Xylazine is an agonist at kappa opioid receptors and exhibits sex-specific responses to opioid antagonism

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Abstract

Xylazine is in the unregulated drug supply at increasing rates, usually combined with fentanyl, necessitating understanding of its pharmacology. Despite commentary from politicians, and public health officials, it is unknown how xylazine impacts naloxone efficacy, and. few studies have examined it alone. Here, we examine the impact of xylazine alone and in combination with fentanyl on several behaviors in mice. Surprisingly, naloxone precipitates withdrawal from xylazine and fentanyl/xylazine coadministration, with enhanced sensitivity in females. Further, xylazine is a full agonist at kappa opioid receptors, a potential mechanism for its naloxone sensitivity. Finally, we demonstrate surprising effects of xylazine to kappa opioid antagonism, which are relevant for public health considerations. These data address an ongoing health crisis and will help inform critical policy and healthcare decisions.

One-sentence summary:

We present surprising new insights into xylazine and fentanyl pharmacology with immediate implications for clinical practice and frontline public health.

Keywords

Xylazine; Opioid; Fentanyl; Mouse; Pharmacology

Introduction

Human exposure to the veterinary anesthetic xylazine has been reported intermittently in Spain [1], Germany [2], Canada [3], and the United States [4,5] since the 1970s, often associated with occupational exposure or intentional self-administration. Sustained use of the liquid veterinary formulation for euphoric effect was documented in Puerto Rico starting around 2001 [6], with sporadic detection in seized street drugs first on the east coast of the United States mainland from 2006 onwards, and in California for at least the last 4 years [7,8]. The complex interplay between illicitly manufactured xylazine, heroin, fentanyl, and methamphetamine supply can be traced to power shifts among drug trafficking organizations, exacerbated by international drug control policies; overseas chemical manufacturers have responded to demand for fentanyl alternatives driven by consumer dissatisfaction with the potent opioid [1,6,7,9,10]. Currently, xylazine is predominantly found in powder forms of unregulated street drugs in many (but not all) regions of the United States, and mostly (but not exclusively) with illicitly manufactured fentanyl [6,7,11]. In recognition, the federal government designated "fentanyl adulterated or associated with xylazine" as an emerging drug threat in April 2023 [7]. However, xylazine and fentanyl co-exposure is not an exclusively American phenomenon: the earliest documented xylazine-fentanyl co-ingestion was accidental in a farm-worker in New Zealand in 1984 [12], and a xylazine-fentanyl-heroin overdose death was reported in the United Kingdom in 2023 [13]. Clinical management of human xylazine exposure is made difficult by disfiguring and lingering skin ulcers, a distinctive agitated withdrawal syndrome, and lack of approved antidote or withdrawal support medications [14,15]. Ultimately, the limited

pharmacological understanding of xylazine, in conjunction with the lack of an approved antidote, has hampered effective clinical responses to this emerging threat [16].

Despite having similar sedative effects, fentanyl and xylazine previously have been thought to act on distinct G protein-coupled receptors (GPCRs). Xylazine purportedly acts on the alpha-2-adrenergic receptor (α_2-AR) whereas fentanyl engages mu, kappa, and delta opioid receptors (μOR, κOR, δOR respectively). As xylazine has been increasingly found in the unregulated drug supply, there have been reports of worsened overdoses attributed to mixtures of fentanyl and xylazine [17–19]. A general assumption has been that due to the presence of xylazine, these overdoses are not as responsive to naloxone [10,20], an opioid receptor antagonist used to alleviate respiratory depression induced by opioids. Some evidence has indicated, however, that xylazine may also act on other receptors [21], though it has not been thoroughly tested *in vitro* nor *in vivo* until now.

Preclinical veterinary research has largely focused on xylazine's sedative effects in combination with ketamine [22,23], and few studies have investigated xylazine alone or in the context of reward learning [24–26]. Additionally, these studies did not account for locomotor effects and the potential sedation induced by α_2-AR agonists which could impede learning mechanisms in rodent models. Recently, Khatri et al. found that xylazine depressed fentanyl self-administration in male and female rats [27]. However, α_2-AR agonists (e.g. clonidine), despite being commonly used to treat opioid withdrawal, may have reinforcing potential themselves [28–36]. Here, we sought to better understand the effects of xylazine alone and determine if it alters the fentanyl-withdrawal experience in both male and female C57BL/6J mice.

Results

Identification of non-sedative doses of xylazine

Few studies in mice have investigated the sedative effects of xylazine administered alone (i.e., without the addition of ketamine or other anesthetics) [24]. Because sedation alters locomotor activity and learning, it is necessary to determine a non-sedative dose for use in behavioral experiments. Typically, a 10 mg/kg IP dose of xylazine is used with ketamine for anesthesia [22,23]. Previous studies have tested doses as low as 2.5–3 mg/kg IP [24,25] We tested a lower range of 0, 0.5, 1, and 3 mg/kg xylazine on locomotor behaviors. We found that 3 mg/kg IP xylazine resulted in decreased distance traveled in both males and females compared to saline (males: $p < 0.0001$; females: $p = 0.0003$) and 0.5 mg/kg (males: $p = 0.0011$; females: $p < 0.0001$), and in females compared to 1 mg/kg ($p = 0.0006$; Fig. 1A). A dose of 3 mg/kg IP also decreased the % ambulatory time compared to the other three doses in both males (3 vs. 0: $p < 0.0001$; 3 vs. 0.5: $p < 0.0001$; 3 vs. 1: $p < 0.0001$) and females (3 vs. 0: $p < 0.0001$; 3 vs. 0.5: $p < 0.0001$; 3 vs. 1: $p < 0.0001$; Fig. 1B). In males but not females, the 1 mg/kg dose decreased % time ambulating compared to saline ($p = 0.012$; Fig. 1B). Additionally, none of the doses resulted in a significant reduction in the average velocity for either sex across the full 60 min trial, although there is a trend for interaction between sex and dosage ($p = 0.0915$).

