is increasingly detected in lethal overdose (Alexander et al. 2022; Cano et al. 2023; Holt et al. 2023; Johnson et al. 2021; Merianne Rose Spencer et al. 2023; Wang et al. 2025; Zhu and Cano 2025), as combinations of fentanyl and xylazine facilitate the use of lower opioid doses to produce subjectively similar effects at lower cost (Justice Department 2022). Nearly all cases of overdose involving xylazine also involve the synthetic μ opioid receptor (MOR) agonist fentanyl (Friedman et al. 2022; Kacinko et al. 2022; Merianne Rose Spencer et al. 2023), suggesting that xylazine-fentanyl coadministration increases the risk of fatal overdose. Illicit use of xylazine in

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North America was first detected in Puerto Rico (Torruella 2011), and quickly became a major adulterant of its heroin supply (Ruiz-Colón et al. 2012). Xylazine use has subsequently been detected in 48 US states and has been detected in over 10% of all overdoses involving fentanyl (Drug Enforcement Agency 2023; Kariisa et al. 2023). Use of xylazine-adulterated fentanyl has further been detected across multiple Canadian provinces (Canada Go 2023; Tobias et al. 2020) and across Europe (Di Trana et al. 2024; Iacopetta et al. 2025; Rock et al. 2023). While xylazine is widely used in veterinary medicine, it is not approved for human use, in whom it induces bradycardia and hypotension (Hoffmann et al. 2001; Ruiz-Colón et al. 2012). Consequently, the effects of xylazine alone or in concert with other drugs of abuse have rarely been investigated in either a clinical or preclinical context (Bedard et al. 2024; Lynch 2025).

Experiments in rodents support the idea that xylazine-fentanyl coadministration increases the lethality of opioid agonists (Acosta-Mares et al. 2023; Smith et al. 2023). However, the mechanisms behind this increase in lethality are poorly understood. Conversely, the lethal effects of exogenous opioid drugs are well-studied, and largely attributable to suppression of breathing (Bateman et al. 2023). Respiratory depression has been observed in human cases of xylazine poisoning (Ayub et al. 2023; Capraro et al. 2001; Gallanosa et al. 1981; Hoffman 2023; Krongvorakul et al. 2018; Liu et al. 2007; Samanta et al. 1990; Spoerke et al. 1986; Velez et al. 2006), suggesting that xylazine-associated increases in opioid lethality may result from exacerbation of opioid-induced suppression of breathing. This possibility is bolstered by observations that xylazine coadministration with fentanyl exacerbates opioid-induced hypoxia in the central nervous system (Choi et al. 2023).

Here we used whole body plethysmography in awake mice to demonstrate that xylazine induces severe respiratory depression at low doses in both male and female mice. Furthermore, we show that the combination of xylazine and fentanyl produces respiratory depression more severe than that induced by fentanyl alone. We demonstrate the pharmacological specificity of xylazine's respiratory effects using the α2AR antagonist atipamezole. We further show that both atipamezole and the MOR antagonist naloxone are unable to reverse the respiratory effects of xylazine-adulterated fentanyl when used alone, while the combination of naloxone with atipamezole was able to rapidly and completely reverse the respiratory suppressive effects of xylazine-adulterated fentanyl.

Materials and methods

Animals

All procedures were approved by the Bloomington Institutional Animal Care and Use Committee of Indiana University. Subjects consisted of 125 male and 36 female C57BL/6 wild type mice (Jackson Laboratories; Bar Harbor, ME) aged 12 weeks at time of testing. Male mice weighed 30–40 g, and female mice weighed 20–30 g prior to testing. Mice were housed in a temperature- and humidity-controlled facility with *ad libitum* access to food and water on a 12:12 h light/dark cycle.

Drugs

Fentanyl citrate and naloxone hydrochloride dihydrate were purchased from Sigma Aldrich (St. Louis, MO). Atipamezole hydrochloride was purchased from Tocris Bioscience (Bristol, UK). Xylazine injectable solution was purchased from Covetrus (Portland, ME). All drugs were administered intraperitoneally in a vehicle of 0.9% saline (Baxter Pharmaceuticals; Bloomington, IN) in a volume of 10 mL/kg.

Assessment of respiratory parameters

Whole body plethysmography (Data Sciences International; St. Paul, MN) was used to assess the respiratory parameters of awake and unrestrained mice as previously described (Watkins et al. 2025). Respiratory metrics evaluated were minute ventilation (total volume of gas exchange, expressed as mL/minute), frequency (breaths per minute), tidal volume (volume of individual breaths in mL), total inspiratory time (ms), total expiratory time (ms), peak inspiratory flow (mL/sec), peak expiratory flow (mL/sec), end inspiratory pause (time between inspiration and expiration; ms), and end expiratory pause (time between respiratory cycles; ms). Data shown are minute ventilation, tidal volume, and respiratory frequency; all other recorded metrics for described experiments are included as supplemental figures.

Mice were habituated to recording chambers within 72 h of initial testing by placing them in recording chambers without experimental manipulation. On the test day, mice received air mixed with 10% CO_2 to standardize hypercapnic responses. Testing consisted of a 50-minute initial recording to establish baseline respiratory parameters, after which mice were briefly removed from test chambers and given an IP injection of drug or vehicle. In experiments involving a single injection, recordings continued for an

additional 60 min without interruption. In experiments involving multiple injections, mice were briefly removed from the chambers after 15 min, administered a second IP injection, and then immediately returned to the chambers for an additional 45 min.

To establish the respiratory effects of xylazine, respiratory parameters were measured before and after a single i.p. injection of xylazine (0.1, 0.3, 1, 3, or 10 mg/kg) in male and female mice ($n = 6–8$ per group). Pharmacological specificity of xylazine's respiratory effects and the role of α2ARs was established by recording respiratory parameters before and after injection of xylazine (3 mg/kg) coadministered with the α2AR antagonist atipamezole (1 mg/kg) in male mice ($n = 4–8$ per group).

To assess the effects of xylazine-fentanyl coadministration, respiratory parameters were measured before and after a single i.p. injection of xylazine (0.1, 0.3, 1, 3, or 10 mg/kg) coadministered with a fixed dose of the synthetic MOR agonist fentanyl (0.2 mg/kg) in both male and female mice ($n = 4–8$ per group). The ability of naloxone (10 mg/kg) and atipamezole (3 mg/kg) to reverse the respiratory effects of fentanyl were evaluated by administering fentanyl (0.2 mg/kg) by i.p. injection to male mice ($n = 4–6$ per group), followed by a second i.p. injection of naloxone or atipamezole after fifteen minutes.

The efficacy of naloxone (10 mg/kg) in reversing the coadministration of fentanyl (0.2 mg/kg) and xylazine (3 mg/kg) was evaluated in male mice ($n = 4–6$ per group) by simultaneously administering xylazine and fentanyl followed by a second i.p. injection of naloxone (10 mg/kg) after fifteen minutes in male mice ($n = 4–6$ per group). We similarly assessed the efficacy of combinations of naloxone (10 mg/kg) and atipamezole (1 mg/kg or 3 mg/kg) in reversing xylazine fentanyl coadministration in male mice ($n = 4–6$ per group).

Data analysis

Statistical analyses were conducted using GraphPad Prism v.10.4 (GraphPad Software; Boston, MA). Plethysmography data were averaged into five-minute bins and represented as mean ± SEM. Two-way repeated measures ANOVA was used to assess the effects of pharmacological manipulations across time and to compare pre-injection baseline respiratory activity between experimental groups. Pre-injection parameters were analyzed to evaluate differences in baseline respiratory activity. Post-injection parameters were analyzed separately from baseline parameters to assess the respiratory effects of administered drugs. Bonferroni's *post hoc* test was used to assess whether pre-injection baseline parameters differed as a function of drug allocation, and whether antagonist conditions differed from active pharmacological manipulations or relevant controls. Dunnett's *post hoc* test was used to compare the effects of active pharmacological manipulations to relevant controls. Dose response curves were generated by taking the difference between the final pre-injection bin and the bin twenty minutes after the first injection (a time point after maximal drug effect and before observed recovery for all drugs evaluated) and fitting a three-parameter logistic regression model using GraphPad Prism.

