American College of Neuropsychopharmacology

www.nature.com/npp

ARTICLE

Check for updates

Combined treatment with naloxone and the alpha2 adrenoceptor antagonist atipamezole reversed brain hypoxia induced by a fentanyl-xylazine mixture in a rat model

Shinbe Choi[1], Matthew R. Irwin[1], Michael R. Noya[1], Yavin Shaham[1] and Eugene A. Kiyatkin[1]✉

Xylazine, a veterinary tranquillizer known by drug users as "Tranq", is being increasingly detected in people who overdose on opioid drugs, indicating enhanced health risk of fentanyl-xylazine mixtures. We recently found that xylazine potentiates fentanyl- and heroin-induced brain hypoxia and eliminates the rebound-like post-hypoxic oxygen increases. Here, we used oxygen sensors coupled with high-speed amperometry in rats of both sexes to explore the treatment potential of naloxone plus atipamezole, a selective α2-adrenoceptor antagonist, in reversing brain (nucleus accumbens) and periphery (subcutaneous space) hypoxia induced by a fentanyl-xylazine mixture. Pretreatment with naloxone (0.2 mg/kg, IV) fully blocked brain and peripheral hypoxia induced by fentanyl (20 µg/kg, IV), but only partially decreased hypoxia induced by a fentanyl-xylazine mixture. Pretreatment with atipamezole (0.25 mg/kg, IV) fully blocked the hypoxic effects of xylazine (1.0 mg/kg, IV), but not fentanyl. Pretreatment with atipamezole + naloxone was more potent than naloxone alone in blocking the hypoxic effects of the fentanyl-xylazine mixture. Both naloxone and naloxone + atipamezole, delivered at the peak of brain hypoxia (3 min post fentanyl-xylazine exposure), reversed the rapid initial brain hypoxia, but only naloxone + atipamezole decreased the prolonged weaker hypoxia. There were no sex differences in the effects of the different drugs and their combinations on brain and peripheral oxygen responses. Results indicate that combined treatment with naloxone and atipamezole is more effective than naloxone alone in reversing the hypoxic effects of fentanyl-xylazine mixtures. Naloxone + atipamezole treatment should be considered in preventing overdoses induced by fentanyl-xylazine mixtures in humans.

Neuropsychopharmacology; https://doi.org/10.1038/s41386-023-01782-2

INTRODUCTION

The illicit use of fentanyl and other structurally similar potent synthetic opioids is a serious health issue marked by the progressive rise of overdose-induced deaths [1–3]. Fentanyl rapidly reaches the brain and induces multiple physiological effects, including respiratory depression followed by brain hypoxia, the primary cause of overdose-induced death [4–6]. The opioid antagonist naloxone is the primary tool to reverse respiratory depression induced by opioid drugs [7–9]. However, this therapeutic strategy is often less effective with fentanyl [10, 11]. Insufficient dose is thought to be the primary factor limiting the therapeutic effectiveness of naloxone [4, 9–11], but the delay between appearance of overdose symptoms and initiation of naloxone treatment is another critical factor [12].

In real-life conditions, pure fentanyl is rarely used alone and is typically co-used with other drugs like benzodiazepines, ketamine, alcohol, and cocaine [13]. More recently, xylazine, a veterinary tranquilizer used for general anesthesia in non-human animals [14], has been found in drug samples obtained from fentanyl overdosed patients [3, 15]. Xylazine is an agonist of α-2 adrenoceptors, and it induces dose-dependent sedation, hypotension, bradycardia, and muscle relaxation, depressing vital functions at higher doses [16, 17]. Xylazine does not bind to opioid receptors but shares many common physiological effects with opioids, resulting in potentiation of opioid-induced sedative and hypoxic effects, posing a greater risk of overdose-induced death from fentanyl-xylazine mixtures.

In a recent electrochemical study in male rats, we examined real-time changes in brain oxygenation induced by xylazine alone and together with fentanyl and heroin [18]. We found that intravenous (IV) injections of low doses of xylazine induce weak and tonic decreases in brain oxygen levels. However, when injected together with IV fentanyl or heroin, it prolonged the hypoxic effect of the opioid drugs.