Xylazine's onset of action is estimated to be about 10–15 min and exploratory behavior naturally declines over time due to intrasession habituation. To examine the temporal effects of xylazine on locomotor activity, we further analyzed the data in both 30 min (Fig. 1D–F) and 10 min time bins (Fig. S1). As expected, distance traveled, % time ambulatory, and velocity generally declined across time for both male and female mice at all doses (male: $F_{(3.439, 123.8)}$ = 86.89 $p < 0.0001$; female: F(3.878, 159.0) = 82.40 $p < 0.0001$; Fig. S1A–C). Males and females differed in distance traveled and velocity (Fig 1D and F), possibly due to an interaction among time, sex, and dosage in the % ambulatory time ($F_{(3, 77)}$ = 2.310 p = 0.0829). Males were more sensitive to the sedative effects of xylazine as their locomotor activity took longer to recover to control levels compared to their female counterparts (S1 A-C). These data confirm that xylazine can exert sedative effects at doses as low as 1 mg/kg in male mice and 3 mg/kg in female mice. Our results reveal sex differences in the time course and recovery from the sedative effects of xylazine. In subsequent experiments, we chose to proceed with 0.5 mg/kg xylazine because it was non-sedative in all measures of both sexes (Fig. 1).

Naloxone- and atipamezole-precipitated withdrawal

Withdrawal from reinforcing substances is a critical component of the addiction cycle [37–39] and xylazine withdrawal has been reported to be particularly severe [14,15]. Previously, we and others have used repeated precipitated morphine withdrawal models to demonstrate that somatic symptoms exacerbate across withdrawal sessions and that interrupted opioid exposure drives behavioral and physiological correlates of addiction [40–45]. Our model emphasizes that the experience of exacerbated withdrawal from low to moderate doses of drug promotes physiological and behavioral adaptations. We have shown that this model results in sleep disturbances, and promotes long-lasting sex-dependent behavioral adaptations in both male and female mice over six weeks into forced abstinence [40,46]. Here we adapted our withdrawal model to fentanyl withdrawal and explored if fentanyl/xylazine co-administration would impact the development and severity of the withdrawal syndrome. We hypothesized that xylazine could potentiate withdrawal from fentanyl and thus we chose doses of fentanyl and xylazine that we did not anticipate would result in maximal withdrawal responsivity in an effort to capture potential synergism. Male and female mice were administered (IP) either saline (equivolume), fentanyl (0.1 mg/kg), xylazine (0.5 mg/kg), or a coadministration of fentanyl/xylazine (0.1 and 0.5 mg/kg respectively). Two hours later, mice received an injection of either naloxone (1 mg/kg SC) or atipamezole (1 mg/kg SC, an α_2-AR antagonist used by veterinarians to reverse xylazine anesthesia; Fig. 2A). We report our data both as z-scores (Fig. 2, S2) of withdrawal symptoms to eliminate the weighting of one symptom over others, and as individual behaviors (Fig. S7)

Surprisingly, and in contrast to the conventional concept that 'xylazine is not affected by naloxone', we found that female mice treated exclusively with xylazine demonstrated significant global somatic withdrawal scores (shown as z-scores, $F_{(1,36)}$=10.80, p = 0.0023, individual withdrawal behaviors Fig. S7 [47]) following naloxone administration, which sensitized over three days (Fig. 2B and C). Indeed, across the 3-day paradigm, xylazine withdrawal was of equal severity to fentanyl withdrawal in females (Day 1: $p > 0.9999$, Day

2: $p = 0.9948$, Day 3: $p = 0.9622$; Fig. 2B). Compellingly, female mice showed the most exacerbated somatic withdrawal when fentanyl and xylazine were combined (Day 3: FX vs. F $p = 0.0925$, FX vs. X $p = 0.0652$, FX vs. S $p < 0.0001$; Fig. 2B). Male mice, however, demonstrated the highest withdrawal scores to fentanyl alone (Day 3: F vs. X $p = 0.0026$, F vs. S $p = 0.0002$), and the fentanyl/xylazine coadministration did not alter the degree of withdrawal experienced (F vs. FX $p = 0.9948$; Fig. 2). We also considered that the sexes and treatment groups might experience different types of somatic withdrawal symptoms. To assess this, we plotted the average z-score for each individual behavior on withdrawal day 3 (Fig. 2, individual scores Fig. S7). Interestingly, the most robust withdrawal symptom for both sexes observed was paw tremors in the fentanyl/xylazine coadministration group. Regardless, females exhibited multiple withdrawal behaviors that were enhanced in the fentanyl/xylazine coadministration group as compared to either the fentanyl or xylazine groups. In both sexes, the addition of xylazine decreased the fecal boli count relative to the fentanyl group (Female FN vs XN $p = 0.0258$, Male FN vs. XN $p = 0.0028$; Fig. 2C). Males displayed a more robust increase in escape jumps due to the fentanyl/xylazine coadministration than females (Female FXN vs XN $p = 0.7992$ and vs. SN $p = 0.6186$, Male FXN vs. XN or SN $p = 0.0160$), but females saw increases in wet dog shakes and abnormal posture between fentanyl/xylazine and fentanyl (Female FN vs XN $p = 0.0258$, Male FN vs. XN $p = 0.0028$; Fig. 2C).

We were surprised to observe that atipamezole was able to induce precipitated withdrawal behaviors from animals exposed to fentanyl alone although without significant sensitization across days (Males-Day 1 vs Day 3: $p = 0.0829$; Females-Day 1 vs. Day 3: $p = 0.2232$). Females exhibited similar levels of withdrawal in fentanyl, xylazine, and fentanyl/xylazine groups in response to atipamezole (Fig. 2D). Males showed reduced atipamezole-induced withdrawal overall compared to females. Interestingly males responded similarly to xylazine and fentanyl (Day 3: $p > 0.9999$), but the coadministration of the two attenuated the effects. Finally, female mice, but not males showed withdrawal symptom sensitization to saline-atipamezole over the three days (Fig. 2D).

These data indicate a hyposensitivity of males to xylazine compared to females (at this dose) and that female responses to fentanyl withdrawal can be enhanced by the addition of xylazine. Further, female mice exhibited increased sensitivity to the α_2-AR antagonist in comparison to males, indicating a sex difference in adrenergic systems. Importantly, the atipamezole data did not replicate the results we observed with naloxone (especially in female mice) suggesting that the naloxone mediated effects were not due to displacement of xylazine from α_2-ARs.