Model asymptotes were constrained to values below 100% inhibition, as inhibition of respiratory parameters over 100% would represent negative respiratory activity. As respiratory activity generally declined over time in all conditions, reflecting decreases in exploratory and anxiety-like behavior in response to a novel environment, the bottom asymptote was constrained to zero inhibition in experiments in which the relevant comparator was a vehicle control, as the lowest tested doses in each experimental group did not significantly differ from vehicle controls for any tested metric. The bottom asymptote was constrained to experimentally observed values in conditions where the relevant comparator was not a vehicle control (e.g., in evaluating the effects of xylazine-fentanyl coadministration, the bottom asymptote of the model was considered to be the inhibition caused by the fixed fentanyl dose alone). Estimation of confidence intervals for slope of respiratory suppression was calculated by simple linear regression in GraphPad Prism v.10.2.

Results

General results

Respiratory parameters generally showed a modest and expected decline across time, consistent with decreasing exploratory behavior and stress resulting from exposure to a novel environment as mice acclimated to recording chambers ($p < 0.0234$ for all conditions). No group differences in baseline minute ventilation were observed, and no group differences in other dependent measures assessing baseline respiratory activity for other metrics were observed at the point of injection except where specifically noted. No fatalities were observed following any experimental manipulations in any study.

Xylazine suppresses respiratory activity in both male and female mice

The α2AR agonist xylazine, administered systemically, markedly decreased minute ventilation in a dose- and time-dependent manner in both male (Drug: $F_{5,32} = 137.7$,

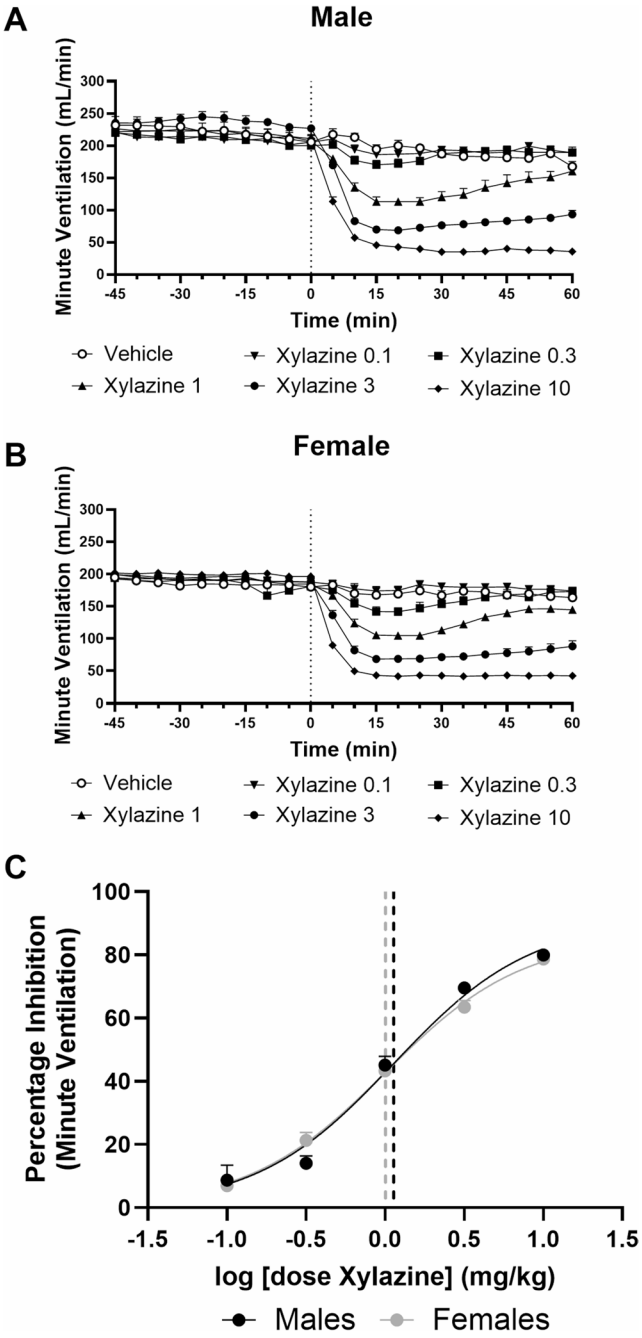


Fig. 1 Xylazine reduces minute ventilation in mice of both sexes. Xylazine reduced minute ventilation in a dose- and time-dependent manner in both (**A**) male and (**B**) female mice. All doses 1 mg/kg and higher depressed minute ventilation in both male and female mice. (**C**) Dose response of xylazine's suppression of minute ventilation in both male and female mice. EC50 (95% CI): Males: 1.133 (0.0854–1.503) mg/kg i.p.; Females: 1.005 (0.8447–1.195) mg/kg i.p. Data were analyzed by two-way ANOVA followed by Dunnett's post hoc test. Data are shown as Mean ± SEM where visible (n = 6–8 per group). Doses are shown as mg/kg, i.p

$p<0.0001$; Interaction: $F_{55,352} = 9.169$, $p<0.0001$) and female (Drug: $F_{5,30} = 109.4$, $p<0.0001$; Interaction: $F_{55,330} = 8.210$, $p<0.0001$) mice (Fig. 1A-B). Xylazine produced similar decreases in minute ventilation in both sexes. *Post hoc* comparisons showed that all doses of xylazine 1 mg/kg and higher depressed minute ventilation in both male and female mice (1–10 mg/kg: $p<0.0001$ for each sex). The EC_{50} (95% Confidence Interval; CI) for xylazine-induced suppression of minute ventilation was 1.133 (0.854–1.503) mg/kg in male mice and 1.005 (0.845–1.195) mg/kg in female mice (Fig. 1C).

Xylazine similarly suppressed respiratory frequency in both male (Drug: $F_{5, 32} = 84.70$, $p<0.0001$; Interaction: $F_{55, 352} = 6.596$, $p<0.0001$) and female (Drug: $F_{5, 30} = 71.16$, $p<0.0001$; Interaction: $F_{55, 330} = 2.826$, $p<0.0001$) mice (Fig. 2A-B). Xylazine additionally suppressed tidal volume in both male (Drug: $F_{5,32} = 71.79$, $p<0.0001$; Interaction: $F_{55,352} = 15.01$, $p<0.0001$) and female (Drug: $F_{5,30} = 63.65$, $p<0.0001$; Interaction: $F_{55,330} = 10.21$, $p<0.0001$) mice (Fig. 2C-D). All doses 1 mg/kg and higher suppressed both frequency (Males: 1–10 mg/kg, $p<0.0001$; Females: 1 mg/kg, $p=0.0001$; 3–10 mg/kg, $p<0.0001$) and tidal volume (Males: 1 mg/kg, $p=0.0002$; 3–10 mg/kg, $p<0.0001$; Females: 1 mg/kg, $p=0.0002$; 3–10 mg/kg, $p<0.0001$) in both male and female mice.

The EC_{50} for xylazine-induced suppression of respiratory frequency was 1.255 (0.900–1.747) mg/kg in male mice and 1.159 (0.766–1.752) mg/kg in female mice (Fig. 2E). The EC_{50} for xylazine-induced suppression of tidal volume was 1.874 (1.284–2.764) mg/kg in male mice and 1.686 (1.333–2.139) mg/kg in female mice (Fig. 2F). In all analyses where dose response curves were generated, the 95% confidence intervals for metrics impacted by xylazine in male and female mice overlapped.

Xylazine suppressed inspiratory flow and expiratory flow while extending inspiratory time, expiratory time, inspiratory pause, and expiratory pause in both male (Supplemental 1 A-F) and female (Supplemental 2 A-F) mice.