The goal of the present study was to explore a potential pharmacological treatment to prevent the severe brain hypoxia induced by the fentanyl-xylazine mixture. Using oxygen sensors coupled with high-speed amperometry, we simultaneously assessed drug-induced changes in oxygen levels in the brain and subcutaneous (SC) space and determined whether a combined treatment with naloxone and atipamezole, a selective α2-adrenoceptor antagonist, will be more effective than naloxone alone in reversing this hypoxia. Brain oxygenation is a functionally important parameter that affects the health and survival of neural

[1]Behavioral Neuroscience Branch, National Institute on Drug Abuse – Intramural Research Program, National Institutes of Health, DHHS, Baltimore, MD 21224, USA.
✉email: ekiyatki@intra.nida.nih.gov

Received: 24 July 2023 Revised: 3 November 2023 Accepted: 28 November 2023
Published online: 20 December 2023

cells, and simultaneous assessment of drug-induced oxygen responses in the periphery makes it possible to examine the relationships between central and peripheral oxygen dynamics and understand the mechanisms underlying brain oxygen responses [19]. As in our previous studies, the nucleus accumbens (NAc), a brain area involved in sensorimotor integration and functioning of motivation-reinforcement circuits [20–22], was the recording brain site.

MATERIALS AND METHODS

Subjects

This study was conducted in 18 adult Long-Evans rats (Charles River Laboratories) of both sexes. Male rats ($n = 12$) weighed 450 ± 40 g and female rats ($n = 6$) weighed 375 ± 25 g at the time of surgery. Rats were individually housed in a climate-controlled animal colony maintained on a 12–12 h light-dark cycle with food and water freely available. Procedures were approved by the NIDA-IRP Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (NIH, Publication 865-23).

Surgical preparations

Surgical preparations were described in detail elsewhere [18, 23, 24]. Under general anesthesia (ketamine 80 mg/kg + xylazine 8 mg/kg with subsequent dosing), each rat was implanted with two oxygen sensors (Model 7002-02; Pinnacle Technology) and jugular catheter. The first sensor was implanted in the medial segment of the NAc [AP + 1.2 mm, ML ± 0.8 mm, and DV + 7.2–7.6 mm from the skull surface [25]. The second sensor was implanted in the SC space in the frontal area of the rat's head. This area is densely vascularized, and a sensor implanted in this area remains stable in freely moving rats, providing an artifact-free and reliable measure of oxygen levels in the periphery [19]. The probes were secured with dental cement to three stainless steel screws threaded into the skull. The jugular catheter ran subcutaneously to a head mount and was secured to the same head assembly. The rats were given 5 or more days to recover from surgery and also given 3 or more daily sessions (~6 h each) for habituation to the recording environment. Jugular catheters were flushed daily with 0.2 ml heparinized saline to maintain patency.

Electrochemical detection of oxygen

Pinnacle oxygen sensors consist of an epoxy-sheathed disc electrode grounded to a fine surface using a diamond-lapping disc. The sensors are prepared from a Platinum-Iridium wire 180 µm in diameter, with a sensing area of 0.025 mm^2 at the tip. The active electrode is incorporated with an integrated Ag/AgCl reference electrode. Dissolved oxygen is reduced on the active surface of these sensors, which is held at a stable potential of −0.6 V versus the reference electrode, producing an amperometric current. The current from the sensor is relayed to a computer via a potentiostat (Model 3104, Pinnacle Technology) and recorded at 1-s intervals, using PAL software utility (Version 1.5.0, Pinnacle Technology).

The oxygen sensors were calibrated at 37 °C by the manufacturer (Pinnacle Technology) according to a standard protocol described elsewhere [26] and their sensitivity was calibrated in-house. The sensors produced incremental current changes with increases in oxygen concentrations within the wide range of previously reported brain oxygen concentrations (0–40 µM). Substrate sensitivity of each sensor varied from 0.57–1.19 nA/1 µM. Oxygen sensors were also tested by the manufacturer for their selectivity toward other electroactive substances, including dopamine (0.4 µM) and ascorbate (250 µM), none of which had significant effects on reduction currents.