Male and female mice exhibit differential c-Fos expression following naloxone-precipitated withdrawal

To begin to probe which brain regions may be differentially activated in the male and female mice following naloxone precipitated withdrawal, we focused on nodes that have been implicated in reward/habit, withdrawal related behaviors, negative reinforcement, and those that contain norepinephrine or receive dense norepinephrine innervation [37]. 75–90 min following naloxone administration on the final day of withdrawal, mice were perfused

for immunohistochemistry. Expression of the immediate early gene, c-Fos was indexed as a measure of activity [48,49]. We analyzed c-Fos levels in brain regions implicated in opioid use disorder that receive input from the locus coeruleus, one of the largest sources of noradrenaline in the brain[50]. Within the regions analyzed, significant differences between treatment groups were observed in the pontine locus coeruleus (LC) region, dorsal bed nucleus of the stria terminalis (dBNST), dorsal medial striatum (DMS), and lateral central nucleus of the amygdala (lCeA) in females, but only in the LC region, DMS, and basolateral amygdala (BLA) in males (Fig. 3).

LC regional c-Fos expression was significantly higher in female mice that received fentanyl alone and xylazine alone compared to female mice that received saline (Fig. 3A). Intriguingly, male mice that received xylazine alone and the fentanyl/xylazine coadministration had significantly higher c-Fos expression than the male mice that received only fentanyl (Fig. 3A). In both sexes, the three treatment groups exhibited significantly higher c-Fos expression than the mice that received saline (Fig. 3).

Female mice displayed significant c-Fos expression differences in a few additional regions of interest. In the dBNST, the female mice that received the coadministration had significantly higher c-Fos expression compared to the female mice that received fentanyl alone. Interestingly, no differences were observed between the saline mice and those that received xylazine, suggesting a dBNST effect that is driven primarily by fentanyl administration (Fig. 3A). Differences in c-Fos expression within the lCeA were also observed in female mice, wherein the coadministration displayed significantly greater c-Fos expression than saline and xylazine groups. In the DMS, the coadministration resulted in increased c-Fos compared to all other female treatment groups and compared to the male coadministration group (Fig. 3A).

In the BLA, males that received fentanyl alone displayed significantly greater c-Fos expression than those that received any other treatment and the coadministration group reduced c-Fos expression to saline levels (Fig. 3A). Fentanyl also increased c-Fos in the DMS of males compared to saline.

Characterization of xylazine pharmacology

Xylazine is canonically believed to be an α_2-AR agonist, though its binding and functional activity at different receptors have not been systematically tested. Because our withdrawal data suggested that xylazine may be targeting other receptors we tested xylazine (10 μM) across a host of common drug targets for radioligand binding activities. Xylazine inhibits radioligand binding by 50 % or more at α_2-ARs, as well as 5-HT$_7$ serotonin receptor (5-HT$_{7A}$R), kappa opioid receptor (κOR), sigma 1 receptor (σ1R), and sigma 2 receptors (σ2R) (Fig. S3). We also tested xylazine in the PRESTO-tango GPCRome screen for potential agonist activity at 320+ human GPCRs. These data indicated xylazine (10 μM) activates α_2-ARs as expected, κOR as well as D2 dopamine receptor (Fig. S4). These binding and functional results at the κOR were validated in additional assays. Xylazine was able to completely displace a radiolabeled κOR agonist ^{3}H-U69593 with a submicromolar binding affinity (Fig. 4A, pK$_i$ = 6.33 ± 0.02, K$_i$ = 0.47 μM). Xylazine showed agonist activity only at the κOR, not the μOR or δOR opioid receptors as shown in concentration response curves

(Fig. S4 D–F). A Gi-GloSensor assay demonstrated that xylazine acts as a full agonist at the κOR with a potency of 1.4 μM (pEC$_{50}$ of 5.86) and was as efficacious (although less potent) as the naturally occurring κOR agonist, salvinorin A (Fig. 4C), as well as a full agonist with a potency of 34 nM at α$_{2A}$-AR as expected (Fig. 4D: pEC$_{50}$ of 7.47). The pK$_i$ values for all competition binding assays can be found in Supplemental Table 1. Potency and efficacy values can be found in Supplemental Table 2. Xylazine was also screened against 97 human kinases in the KINOMEScan™ profiling assays (Eurofins) and results showed that at 10 uM it had little or no inhibitory activity at any tested kinases (supplementary excel sheet).

The major metabolites of xylazine, 3-hydroxy- and 4-hydroxy-xylazine [51,52], were also tested for activity in GPCRome agonist activity screening, and at μOR, δOR, κOR, nociception receptor, and D2R (Figs. 4 and S4,5). Xylazine and both metabolites showed Gi agonist activity at κOR but not the other opioid receptors in these assays. 3-hydroxy-xylazine was as efficacious, though less potent, as xylazine and salvinorin A. 4-hydroxy-xylazine was less efficacious at κOR overall. As these functional assays could over-estimate agonist activity due to the signal amplification nature of the assays, we turned to Bioluminescence Resonance Energy Transfer 2 (BRET2) assays (TRUPATH [53]) to identify potential bias activity among inhibitory G proteins (G$_{i1}$, G$_{oA}$ and G$_z$) and β-arrestin signaling pathways by xylazine and the metabolites. Xylazine and 3-hydroxy-xylazine showed similar activation of κOR, G$_{i1}$ and G$_{oA}$, G$_z$ pathways while 4-hydroxy showed no activity (Fig. 4E, G, I). Xylazine, its metabolites, as well as other relevant adrenergic agonists exhibited agonist activity at α$_{2A}$-AR G$_{i1}$, G$_{oA}$, and G$_z$ (Fig. 4F, H, J). Interestingly, xylazine and the metabolites showed no activity in ß-arrestin 1 (Fig. S5G) or ß-arrestin 2 recruitment (Fig. 4K). Xylazine and the metabolites also had minimal α$_{2A}$-AR ß-arrestin 2 recruitment activity, though slightly more efficacious than at κOR (Fig. 4L). Importantly, these are the first findings to our knowledge indicating xylazine and 3-hydroxy-xylazine are both G protein biased agonists at κOR and α$_{2A}$-AR.

Assessment of potential for therapeutic benefit of κOR-antagonism

Following identification of xylazine as an agonist at the κOR, we tested the ability of the κOR-selective antagonist nor-binaltorphimine (nor-BNI) to mitigate withdrawal symptoms. Given nor-BNI's long lasting effects due to receptor modification/inactivation [54–56], mice were injected with fentanyl, xylazine, the combination, or saline for three consecutive days. On the third day only, mice were injected with nor-BNI and withdrawal was scored for the next 10 min (Fig. 5A). There was a main effect of treatment group regardless of sex (F$_{(3,112)}$=3.614, $p = 0.0155$; Fig. 5C). Additionally, there was a main effect of sex in which female mice exhibited increased withdrawal z-scores compared to males (F$_{(1,112)}$=38.79, $p < 0.0001$; Fig. 5C). These differences were significant in the saline ($p = 0.0019$), fentanyl ($p = 0.0004$), and fentanyl/xylazine ($p = 0.0001$) conditions, but only trending in the xylazine condition ($p = 0.0988$; Fig. 5C). Female fentanyl mice demonstrated a range of different withdrawal behaviors but exhibited increased instances of abnormal posture compared to the xylazine alone females ($p = 0.0262$; Fig. 5D).