Xylazine-induced suppression of respiratory activity is completely prevented by coadministration of the α2AR antagonist atipamezole

The α2AR antagonist atipamezole (1 mg/kg, i.p.) completely blocked the respiratory suppressive effects of a coadministered dose of xylazine (3 mg/kg, i.p.) on minute ventilation (Drug: $F_{3, 20} = 52.58$, $p<0.0001$; Interaction:

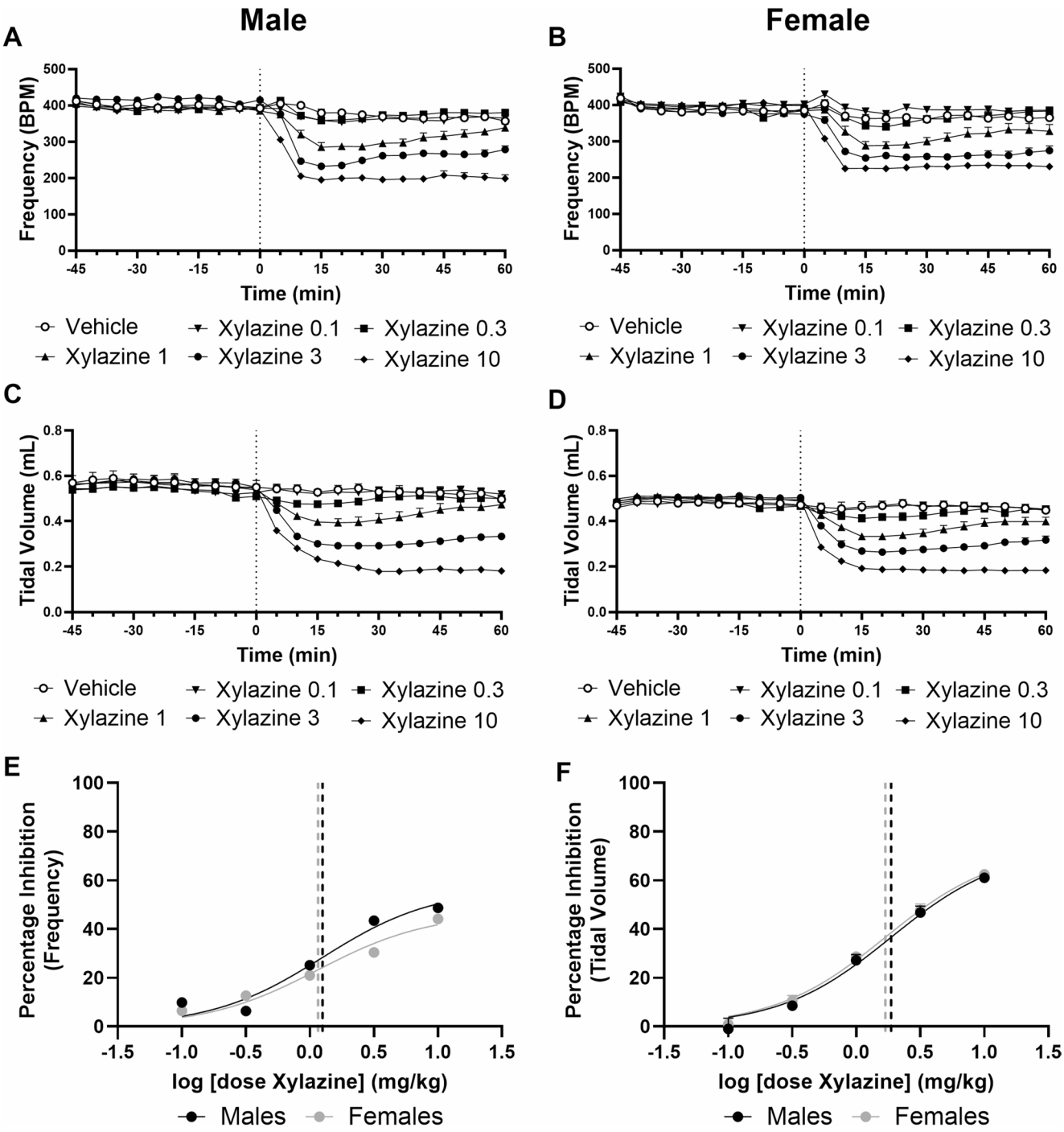


Fig. 2 Xylazine reduced respiratory frequency in both (**A**) male and (**B**) female mice. Doses 1 mg/kg and higher suppressed frequency in both male and female mice. Xylazine similarly reduced tidal volume in a dose- and time-dependent manner in both (**C**) male and (**D**) female mice. All doses 1 mg/kg and higher depressed tidal volume in both male and female mice. (**E**-**F**) Dose response of xylazine's suppression of (**E**) frequency and (**F**) tidal volume in mice of both sexes. EC50 (95% CI) tidal volume: Males: 1.876 (1.284–2.764) mg/kg i.p.; Females: 1.686 (1.333–2.139) mg/kg i.p. EC50 (95% CI) frequency: Males: 1.255 (0.090–1.747) mg/kg i.p.; Females 1.159 (0.766–1.752) mg/kg i.p. Data were analyzed by two-way ANOVA followed by Dunnett's post hoc test. Data are shown as Mean ± SEM where visible (n = 6–8 per group). Doses are shown as mg/kg, i.p

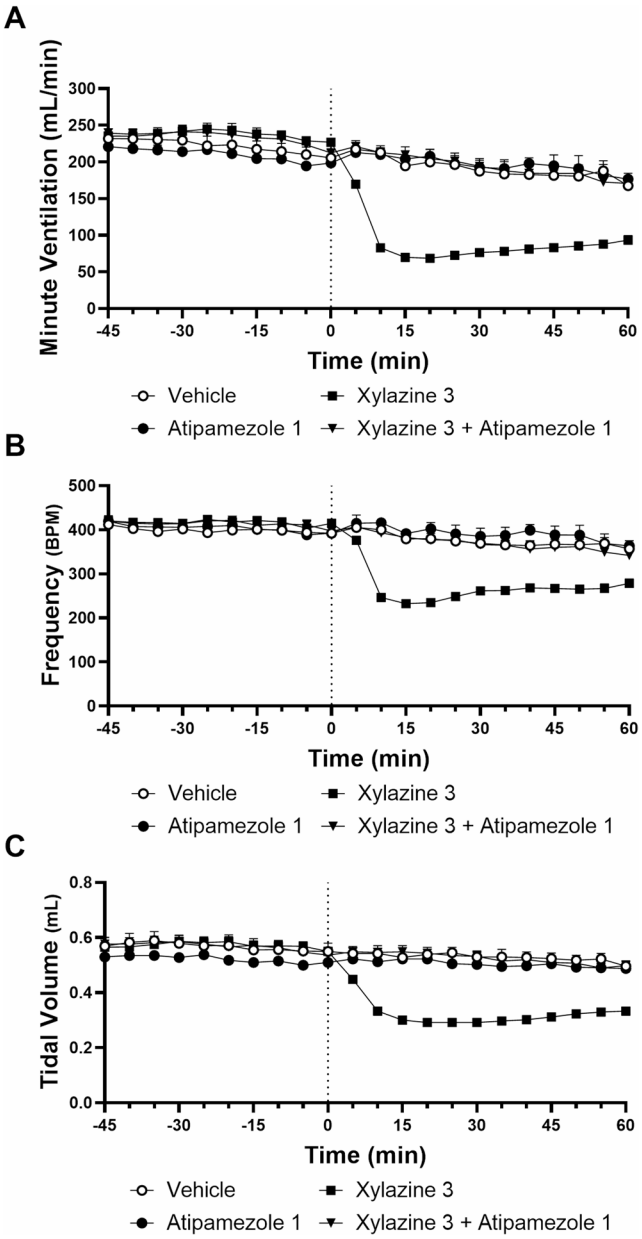


Fig. 3 The α2AR antagonist atipamezole prevents the respiratory effects of xylazine. Coadministration of xylazine and atipamezole completely prevents reduction of (**A**) minute ventilation, (**B**) frequency, and (**C**) tidal volume in male mice. Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. Data are shown as Mean ± SEM where visible (n = 6–8 per group). Doses are shown as mg/kg, i.p

$F_{33, 220}$ = 7.187, p < 0.0001; Fig. 3A), respiratory frequency (Drug: $F_{3, 20}$ = 29.89, p < 0.0001; Interaction: $F_{33, 220}$ = 8.480, p < 0.0001; Fig. 3B), and tidal volume (Drug: $F_{3, 20}$ = 36.63, p < 0.0001; Interaction: $F_{33, 220}$ = 8.450, p < 0.0001; Fig. 3C). Respiratory parameters were lower in groups receiving xylazine (3 mg/kg i.p.) alone compared to all other groups (p < 0.0001 for all metrics). Respiratory parameters of mice injected with only vehicle did not differ from those injected with both xylazine and atipamezole (p > 0.9999 for all metrics; Fig. 3A-C) or atipamezole alone (p > 0.9999 for all metrics; Fig. 3A-C).