Experimental procedures

The rats were habituated to the recording cages for 4–6 h over three days prior to surgery. At the beginning of each recording session, the rats were minimally anesthetized (<2 min) with isoflurane and sensors were connected via an electrically shielded flexible cable and a multi-channel electrical swivel to the recording instruments. The injection port of the jugular catheter on the head mount was connected to a plastic catheter extension that allows stress- and cue-free drug delivery from outside the cage. When the rats received two different drugs within one recording session, two catheter extensions mounted on the recording cable were used to minimize any contamination of one drug by another drug. Testing began a minimum of 60 min after connecting the sensors to the recording instruments, when baseline values of electrochemical currents stabilized. For the next 4–6 h, the rats received several drug treatments (see below). Upon completion of the drug treatments, they were removed from the cages and briefly anesthetized by isoflurane to disconnect them from the recording instruments. Then, the catheters were flushed with heparinized saline before the rats returned to the animal colony. The recordings of each rat were conducted for 2–5 sessions, and the number of sessions in each experiment was determined by the quality of electrochemical recording and patency of the IV catheter. The drugs were delivered via slow IV injections (~0.2 ml/10 s) to freely moving rats in quiet resting conditions.

Fentanyl (Fentanyl Citrate Injection 50 µg/mL; Hospira Inc.) was delivered at a 20 µg/kg dose, which is larger than that for clinical use as an analgesic drug and for maintaining self-administration behavior in rats (typically 1–10 µg/kg; [27–29] but is within the limits of possible human consumption, which can exceed 1–2 mg [4].

Xylazine (Xylazine HCl, MP) was used at a 1 mg/kg dose, which is much lower than the LD50 for IV administration in rats (22–43 mg/kg; [30]). Based on our previous study, xylazine at this dose modestly decreases locomotor activity, brain and body temperature, and NAc oxygen levels [18]. Naloxone (naloxone HCl, Sigma) was used at a 0.2 mg/kg dose, which is higher than the doses used clinically (2–8 mg/70 kg; [10, 11] but relatively low in preclinical studies [31]. Atipamezole (Atipamezole HCl, Antisedan, Zoetis) was used at a 0.25 mg/kg dose, a dose known to reverse the sedative and analgesic effects of xylazine [32]. This dose is much lower than LD50 for IV administration in rats (>30 mg/kg; [32]. In humans, 30 mg of atipamezole has no cardiovascular and subjective effects, and 100 mg of the drug induces motor restlessness and weak hypertension [32].

Our study combines the results obtained in six experiments, shown schematically in Supplementary Materials (Supplementary Fig. S1). Chronologically, our tests were initially conducted in male rats and then in female rats. In the first two experiments, we tested the effect of naloxone or atipamezole on changes in oxygen levels in the NAc and SC space induced by fentanyl alone or xylazine alone, respectively. Fentanyl or xylazine were injected twice: first alone and then 10 min after an injection of the respective antagonist. In the next two experiments, we tested the effect of pretreatment with naloxone or naloxone + atipamezole on oxygen responses induced by the fentanyl-xylazine mixture. As in the previous tests, the fentanyl-xylazine mixture was delivered twice: first alone and then 10 min after an injection of naloxone or naloxone + atipamezole. In the last two tests, we examined the effect of naloxone or naloxone + atipamezole on oxygen responses induced by the fentanyl-xylazine mixture. In contrast to the pretreatment procedure used in the previous tests, the antagonists were injected 3 min after the injection of the fentanyl-xylazine mixture, when brain oxygen level showed a maximal decrease. The order of all tests was counterbalanced. As in our previous study [12], in all six experiments, the first injection of fentanyl, xylazine, or fentanyl-xylazine served as the comparison/reference point for the subsequent tests for the effects of naloxone, atipamezole, or naloxone + atipamezole on the hypoxic effects of fentanyl, xylazine, or fentanyl-xylazine. We used this within-subjects repeated-measures approach because in our previous study, we found stable oxygen responses to three repeated IV fentanyl (20 µg/kg) injections when they were given at 60–90 min intervals [12].

Histological verification of electrode placements

After the completion of the experiments, the rats were euthanized with isoflurane, decapitated, and their brains were extracted and placed in 10% formalin. The brains were sliced on a cryostat and analyzed for verification of the location of implants and possible tissue damage around the recording site.