Given the sex differences in nor-BNI-precipitated withdrawal, we also investigated the ability of nor-BNI to alter the naloxone-precipitated withdrawal experience (Fig. 5B). We

hypothesized that pretreatment with nor-BNI 7-days prior to drug exposure might mitigate the severity of xylazine withdrawal we previously saw in female mice. Both sexes showed a main effect of day (Females: $F_{(1.563, 26.56)}$ = 4.738, p = 0.0242; Males: $F_{(1.436, 25.85)}$ = 47.05, $p < 0.0001$), while males also showed an interaction of day and pretreatment group ($F_{(2, 36)}$ = 3.823, p = 0.0312; Fig. 5E). Surprisingly, female mice who received nor-BNI 7 days prior to the withdrawal paradigm had higher withdrawal z-scores by day 3 than mice who were pretreated with saline (p = 0.0389; Fig. 5E). Despite showing cumulative differences in withdrawal z-scores, there were no post-hoc differences between pretreatments in specific behaviors (Fig. 5F).

Discussion

Cycles of drug exposure and withdrawal are critical to the development of substance use disorders [37,38]. The increase of xylazine in the North American drug supply in recent years prompts the need to understand how xylazine may interact both alone and in conjunction with fentanyl to alter behavioral and physiological responses. Here, we report the first xylazine dose-response locomotor study in male and female mice as well as the first assessment of adrenergic- and opioid-receptor antagonist-precipitated withdrawal symptoms following, xylazine, fentanyl, and xylazine/fentanyl administration in mice. These experiments show that male and female mice are differentially sensitive to xylazine. We find female mice are less sensitive to the motor-suppressing effects of xylazine contrary to the recent findings in rats reported by Khatri et al. (2023), potentially due to their use of repeated dosing of xylazine or species differences [27]. Using a modified version of our 3-day precipitated withdrawal model [40,41,46], we show xylazine is indeed responsive to naloxone, contrary to common assumptions made by both health professionals and in the media [7]. Both sexes exhibited some level of somatic withdrawal behaviors to xylazine and naloxone, though females showed sensitized behavioral responding. Indeed, females appear to be as sensitive, if not more sensitive to xylazine withdrawal than fentanyl withdrawal at tested doses, while males remain much more responsive to fentanyl withdrawal conditions. At the doses tested in our study, the effect of naloxone precipitated withdrawal on xylazine/fentanyl combination was synergistic as compared to each drug in isolation. This was especially apparent when examining increased bouts of paw tremors, which may represent a more passive coping behavior that we have previously observed is sexually dimorphic in opioid withdrawal [41]. In contrast, we did not observe similar findings when withdrawal was precipitated by atipamezole, an α_2-AR antagonist used anesthesia reversal in veterinary medicine. These intriguing findings led us to consider the possibility of direct xylazine activity on opioid receptors. Previous studies have shown that xylazine is antinociceptive, results in a cross-tolerance to some mechanisms of opioid induced antinociception, and that these effects are naloxone-sensitive, but surprisingly not sensitive to the κOR selective antagonist nor-BNI [57–60]. Congruent with this data, we did not observe significant expression of withdrawal behavior to nor-BNI precipitated withdrawal, and pretreatment with nor-BNI exacerbated naloxone precipitated withdrawal in female mice. Until now, xylazine was thought to exert these effects through promotion of endogenous opioid release and xylazine has not been directly tested as a potential opioid agonist. We are the first to

report definitive evidence that xylazine acts as a full agonist at κOR and is biased towards G-protein signaling pathways.

Xylazine has complex pharmacological targets

The synergism of xylazine and fentanyl on withdrawal behavior in female mice is intriguing also, because we and others have previously shown that α_2-ARs are subject to dysregulation by opioid administration [42,61]. As norepinephrine (NE) in the ventral noradrenergic bundle is critical for opioid reward learning [62], the activation of these critical circuits by both opioids and xylazine are targets for future experiments. These data, along with others [61,63], strongly suggest that there is extensive crosstalk between the α_2-AR and opioid receptor systems [42]. Because of this, it is critical to understand how, and if, effects are compounded when agonists target both α_2-AR and opioid receptors simultaneously. Indeed, recent studies examining hypoxia have demonstrated that combined treatment with atipamezole and naloxone reduces the prolonged oxygen deprivation induced by xylazine/ fentanyl administration [64]. In contrast, only naloxone, and not the α_2-AR antagonist yohimbine, prevented fatal overdose by the combination [65]. Furthermore, we identified that sigma receptors are also impacted by xylazine. These intracellular receptors are known to complex with both opioid receptors and the dopamine transporter. Understanding how sigma receptors may compound with κORs in critical brain circuits for reward and withdrawal will be important to understand the impact of xylazine on addictive behaviors. When we tested the ability of atipamezole to evoke somatic withdrawal behaviors akin to precipitated opioid withdrawal, we found that again females were more responsive this manipulation, even showing withdrawal sensitization over days to saline-atipamezole alone. Despite potential differences in responsivity, we found that neither sex was sedated or showed decreased ambulatory activity at the selected dose of 0.5 mg/kg xylazine. Given evidence in the human population that women experience exacerbated withdrawal symptoms [66], and that female rats self-administer higher levels of fentanyl [67], future studies should consider the influence of sex differences on adrenergic and opioid system interactions.

Sex as a biological variable in adrenergic and opioid systems

Both the adrenergic systems and the κOR system are known to have sex differences in rodent models [68–71]. In our study we found sex differences in locomotion, precipitated withdrawal behavior, and in immediate early gene activation by withdrawal. Female rats are less sensitive to the depressive effects, and show differential c-Fos activation in the dBNST to κOR agonism when compared with male rats [72]. Here we also found that withdrawal from fentanyl/xylazine coadministration increased c-Fos in the dBNST of female but not male mice. It would be interesting to know which cell types in the dBNST were activated by each of these treatments. In clinical reports, women tend to report enhanced analgesia from mixed κOR/μOR agonists, while rodent models show males with enhanced analgesia to κOR agonism [73,74]. These differences may be partially explained by the melanocortin 1 receptor gene and sex differences as related to α-melanocyte-stimulating-- hormone (α-MSH) release via κOR dependent mechanisms [75,76]. Here we demonstrated sex differences in responsivity to the κOR-antagonist, nor-BNI. Females showed significant withdrawal symptoms following nor-BNI injections under multiple treatment conditions, indicating an engagement of the kappa system that was not seen in males to the same degree.