Atipamezole similarly blocked reductions of inspiratory flow and expiratory flow and extensions of inspiratory time, expiratory time, and inspiratory pause (Supplemental 3 A-F).

Fentanyl-xylazine coadministration enhances suppression of respiratory activity by fentanyl

Fentanyl (0.2 mg/kg, i.p.) suppressed minute ventilation in the presence and absence of xylazine (0.1–10 mg/kg i.p.) and these effects were both dose- and time-dependent (Drug: $F_{6, 25}$ = 40.80, p < 0.0001; Interaction: $F_{66, 275}$ = 3.603, p < 0.0001; p = 0.0092 vs. vehicle) (Fig. 4A). Fentanyl (0.2 mg/kg, i.p.) suppressed minute ventilation relative to vehicle (p = 0.0092) whereas the same fentanyl dose coadministered with xylazine (1–10 mg/kg, i.p.) produced greater suppression of minute ventilation than fentanyl alone (1 mg/kg: p = 0.0440; 3–10 mg/kg: p < 0.0001; Fig. 4A). Coadministration of lower xylazine doses (0.1–0.3 mg/kg) did not enhance fentanyl-induced suppression of minute ventilation relative to fentanyl alone (p > 0.9999; Fig. 4A).

Fentanyl (0.2 mg/kg, i.p.) also suppressed respiratory frequency (Drug: $F_{6, 25}$ = 48.07, p < 0.0001; Interaction: $F_{66, 275}$ = 3.123, p < 0.0001; p < 0.0001 vs. vehicle; Fig. 4B) in male mice. Doses of xylazine 3 mg/kg and above produced greater suppression of frequency when coadministered with fentanyl than fentanyl produced alone (3–10 mg/kg, p < 0.0001; Fig. 4B). Fentanyl (0.2 mg/kg, i.p.) did not reliably suppress tidal volume (Drug: $F_{6, 25}$ = 23.99, p < 0.0001; Interaction: $F_{66, 275}$ = 8.281, p < 0.0001; p > 0.9999 vs. vehicle; Fig. 4C) in male mice. Doses of xylazine 3 mg/kg and above produced greater suppression of respiratory frequency when coadministered with fentanyl than fentanyl produced alone (3–10 mg/kg, p < 0.0001; Fig. 4C).

The EC_{50} dose for suppression of minute ventilation by xylazine in combination with fentanyl (0.2 mg/kg, i.p.) was 1.393 (0.855–2.292) mg/kg with a slope of 26.48 (22.38–30.57). The EC_{50} dose for xylazine alone was 1.133 (0.854–1.503) mg/kg with a slope of 39.59 (34.90–44.29) (Fig. 4D). The EC_{50} dose for suppression of frequency by xylazine in combination with fentanyl (0.2 mg/kg, i.p.) was 2.080 (1.120 to 4.010) mg/kg with a slope of 17.83 (14.09–21.58). The EC_{50} dose for xylazine alone was 1.255 (0.8999–1.747) mg/kg with a slope of 22.95 (19.45–26.44) (Fig. 4E). The EC_{50} dose for suppression of tidal volume by xylazine in combination with fentanyl (0.2 mg/kg, i.p.) was 1.787 (1.032–3.170) mg/kg with a slope of 25.26 (21.98–29.14). The EC_{50} dose for xylazine alone was 1.874 (1.284–2.764) mg/kg with a slope of 32.45 (28.84–36.06) (Fig. 4F).

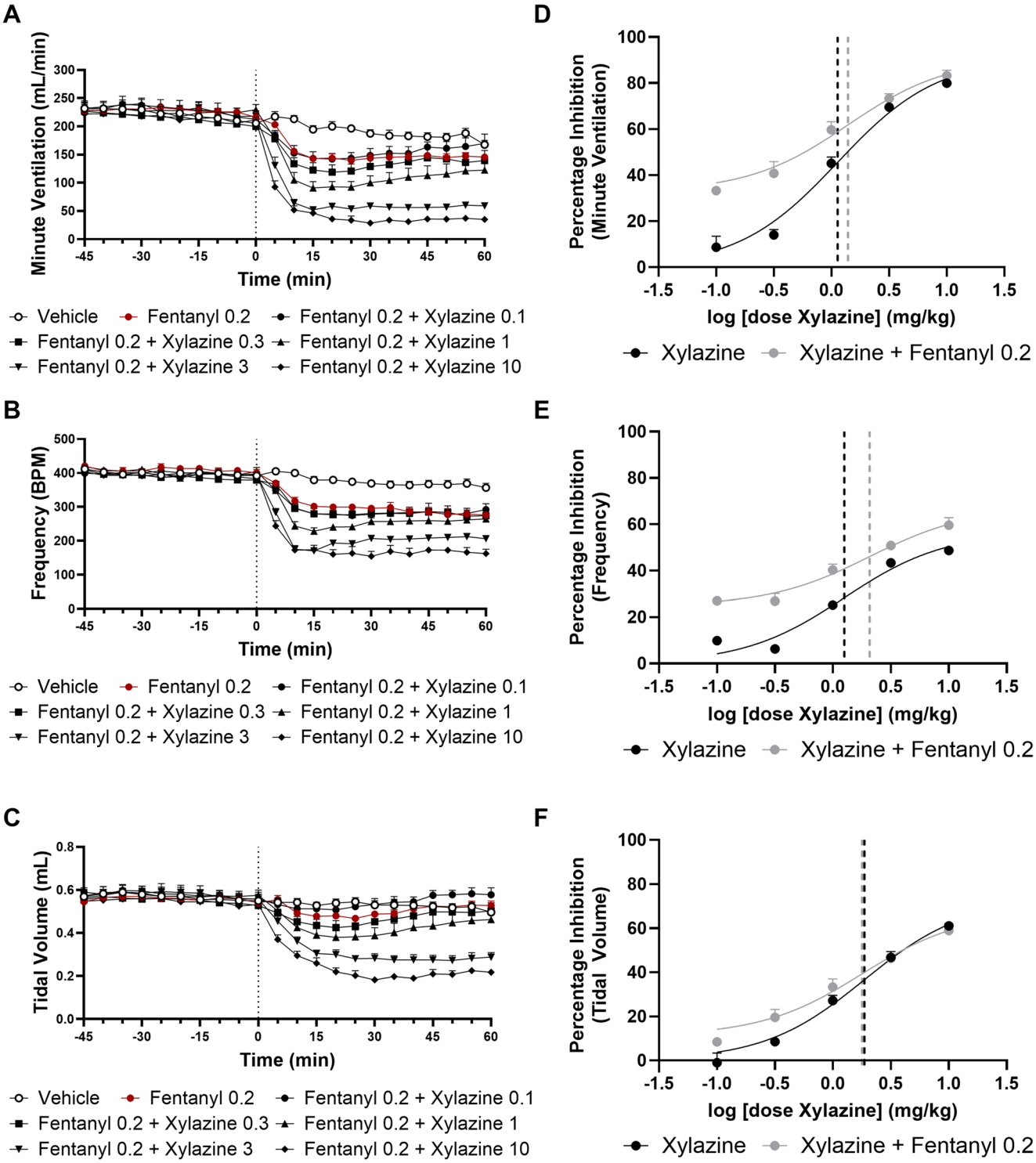


Fig. 4 The combination of xylazine with a dose of the synthetic MOR agonist fentanyl sufficient to produce modest respiratory depression produced greater suppression of (**A**) minute ventilation, (**B**) frequency, and (**C**) tidal volume than fentanyl alone. Minute ventilation was suppressed to a greater degree by xylazine doses 1 mg/kg and above, frequency by doses 3 mg/kg and above, and tidal volume by doses 3 mg/kg and above. (D-F) Dose response of xylazine and xylazine-fentanyl combination in male mice. EC50 (95% CI) xylazine: minute ventilation: 1.133 (0.8542–1.503) mg/kg i.p.; frequency: 1.255 (0.8999–1.747) mg/kg i.p.; tidal volume: 1.874 (1.284–2.764) mg/kg, i.p. EC50 (95% CI) xylazine-fentanyl coadministration minute ventilation: 1.393 (0.8552–2.292) mg/kg i.p.; frequency: 2.080 (1.120 to 4.010) mg/kg, i.p.; tidal volume: 1.787 (1.032 to 3.170) mg/kg, i.p. Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. Data are shown as Mean ± SEM where visible (n = 6–8 per group). Doses are shown as mg/kg, i.p

Coadministration of fentanyl and xylazine similarly suppressed both inspiratory flow and expiratory flow while extending inspiratory time, expiratory time, inspiratory pause, and expiratory pause (Supplemental 4 A-F).