Data analysis

Electrochemical data were sampled at 1 Hz using PAL software utility (Pinnacle Technology) and analyzed with 1-min time resolution. Electrochemical data were first analyzed as raw currents. Because each individual sensor differed slightly in background current and substrate sensitivity in vitro, the currents were transformed into concentrations and represented as relative changes, with the pre-stimulus baseline set at 100%. One-way repeated measures ANOVAs (followed by Fisher LSD post-hoc tests) were used to evaluate statistical significance of drug-induced changes in brain oxygen levels. Two-way repeated-measure ANOVAs were used to analyze between-group differences in drug effects. Fentanyl,

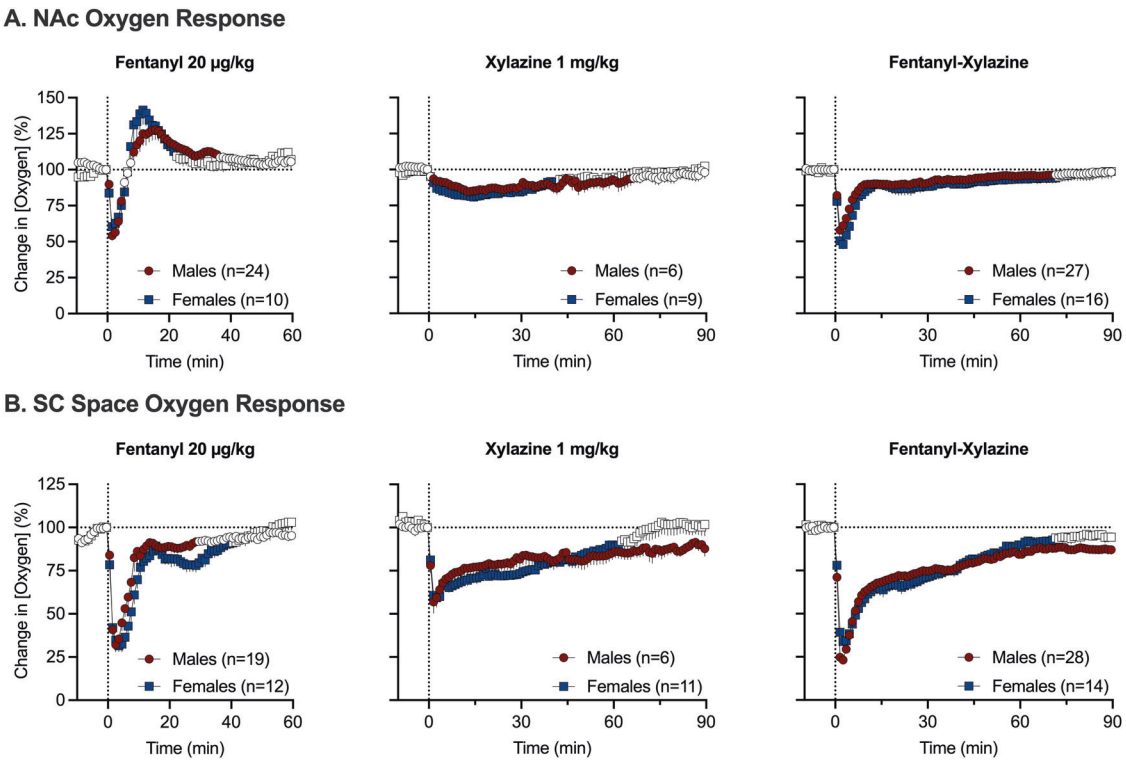

Fig. 1 Oxygen responses induced by fentanyl, xylazine, and their mixture in male and female rats. Graphs show mean (±SEM) oxygen changes in the NAc (**A**) and SC space (**B**) shown in percent vs. pre-injection baseline. XYL=xylazine; NAL=naloxone, ATI=atipamezole. n represents the number of averaged responses obtained in 12 male and 6 female rats. Filled symbols represent values significantly different from pre-injection baseline.

xylazine, and fentanyl-xylazine effects on oxygen responses were similar and statistically non-significant between males and females (see Fig. 1). There were also no statistically significant differences between males and females for the data presented in Figs. 2–4. Thus, we combined the male and female data for the figures and statistical analyses. For a complete statistical reporting, see Supplementary Materials.