Pretreatment with nor-BNI resulted in exacerbated naloxone-precipitated withdrawal from xylazine in females, but not males. These results indicate that κOR-antagonism might be a beneficial addition to overdose and/or recovery treatments for some people, but could make withdrawal worse in others, promoting increased opioid administration due to negative reinforcement. Future studies will need to examine this circuitry with a more focused lens to determine the role of κOR and the adrenergic systems in mediating the response to fentanyl, xylazine and in combination.

Contextualizing our findings in the current public health emergency

Our findings carry important clinical and public health implications. Considering that xylazine is a full κOR agonist, we note two prominent historical and international examples of non-medical use of the κOR agonist pentazocine: the "Ts and Blues" (pentazocine and tripelennamine) outbreak in the midwestern United States from 1977 to 1981 [77–79], and pentazocine injection in Nigeria [80] and India [81–84]. In both settings, characteristic skin lesions beyond the site of injection, eschar formation, and wound cratering were observed [85–90], with morphological similarity [91] to reports involving xylazine from Puerto Rico [92], the Philadelphia area [9,93], and New Haven, Connecticut [94]. κOR distribution in human skin has led to its study as a therapeutic target [95–97], suggesting new directions for research into wound etiology. Separately, withdrawal symptoms specific to pentazocine include heightened anxiety, agitation, and paranoia [98]; these are also cited by clinicians and people who use drugs to be distinguishing presentations of xylazine withdrawal, increasing the difficulty of initiating medication assisted therapy for opioid dependence [14,99,100]. Further investigations are needed to establish if similarities to skin ulcers and withdrawal are coincidental or may be mediated in part by κOR. It is also worth noting that pentazocine, akin to xylazine, also targets sigma receptors. In addition, existing human pharmaceutical κOR agonists (pentazocine, butorphanol, nalbuphine) could be investigated immediately to alleviate xylazine withdrawal, which is difficult to manage in clinical settings [7]. Dexmedetomidine, another α_2-AR agonist approved for human use for other indications in the United States, could logically be considered a candidate medication to be investigated for its potential to manage xylazine withdrawal, although it would be considered off-label at the current time. (Note: we are not endorsing off-label use.) Current public health and harm reduction messaging makes claims that naloxone is ineffective in reversing the effects of xylazine [7,101]. This is problematic because this messaging may lead people to not use naloxone in an overdose scenario when xylazine is suspected in conjunction with fentanyl. In mice, we found that xylazine is responsive to naloxone both in cases where xylazine is administered alone, and in combination with fentanyl. While our findings do not address if xylazine impacts opioid-induced respiratory depression, nor if the presence of xylazine mediates naloxone's ability to rescue opioid-induced respiratory depression, they do suggest that more nuanced health messaging is warranted in community-based naloxone distribution settings. Opioid-induced respiratory depression is thought to be due to activity at μOR [102]. Our current data suggest a lack of direct xylazine activity at μOR, however, it is possible that although it has no direct effect on opioid-induced respiratory depression, crosstalk between the two systems or allosteric modulation of μOR might still play a role. The U.S. has seen a recent increase of overdose deaths in which xylazine was identified as contributory to death [103]; however attribution of causation by

medical examiners is inconsistent in practice, and many states do not assay for or report xylazine when present in overdose [18]. Our findings suggest the urgent need to understand the mechanisms by which xylazine may be implicated in opioid-related overdose, with implications for reporting by forensic medical toxicologists. Our work, and others, seeks to bridge the gap in translatability to continue to provide meaningful animal models of contaminants in the drug supply, providing physicians and regulatory agencies with data to make rapid and effective decisions for public health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Data availability

Data will be made available on request.

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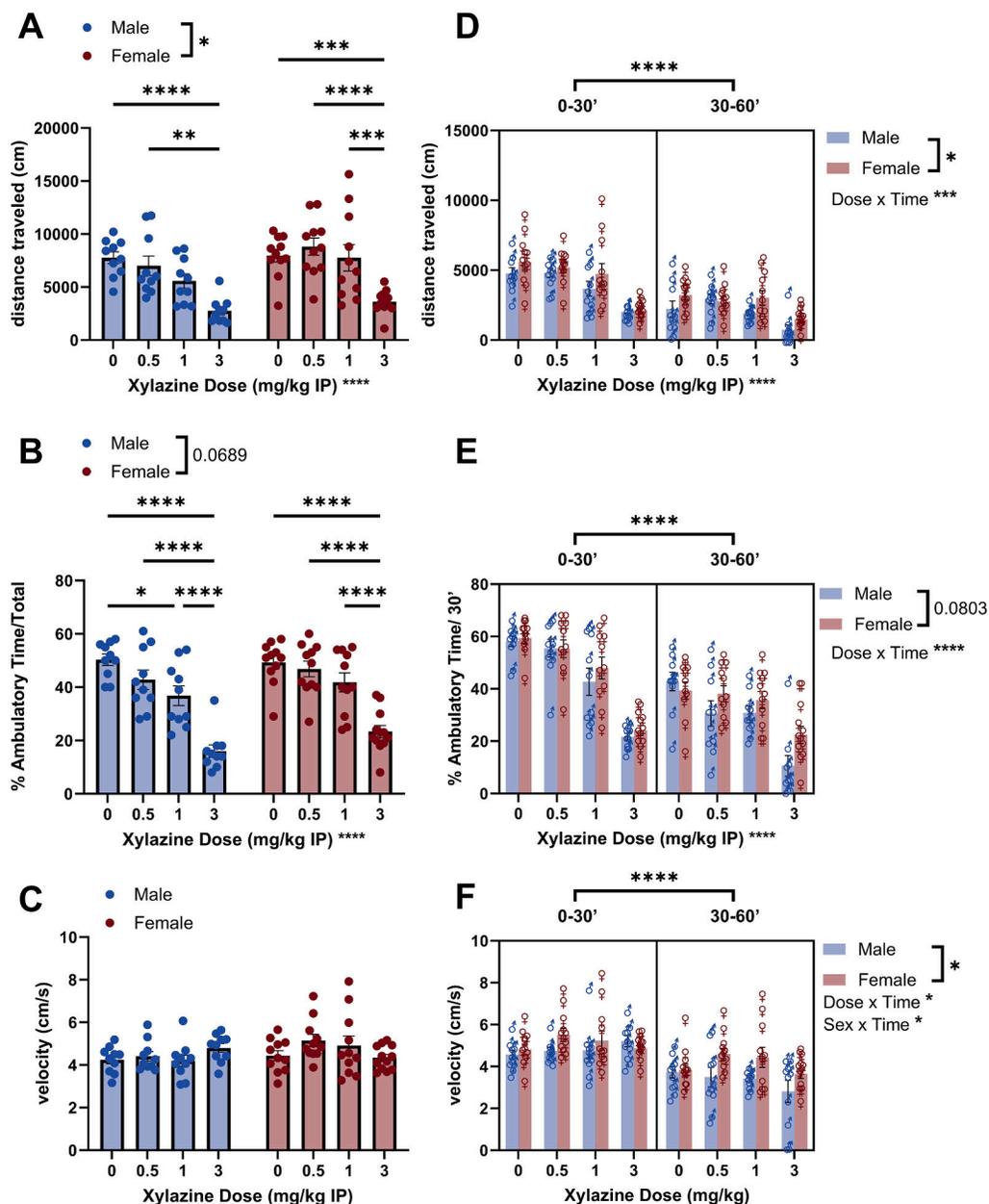
Fig. 1.