Both naloxone and atipamezole reverse fentanyl-induced respiratory depression

The MOR antagonist naloxone (10 mg/kg, i.p.), administered 15 min after fentanyl (0.2 mg/kg, i.p.), completely reversed the opioid-induced suppression of minute ventilation overall and in a time-dependent manner (Drug: $F_{2, 14}$ = 10.11, p=0.0019; Interaction: $F_{16, 112}$ = 6.627, p<0.0001; Fig. 5A). Minute volume was lower overall in groups that received fentanyl followed by vehicle treatment, compared to groups receiving either fentanyl followed by naloxone rescue (p=0.0217) or vehicle followed by vehicle (p=0.0021). Naloxone rescue completely reversed fentanyl-induced suppression of minute ventilation (p=0.612 vs. vehicle followed by vehicle).

Similarly, fentanyl (0.2 mg/kg i.p.) followed by vehicle challenge reduced respiratory frequency in a naloxone-reversible manner (Drug: $F_{2, 14}$ = 24.43, p<0.0001; Interaction: $F_{16,112}$ = 2.951, p=0.0004; Fig. 5B). Respiratory frequency was lower in groups that received fentanyl followed by vehicle treatment compared to groups receiving fentanyl followed by naloxone rescue (p=0.0005) or vehicle followed by vehicle (p<0.0001). Naloxone rescue completely reversed fentanyl-induced suppression of respiratory frequency (p=0.274 vs. vehicle followed by vehicle). This dose of fentanyl did not suppress tidal volume overall although the interaction was significant (Drug: $F_{2,14}$ = 0.0281, p=0.9724; Interaction: $F_{16,112}$ = 5.221, p<0.0001; Fig. 5C). *Post hoc* comparisons failed to reveal any differences in tidal volume between groups at any post-injection timepoint (p≥0.1043).

The α2AR antagonist atipamezole (3 mg/kg, i.p.) partially reversed the fentanyl-induced suppression of minute ventilation and these effects were time dependent (Drug: $F_{2,12}$ = 13.63, p=0.0008; Interaction: $F_{16,96}$ = 7.019, p<0.0001; Fig. 5D). Minute ventilation was lower overall in groups receiving either fentanyl followed by vehicle (p=0.0007) or fentanyl followed by atipamezole (p=0.0234) compared to the vehicle-vehicle group. Atipamezole partially and transiently reversed fentanyl-induced suppression of minute ventilation at a subset of post-injection timepoints following injection of the α2AR antagonist (5 min: p=0.0230; 10 min: p=0.0440 vs. fentanyl followed by vehicle). Fentanyl-vehicle reduced minute ventilation compared to vehicle-vehicle treatment over fifty minutes after initial injection of fentanyl (p<0.0209) and again at sixty minutes (p=0.0178). Fentanyl-atipamezole treatment showed lower minute ventilation compared to vehicle-vehicle treatment for 45 min following initial injection of fentanyl (p<0.0353) and again at 50 min (p=0.0360) but was no longer present at 55–60 min (p≥0.2307) post-injection.

Atipamezole (3 mg/kg, i.p.) challenge, administered fifteen minutes after fentanyl (0.2 mg/kg, i.p.), also produced a modest but reliable blunting of fentanyl-induced suppression of respiratory frequency and these effects were time-dependent (Drug: $F_{2,12}$ = 27.97, p<0.0001; Interaction: $F_{16,96}$ = 3.649, p<0.0001; Fig. 5E). Respiratory frequency was lower across the observation interval in both the fentanyl-vehicle group (p<0.0001) and the fentanyl-atipamezole group (p=0.0010) compared to the vehicle-vehicle group and the effects of fentanyl-vehicle did not differ from fentanyl-atipamezole treatment overall (p=0.3206). Respiratory frequency was transiently higher in the fentanyl-atipamezole group compared to the fentanyl-vehicle group at a subset of post-injection timepoints (5 min: p=0.0163; 10 min: p=0.0117). By contrast, respiratory frequency was reduced in both the fentanyl-vehicle group (p<0.0001) and the fentanyl-atipamezole group (p≤0.0002) compared to the vehicle-vehicle group at all post-injection timepoints.

In fentanyl-treated groups that were subsequently challenged with either vehicle or atipamezole, tidal volume was not altered overall but the interaction across time was significant (Drug: $F_{2,12}$ = 0.0976, p=0.9077; Interaction $F_{16,96}$ = 5.006, p<0.0001; Fig. 5F). However, *post hoc* comparisons failed to reveal any differences between any groups at any post-injection time point (p≥0.0712 fentanyl-vehicle vs. vehicle-vehicle; p>0.9999 fentanyl-atipamezole vs. vehicle-vehicle; p≥0.5446 fentanyl-vehicle vs. fentanyl-atipamezole).

Naloxone reversed fentanyl-induced suppression of inspiratory flow and extensions of inspiratory time, expiratory time, and inspiratory pause. This dose of fentanyl did not suppress expiratory flow or extend expiratory pause (Supplemental 5 A-F). Atipamezole did not reverse fentanyl-induced suppression of inspiratory flow or expiratory flow, or extensions of inspiratory time, expiratory time, or inspiratory pause (Supplemental 6 A-F).

Combinations of naloxone and atipamezole – but not naloxone alone – reverse respiratory suppression by xylazine-fentanyl coadministration

Fentanyl (0.2 mg/kg, i.p.) coadministered with xylazine (3 mg/kg, i.p.) suppressed minute ventilation in a manner that was preferentially reversed by the combination of naloxone (10 mg/kg., i.p.) and atipamezole (1–3 mg/kg, i.p.) but not by naloxone (10 mg/kg, i.p.) alone and only partially by atipamezole (3 mg/kg) (Drug: $F_{5,23}$ = 36.16, p<0.0001; Interaction: $F_{40,184}$ = 9.499, p<0.0001). *Post*