RESULTS

Fentanyl (20 µg/kg)

The central (NAc) effect of fentanyl in rats of both sexes was biphasic: a rapid, robust (~50–60% of pre-injection baseline), and transient (6–8 min) decrease followed by a weaker and prolonged increase (Fig. 1A). In contrast, oxygen changes in the SC space were monophasic: a strong (~30% of baseline) and prolonged decrease (~50 min) (Fig. 1B). The fentanyl-induced oxygen responses in the NAc and SC space were similar in males and females.

Xylazine (1 mg/kg)

The drug induced significant changes in oxygen levels, with weak (~80–90% of baseline), slow, and prolonged oxygen decreases in the NAc and more rapid and stronger (~60%) decreases in the SC space (Fig. 1). These between-site differences were significant for ~10 min post-injection.

Fentanyl-xylazine mixture

The mixture induced an initial brain oxygen decrease that was similar in magnitude to fentanyl alone, but the effect was monophasic, and the duration of hypoxia was longer than that with fentanyl alone (40–50 min vs. 8 min) (Fig. 1). The changes in SC space were also monophasic and more prolonged, exceeding those in the brain for the entire 90 min post-injection (Fig. 1).

Together, the addition of xylazine to fentanyl had a minimal effect on the initial phasic brain and peripheral hypoxia but significantly prolonged the subsequent tonic hypoxia. These patterns of drug-induced oxygen fluctuations were sex-independent.

Effect of pretreatment with naloxone or atipamezole on fentanyl- or xylazine-induced hypoxia. Naloxone (0.2 mg/kg) injected 10 min before fentanyl blocked the decreases in oxygen levels induced by fentanyl in both recording locations (Fig. 2Aa). In contrast to the biphasic oxygen response induced by the first fentanyl injection, after pretreatment with naloxone, NAc oxygen levels did not change after the second fentanyl injection (Fig. 2Ab). A similar blockade was seen in the SC space (2Ac). Naloxone alone had a smaller, inconsistent changes in oxygen in both the brain and SC space (Fig. 2Aa).

Pretreatment with atipamezole (0.25 mg/kg) blocked oxygen responses induced by xylazine (Fig. 2Ba). The differences between the first and second xylazine injections were significant for both NAc (Fig. 2Bb) and SC space (Fig. 2Bc). Atipamezole also increased oxygen levels in NAc but not the SC space.

Together, pretreatment with naloxone or atipamezole reversed hypoxia induced by fentanyl or xylazine.

Effect of pretreatment with naloxone or naloxone + atipamezole on fentanyl-xylazine mixture-induced hypoxia. Naloxone pretreatment only partially blocked hypoxia induced by the fentanyl-xylazine mixture (Fig. 3Aa). In the NAc, naloxone modestly weakened the phasic oxygen decrease. The between-group difference was significant only at 6 min post-injection (Fig. 3Ab). The inhibitory effect of naloxone on oxygen changes in the SC space was also minimal (Fig. 3Ac): it modestly weakened the initial phasic oxygen decrease and modestly shortened the duration of