Effect of acute IP xylazine administration on locomotor activity. (A) Cumulative distance traveled, (B) % ambulatory time, and (C) average velocity of male and female mice administered saline or xylazine (0.5, 1.0, or 3.0 mg/kg). (D) Distance traveled, (E) % ambulatory time, and (F) velocity split into 30 min bins. (D-F) 3-way ANOVAs (Time x Sex x Dose).
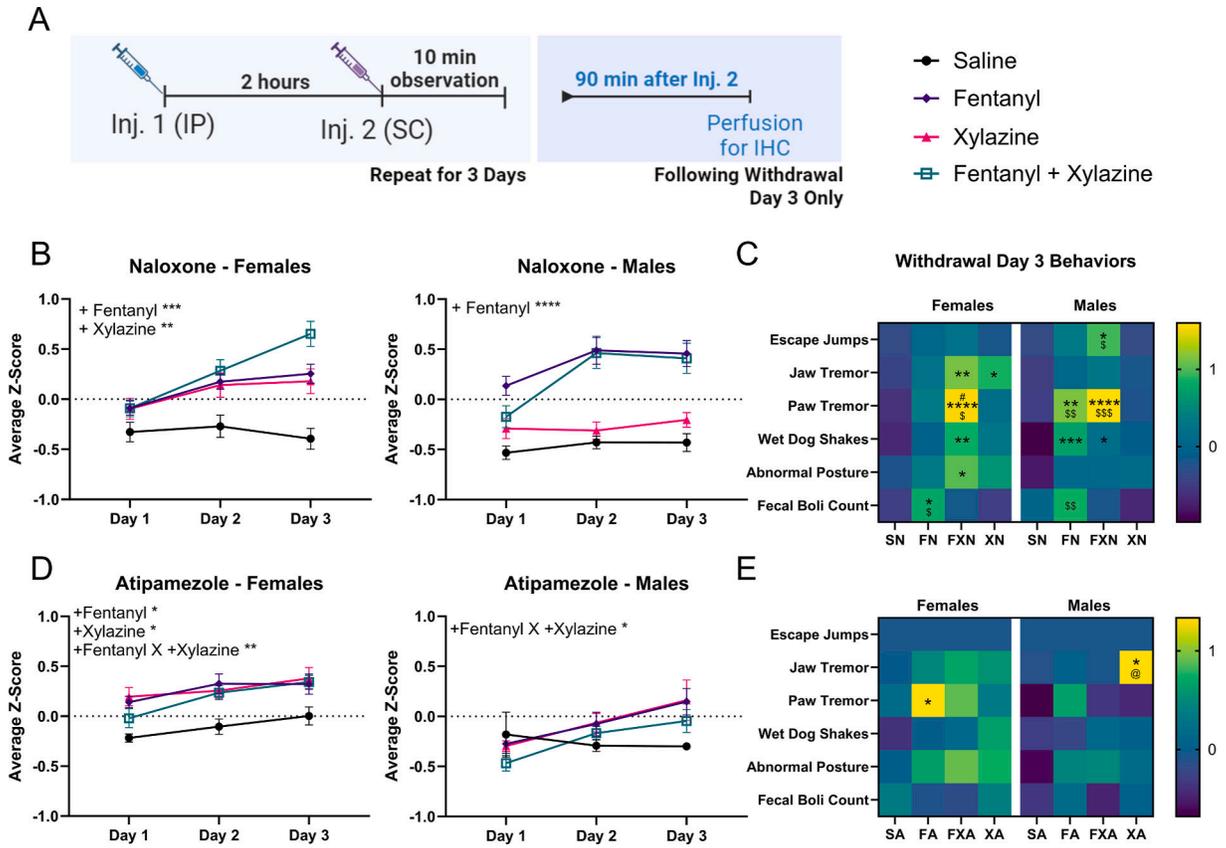
Fig. 2.