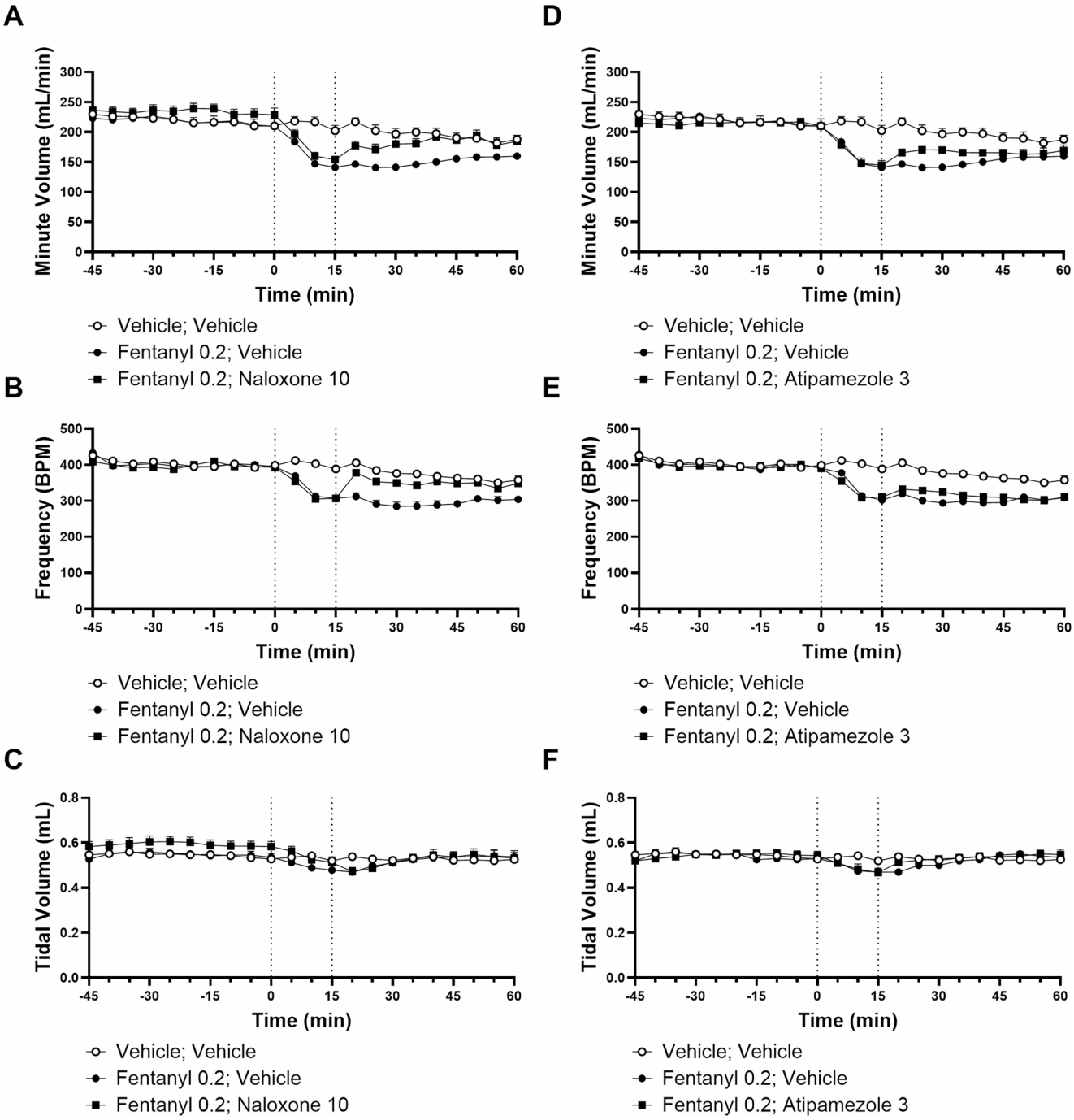


Fig. 5 The MOR antagonist naloxone reverses the respiratory effects of fentanyl. Administration of naloxone fifteen minutes after administration of fentanyl completely reversed fentanyl-induced reduction of (A) minute ventilation and (**B**) frequency. Neither metric differed from mice exposed to vehicle. (**C**) Tidal volume was not altered by this dose of fentanyl, and no group differed from vehicle controls. Atipamezole (3 mg/kg, i.p.), administered fifteen minutes after fentanyl (0.2 mg/kg, i.p.) produced a modest reversal of fentanyl-induced suppression of (**D**) minute ventilation and (**E**) frequency. This reversal was limited to timepoints 10 and 15 min after the injection of atipamezole. This dose of fentanyl did not depress tidal volume (**F**). Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. Data are shown as Mean ± SEM where visible (n = 4–6 per group). Doses are shown as mg/kg, i.p

hoc comparisons revealed that coadministered fentanyl and xylazine reduced minute ventilation overall compared to either vehicle-vehicle treatment ($p < 0.0001$) or the same concentrations of xylazine-adulterated fentanyl that were subsequently challenged with atipamezole (3 mg/kg, i.p.), naloxone (10 mg/kg, i.p.), or a combination of naloxone (10 mg/kg, i.p.) and atipamezole (1 or 3 mg/kg, i.p.; $p < 0.0025$ for each comparison; Fig. 6A).

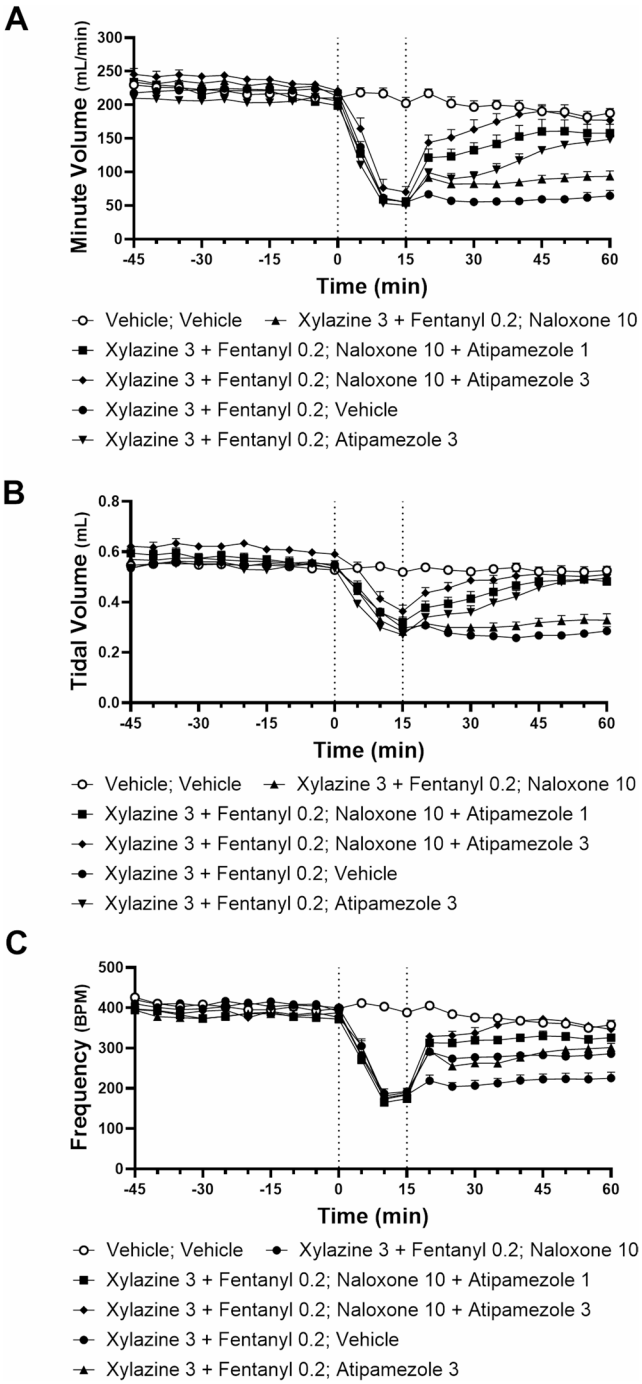


Fig. 6 Naloxone failed to reverse the respiratory effects of xylazine-fentanyl coadministration. Naloxone (10 mg/kg, i.p.), administered fifteen minutes after the injection of a combination of xylazine (3 mg/kg, i.p.) and fentanyl (0.2 mg/kg, i.p.), did not reverse suppression of minute ventilation (**A**). Naloxone produced a modest but consistent increase in frequency (**B**) but failed to return to levels observed in vehicle controls. Naloxone did not reverse suppression of tidal volume (**C**). The combination of atipamezole (1 mg/kg) and naloxone (10 mg/kg) successfully reversed the respiratory effects of xylazine-fentanyl coadministration on (**A**) minute ventilation, (**B**) frequency and (**C**) tidal volume. Minute ventilation and frequency remained suppressed in comparison to vehicle controls, while tidal volume did not differ in comparison to mice receiving only vehicle. The combination of naloxone (10 mg/kg) and atipamezole (3 mg/kg) completely reversed the effects of xylazine-fentanyl coadministration at the same doses. All metrics did not differ from vehicle controls. Atipamezole (3 mg/kg) partially reversed the effects of xylazine-fentanyl coadministration of (**A**) minute ventilation, (**B**) frequency and (**C**) tidal volume but failing to restore levels observed in vehicle controls. Modest differences in baseline values for tidal volume (Drug: F5,23 = 5.584, p = 0.0016; Interaction: F45,207 = 1.212, p = 0.0587) and respiratory frequency (Drug: F5,23 = 5.691, p = 0.0015; Interaction: F45,207 = 1.932, p = 0.0011) were detected between some experimental groups. Post hoc tests revealed differences between mice receiving fentanyl and xylazine followed by naloxone and high dose atipamezole, which displayed higher tidal volume at the timepoint of injection than those receiving vehicle followed by vehicle ($p < 0.0001$). Mice receiving fentanyl and xylazine followed by naloxone and low dose atipamezole displayed higher frequency than those receiving atipamezole alone ($p = 0.0162$) or naloxone combined with low dose of atipamezole ($p = 0.0093$). Data are shown as Mean ± SEM where visible (n = 4–6 per group). Doses are shown as mg/kg, i.p

The combination of naloxone with the high ($p = 0.8549$) but not the low ($p = 0.0042$) dose of atipamezole reversed reductions in minute ventilation induced by xylazine-adulterated fentanyl to levels exhibited by mice receiving only vehicle (Fig. 6A). Notably, xylazine-adulterated fentanyl reduced minute ventilation similarly in groups challenged only with naloxone or with vehicle ($p = 0.4334$), showing the failure of naloxone rescue. Administration of the high dose of atipamezole (3 mg/kg, i.p.) alone induced partial reversal ($p = 0.0025$) but did not restore minute ventilation to levels seen in mice receiving only vehicle ($p < 0.0001$).