Naloxone- and atipamezole-precipitated withdrawal. (A) 3-day precipitated withdrawal paradigm. Global scores are shown as average z-score ± SEM. (B) Average z-scores of female and male mice over three days of naloxone-precipitated withdrawal. (C) Heatmap of average z-scores on day three of withdrawal for individual behaviors. (D) Average z-scores of female and male mice over three days of atipamezole-precipitated withdrawal. (E) Heatmap of average z-scores on day three of atipamezole-precipitated withdrawal for individual behaviors. (B&D) 3-way ANOVAs (Day X Addition of Fentanyl X Addition of Xylazine) $P = 0.05^*$, 0.01^{**}, 0.001^{***}, 0.0001^{****}. Main effects p-values and Tukey's post-hoc shown in Fig. S2. (C&E) 2-way ANOVAs (Tx Group X Behavior) with Tukey's post-hoc, $P = 0.05$ where * (vs. saline), # (vs. fentanyl), @ (vs. fentanyl/xylazine), and $ (vs. xylazine). SN=saline-naloxone; FN=fentanyl-naloxone; FXN=fentanyl/xylazine-naloxone; XN=xylazine-naloxone; SA=saline-atipamezole; FA=fentanyl-atipamezole; FXA=fentanyl/xylazine-atipamezole; XA=xylazine-atipamezole.
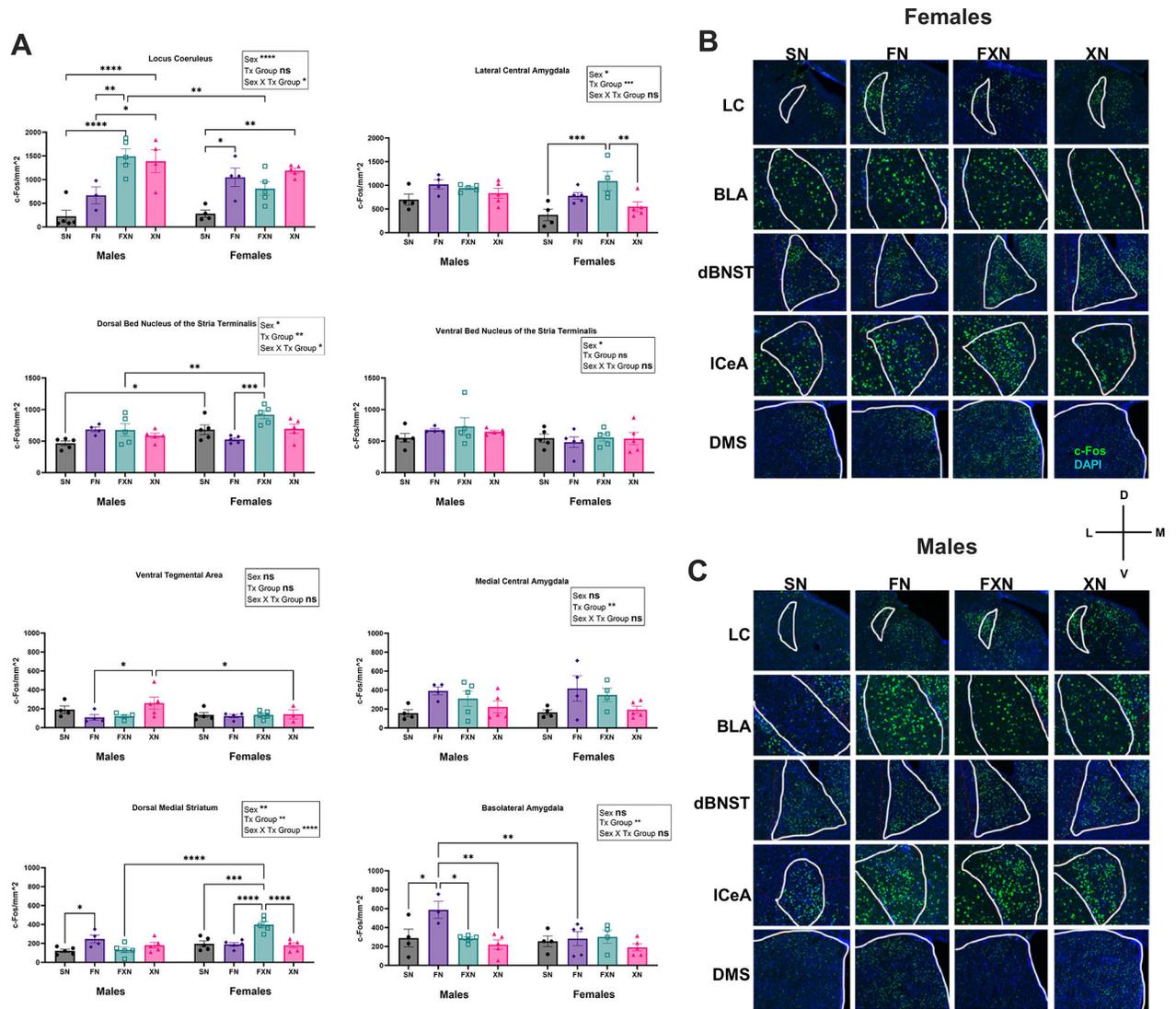
Fig. 3.
Quantification of c-Fos expression following naloxone-precipitated withdrawal. (A) Female and male c-Fos expression displayed as number of positive cells per mm2 in various regions of interest. (B) Representative images of female regions of interest. (C) Representative images of male regions of interests. (A) 2-way ANOVAs (Tx group X Sex) with Tukey's post-hoc. P= 0.05*, 0.01**, 0.001***, 0.0001****.
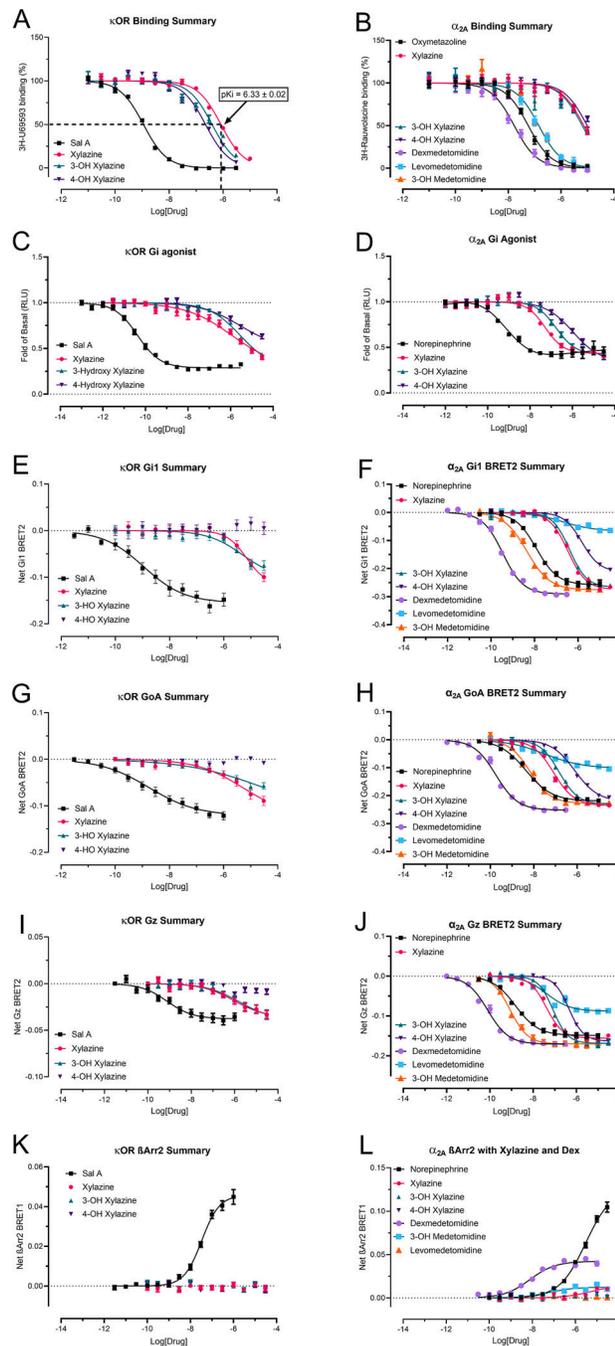
Fig. 4.