Fentanyl (0.2 mg/kg, i.p.) coadministered with xylazine (3 mg/kg, i.p.) suppressed respiratory frequency in a manner that was preferentially reversed by the combination of naloxone (10 mg/kg., i.p.) and atipamezole (1–3 mg/kg, i.p.) but only partially reversed by naloxone (10 mg/kg, i.p.) or atipamezole (3 mg/kg, i.p.) rescue alone (Drug: $F_{5,23} = 53.27$, $p < 0.0001$; Interaction: $F_{40,184} = 3.903$, $p < 0.0001$: Fig. 6B). Xylazine-fentanyl coadministration reduced respiratory frequency relative to vehicle-vehicle treatment ($p < 0.0001$) or groups receiving the same concentration of xylazine-adulterated fentanyl that were subsequently challenged either with atipamezole or naloxone only, or naloxone in combination with either low (1 mg/kg, i.p.) or high (3 mg/kg, i.p.) dose atipamezole ($p < 0.0001$ for each comparison).

Naloxone (10 mg/kg i.p.) challenge increased respiratory frequency in groups receiving xylazine-adulterated fentanyl ($p < 0.0001$) but failed to restore levels to those observed in vehicle controls ($p < 0.0001$) (Fig. 6B). The combination of naloxone with the high ($p > 0.9999$) but not the low ($p = 0.0012$) dose of atipamezole completely reversed the reductions in respiratory frequency induced by xylazine-adulterated fentanyl to levels observed in mice receiving only vehicle. Administration of the high dose of atipamezole

(3 mg/kg, i.p.) alone induced partial reversal ($p=0.0002$) but did not restore minute ventilation to levels seen in mice receiving only vehicle ($p<0.0001$).

Fentanyl (0.2 mg/kg, i.p.) coadministered with xylazine (3 mg/kg, i.p.) suppressed tidal volume in a manner that was preferentially reversed by the combination of naloxone (10 mg/kg., i.p.) and atipamezole (1–3 mg/kg, i.p.) but not by naloxone (10 mg/kg, i.p.) and only partially reversed by atipamezole (3 mg/kg i.p.) rescue alone (Drug: $F_{5,23} = 24.14$, $p<0.0001$; Interaction: $F_{40,184} = 9.652$, $p<0.0001$; Fig. 6C). *Post hoc* comparisons revealed that xylazine-fentanyl coadministration reduced tidal volume overall relative to either vehicle-vehicle treatment, or groups receiving the same concentrations of xylazine-adulterated fentanyl that were subsequently challenged with atipamezole (3 mg/kg, i.p.) or a combination of atipamezole (1 or 3 mg/kg, i.p.) and naloxone ($p<0.0015$ for each comparison).

The combination of naloxone with either the high ($p>0.9999$) or the low ($p=0.0791$) dose of atipamezole reversed reductions in tidal volume induced by xylazine-adulterated fentanyl to levels observed in groups receiving only vehicle (Fig. 6C). Xylazine-adulterated fentanyl reduced tidal volume similarly in groups were challenged with vehicle or naloxone only ($p>0.9999$), showing failure of naloxone rescue (Fig. 6C). Atipamezole (3 mg/kg, i.p.) challenge alone reversed suppression of tidal volume ($p=0.0015$) but did not restore levels to those observed in mice receiving vehicle ($p=0.0239$).

Naloxone (10 mg/kg) rescue partially reversed fentanyl-xylazine coadministration's suppression of inspiratory flow and expiratory flow, and partially reversed elongation of inspiratory time, expiratory time, and inspiratory pause. Low dose (1 mg/kg) atipamezole partially reversed extensions of inspiratory flow and completely reversed reduction of expiratory flow and extension of expiratory flow, inspiratory time, expiratory time, and inspiratory flow. High dose (3 mg/kg) atipamezole completely reversed suppression of inspiratory and expiratory flow and extensions of inspiratory time, expiratory time, and inspiratory pause. Expiratory pause was unaffected by doses of fentanyl and xylazine in these experiments (Supplemental 7 A-F). Administration of vehicle followed by naloxone (10 mg/kg) or vehicle followed by a combination of naloxone and atipamezole (3 mg/kg) did not affect any tested metric (Supplemental 8 A-C; Supplemental 9 A-F).

Discussion

Forensic drug reports increasingly link xylazine, a common veterinary sedative, to lethal fentanyl overdose in humans (Alexander et al. 2022; Cano et al. 2023, 2024; Friedman et al. 2025; Holt et al. 2023; Johnson et al. 2021; Merianne Rose Spencer et al. 2023; Wang et al. 2025; Zhu and Cano 2025). No antidote specific to xylazine-induced lethality exists for human overdose (Kariisa et al. 2021) and it is not clear whether any effective pharmacological tools currently exist to reverse overdoses inflicted by xylazine-adulterated opioids. Here, we examined the effects of xylazine, both alone and in combination with fentanyl, on breathing using whole body plethysmography in adult C57Bl/6 mice of both sexes and explored mechanisms to reverse observed respiratory suppression. Xylazine suppressed breathing on its own and exacerbated fentanyl-induced respiratory depression. Furthermore, a combination of both a MOR and a α2AR antagonist was capable of reversing respiratory suppressive effects of xylazine-adulterated fentanyl, whereas either antagonist alone was ineffective.

Xylazine, at relatively low doses, decreased minute ventilation by suppressing both respiratory frequency and tidal volume and enhanced the efficacy of a low dose of the potent MOR agonist fentanyl in suppressing all three respiratory parameters. Xylazine's respiratory effects were mediated by α2ARs and were prevented by coadministration of the α2AR antagonist atipamezole. Moreover, atipamezole partially reversed fentanyl-induced respiratory depression, albeit with limited efficacy. Most relevant to clinical cases of human overdose, the MOR antagonist naloxone did not reverse respiratory depression induced by xylazine-fentanyl coadministration. By contrast, mixtures of naloxone and atipamezole rapidly (within five minutes of administration) and, ultimately, completely reversed the effects of xylazine-adulterated fentanyl. High dose atipamezole produced only delayed and partial reversal, with meaningful recovery occurring only 20 min after injection.

Xylazine dose-dependently suppressed respiratory activity in both male and female mice at doses lower than those commonly used in anesthesia (Massey and Richerson 2017). Xylazine depressed both respiratory frequency and tidal volume, producing rapid and persistent depression of breathing. These effects were observed at low doses with human relevance (Ruiz-Colón et al. 2014). Furthermore, the combination of fentanyl with doses of xylazine that intrinsically suppressed breathing produced greater suppression of breathing than fentanyl alone, mirroring the use of xylazine as an adulterant to illicit synthetic opioids.

Respiratory effects of xylazine were not sex-specific. Confidence intervals for metrics impacted by xylazine and xylazine-fentanyl coadministration overlapped in our dose response curves, suggesting that drug effects did not differ between sexes. This observation aligns with previous reports that xylazine-induced lethality is generally similar between sexes (Smith et al. 2023).

The sedative effects of xylazine are primarily attributed to activity at α2AR, at which xylazine functions as a

nonselective agonist of all receptor subtypes (Schwartz and Clark 1998). α2ARs are inhibitory Gi/Go-coupled receptors whose endogenous ligands are epinephrine and norepinephrine (Bylund 2007; Lomasney et al. 1991; Wu et al. 2021). These receptors are expressed in both the periphery and central nervous system, including in the brainstem, where breathing is primarily controlled (Mansouri et al. 2001; Ruffolo 1985; Scheinin et al. 1994; Wu et al. 2021). Xylazine's depression of breathing was dependent on α2ARs, and the α2AR antagonist atipamezole completely prevented the effect of xylazine on breathing. Combinations of naloxone and atipamezole reversed the respiratory effects of xylazine-fentanyl coadministration in a subset of tested doses.