Xylazine acts as a G-protein biased agonist at κOR and α$_{2A}$-AR. (A-B) Radioligand competitive binding assay confirms xylazine activity at κOR (A) and α$_2$-AR (B), shown with known reference agonists. (C-D) Gi-GloSensor cAMP assays at κOR (C) and α$_2$-AR (D). (E-L) TRUPATH BRET2 assays for Gi1 (κOR (E) and α$_2$-AR (F)), GoA (κOR (G) and α$_2$-AR (H)), Gz (κOR (I) and α$_2$-AR (J)), and Barr2 (κOR (K) and α$_2$-AR (L)).
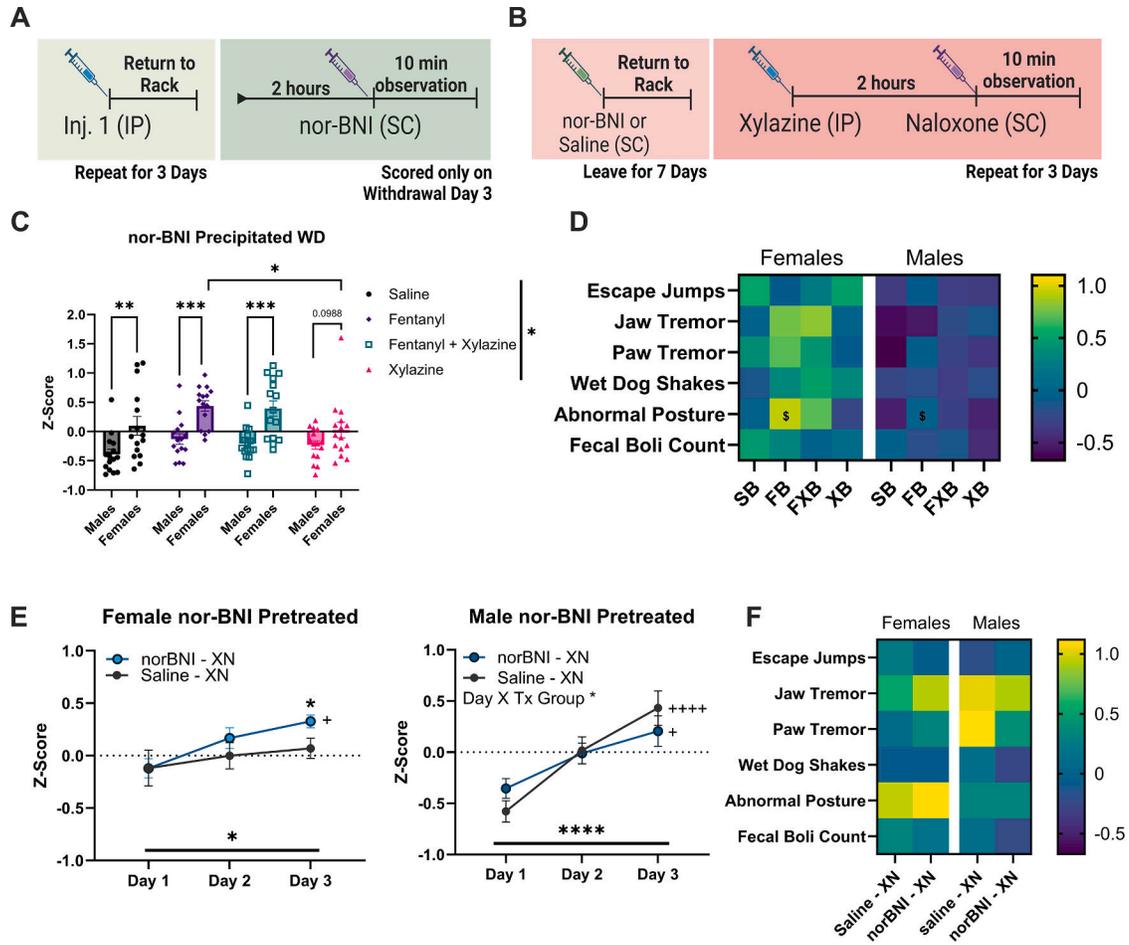
Fig. 5.
Female mice exhibit increased responses to kOR antagonism. (A) nor-BNI-precipitated withdrawal paradigm. Mice received agonist injections for 3 days and nor-BNI 2 h later only on the 3rd day. (B) nor-BNI pretreated withdrawal paradigm. Mice received nor-BNI or saline 7 days prior to xylazine and naloxone-precipitated withdrawal. (C) Average z-score of nor-BNI precipitated withdrawal for female and male mice. 2-way ANOVA (Treatment Group X Sex) (D) Average z-score of nor-BNI pretreated naloxone withdrawal for individual behaviors. (E) Average z-score of nor-BNI pretreated naloxone withdrawal for female and male mice across 3 days. 2-way ANOVAs (Day X Pretreatment Condition). Tukey's post-hoc Day 1 vs Day 3: $P = P = 0.05^{+}, 0.01^{++}, 0.001^{+++}, 0.0001^{++++}$. (F) Average z-score of nor-BNI pretreated naloxone withdrawal for individual behaviors on day 3. $P = 0.05^{*}, 0.01^{**}, 0.001^{***}, 0.0001^{****}$. Main effects p-values and Tukey's post-hoc shown in Fig. S6. (D & F) 2-way ANOVAs (Tx Group X Behavior) with Tukey's post-hoc, $P = 0.05$ where * (vs. saline), # (vs. fentanyl), @ (vs. fentanyl/xylazine), and $ (vs. xylazine). XN=xylazine-naloxone; SB=saline-nor-BNI; FB=fentanyl-nor-BNI; FXB=fentanyl/xylazine-nor-BNI; XB=xylazine-nor-BNI.