Suppression of breathing is observed in cases of human xylazine overdose, which often require mechanical ventilation, and in veterinary use of xylazine-ketamine anesthesia (Ayub et al. 2023; Capraro et al. 2001; Gallanosa et al. 1981; Hoffman 2023; Krongvorakul et al. 2018; Liu et al. 2007; Massey and Richerson 2017; Samanta et al. 1990; Sanford and Colby 1980; Spoerke et al. 1986; Velez et al. 2006). Xylazine doses ranged from 0.73 to 22 mg/kg in a meta-analysis examining case reports of nonlethal human xylazine overdose where respiratory depression was observed (Ruiz-Colón et al. 2014). Similarly, xylazine doses 1 mg/kg and above induce respiratory depression in mice in our experiments.

Clinical literature is similarly replete with case studies in which intravenous naloxone was administered to no apparent effect (Ayub et al. 2023; Ball et al. 2022; Capraro et al. 2001; Duong et al. 2023; Gallanosa et al. 1981; Samanta et al. 1990; Spoerke et al. 1986). Our results align with human clinical reports indicating that naloxone is not an effective reversal agent in cases of xylazine overdose. Respiratory suppression induced by xylazine-adulterated fentanyl was not reversed by naloxone. A dose of naloxone that completely reversed the respiratory suppressive effects of fentanyl alone did not reverse respiratory effects of the same fentanyl dose mixed with xylazine. Only two drugs – naloxone and nalmefene – are approved by the United States Federal Drug Administration for countering respiratory effects of drug-induced overdose (Crystal et al. 2024; Ellison et al. 2024). Both drugs function as competitive antagonists at MOR. However, we show that xylazine-induced respiratory suppression requires α2ARs and antagonism by naloxone was ineffective in reversing xylazine-involved respiratory depression in our studies. It is likely that only the opioid component of xylazine-adulterated fentanyl is affected by naloxone.

It has been suggested (Choi et al. 2023, 2024) that atipamezole may serve as a tool to reverse xylazine-induced lethality. While the ability of atipamezole to reverse the respiratory effects of xylazine in humans has, to our knowledge, never been demonstrated, atipamezole reverses the sedative and cardiovascular effects of α2AR agonists in humans (Karhuvaara et al. 1991) and α2AR antagonists reverse respiratory effects of MOR agonists in rats (Vonhof and Sirén 1991). In our studies, atipamezole blocked xylazine's suppression of breathing, and partially rescued fentanyl-induced respiratory depression. In mice exposed to both xylazine and fentanyl, a combination of atipamezole and naloxone produced rapid and complete reversal of respiratory suppression. The dose of atipamezole required to completely reverse respiratory suppression by fentanyl-xylazine coadministration (3 mg/kg) was higher than that required to prevent respiratory suppression by the same dose of xylazine administered alone (1 mg/kg). Minute ventilation was immediately improved by both the high and low dose of atipamezole in combination with naloxone, and the high dose of atipamezole restored breathing to levels observed in mice exposed to vehicle within 25 min of naloxone-atipamezole administration. Thus, combinations of naloxone and atipamezole may effectively reverse the respiratory effects of xylazine-fentanyl coadministration in cases of human overdose.

In contrast to naloxone, which rarely causes serious adverse effects in human clinical use (Buajordet et al. 2004; Wermeling 2015), atipamezole increases arterial blood pressure and heart rate in humans but appears to be well-tolerated at doses analogous to those required here to fully reverse the effects of xylazine-fentanyl coadministration (Karhuvaara et al. 1990; Penttilä et al. 2004; Pertovaara et al. 2005). Nevertheless, precipitated opioid withdrawal can also induce cardiovascular effects, which may impact the safety profile of interventions involving both naloxone and atipamezole (Buajordet et al. 2004; Wermeling 2015). Given the extensive and lethal nature of human use of adulterated illicit opioids and lack of effective reversal agents, potential benefits of therapeutic use of atipamezole in suspected cases of xylazine overdose may outweigh such risks. We did not observe fatalities in experimental animals but did not evaluate cardiovascular effects of atipamezole-naloxone coadministration. Caution must be exercised in extrapolating these results to humans. To our knowledge, the potential cardiovascular interactions of naloxone-atipamezole coadministration remain unaddressed in current literature.

Functional interactions between MOR and α2ARs have been reported. Synergy and allosteric modulation between the analgesic effects of receptor agonism are implicated in analgesia and antinociception (Chabot-Doré et al. 2015a, b), and the receptors dimerize with functional interactions in cell culture (Vilardaga et al. 2008). Several canonical α2AR agonists show affinity for various opioid receptors (Bedard et al. 2024; Monroe et al. 1991; Root-Bernstein et al. 2018). Furthermore, α2ARs are co-expressed with MORs in brain regions that influence breathing, including

the nucleus tractus solitarius (Glass and Pickel 2002), and play a functional role in regulation of breathing by the retrotrapezoid nucleus (Oliveira et al. 2016) and locus coeruleus (Biancardi et al. 2008). The receptors share a common signaling pathway, both coupling to Gi, and interacting with G protein-coupled inwardly rectifying potassium channels (Bünemann et al. 2001; Tsentsevitsky et al. 2024), a key downstream target of MOR activation implicated in opioid-induced respiratory depression (Levitt et al. 2015; Liang et al. 2018; Montandon et al. 2016), although evidence for this effect is mixed in vivo (Li and Bayliss 1997). Viewed holistically, these interactions suggest potential pathways for the development of pharmacological interventions that simultaneously target respiratory depression induced by both drug classes. Respiratory stimulants agnostic to either receptor may represent a potential strategy to combat polydrug overdose resistant to naloxone reversal (James et al. 2025; van Lemmen et al. 2025). This approach has shown promise in reversing CNS hypoxia inflicted by xylazine-fentanyl coadministration (Miller et al. 2024). More work is necessary to determine the co-localization of α2ARs and MORs within respiratory circuits and the mechanisms underpinning interactions between the respiratory effects of the two receptors.

Xylazine induces CNS hypoxia at doses similar to those that depress respiration in our studies (Choi et al. 2023). However, doses of xylazine that induce respiratory depression in our preclinical studies or in human overdose cases are far lower than those which induce lethality in mice. We did not observe lethality in any experimental animals despite significant suppression of respiratory activity. In studies examining the lethality of xylazine in mice, deaths are not observed until xylazine doses surpassed 60 and 100 mg/kg, i.p. (Acosta-Mares et al. 2023; Smith et al. 2023). While the hypoxic effects of xylazine have been hypothesized (Choi et al. 2023, 2024) to be independent of respiratory depression and to instead result from cerebral vasoconstriction or other well-defined effects of α2AR activity, our results suggest that xylazine-induced CNS hypoxia is a result of respiratory depression, given the close coupling of doses demonstrated to depress breathing in our studies and the doses demonstrated to reduce partial oxygen in the CNS. However, given the disparity between lethal doses of xylazine and doses shown here to elicit extreme respiratory suppression, whether non-respiratory effects of xylazine play a role in promoting xylazine-induced lethality remains to be determined. Alternatively, sustained interruptions of breathing, which we did not measure, may only appear at doses of fentanyl and xylazine significantly higher than those examined here.

In conclusion, xylazine induces severe respiratory depression at subanesthetic doses in awake mice. This effect was dependent on α2ARs and was fully blocked by the coadministration of the α2AR antagonist atipamezole. Xylazine-induced respiratory depression was observed at doses that were significantly lower than those previously shown to increase fentanyl-mediated lethality in mice. We further show that combinations of xylazine with low doses of fentanyl produce severe respiratory depression beyond that produced by either drug alone. Respiratory suppression inflicted by xylazine-fentanyl coadministration was not reversed by naloxone administration. In contrast, combined injection of naloxone and atipamezole fully reversed the respiratory effects of xylazine-fentanyl coadministration. Taken together, suppression of breathing by xylazine may enhance lethality in humans by contributing to the respiratory suppression observed in opioid overdose. Our studies also suggest that widely distributed pharmacological tools used to reverse lethal overdose may be ineffective in cases involving xylazine-adulterated fentanyl, and identity a mechanistic basis by which this suppression of breathing may be successfully reversed.

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Declarations

Clinical trial number Not applicable.

Competing interests The authors declare no competing interests.

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