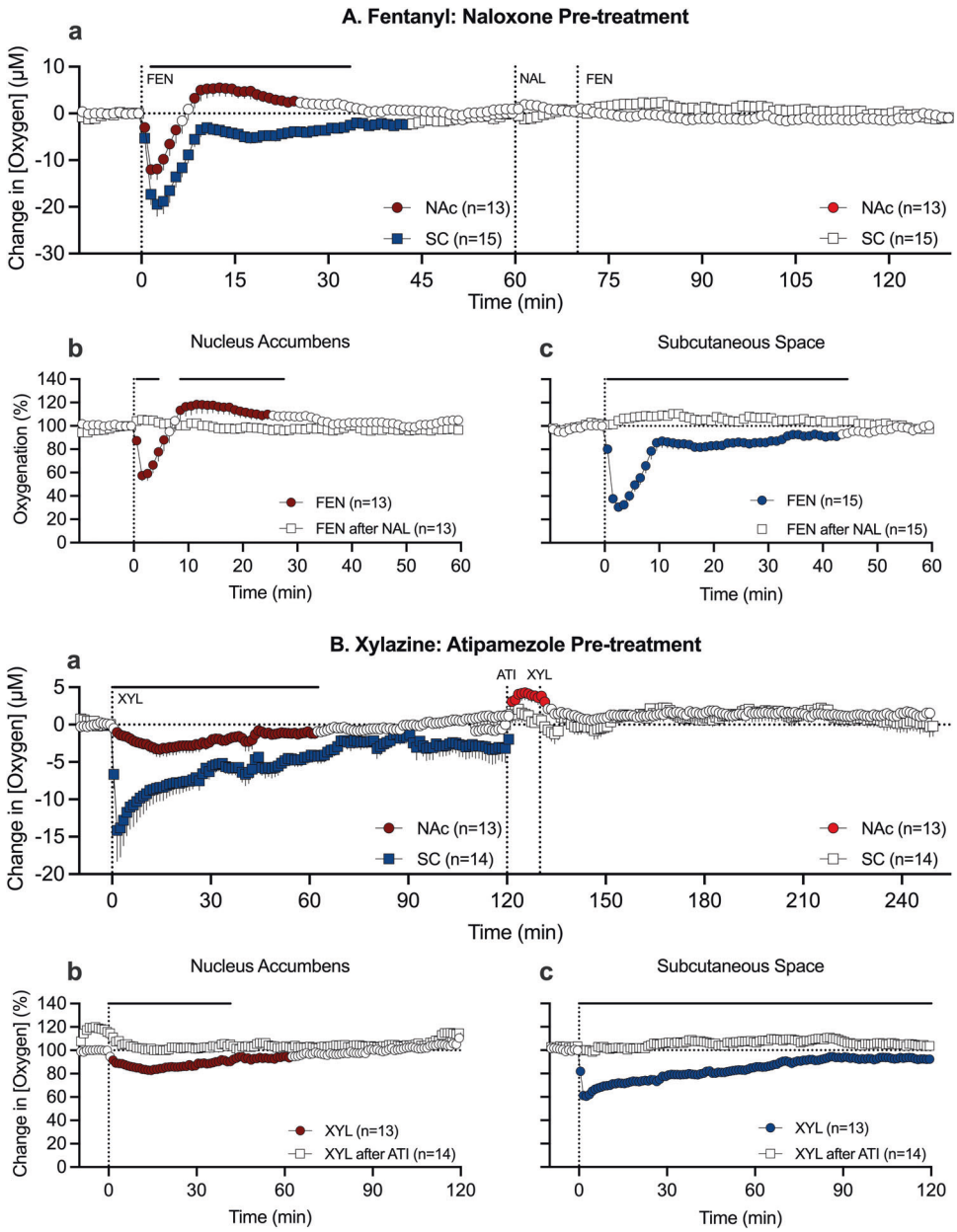

Fig. 2 The effects of naloxone or atipamezole on oxygen responses induced by fentanyl or xylazine in male and female rats. A Mean (±SEM) changes in oxygen levels in the NAc and SC space induced by fentanyl (20 μg/kg, IV) before and after naloxone pretreatment (0.2 mg/kg, IV). **B** Mean (±SEM) changes in oxygen levels in the NAc and SC space induced by xylazine (1 mg/kg, IV) before and after atipamezole pretreatment (0.25 mg/kg, IV). In both panels, **a** = changes for the entire recording session calibrated in μM, **b** and **c** = changes for the 1st and 2nd drug injection shown as percent vs. pre-injection baseline (=100%). Filled symbols show values significantly different from pre-injection baseline ($p < 0.05$). *n* = numbers of averaged responses obtained in 5 male and 5 female rats. Bold horizontal lines show time intervals during which mean oxygen values between the 1st and 2nd injection of fentanyl or xylazine are significant ($p < 0.05$).

the tonic decrease. Naloxone + atipamezole pretreatment had a much stronger effect on the oxygen response (Fig. 3Ba): the antagonist combination blocked the oxygen decreases in both NAc (Fig. 3Bb) and SC space (Fig. 3Bc).

Together, pretreatment with naloxone + atipamezole but not naloxone alone fully reversed hypoxia induced by the fentanyl-xylazine mixture.

Effect of treatment with naloxone or naloxone + atipamezole on fentanyl-xylazine mixture-induced hypoxia. The experiments described above showed that pretreatment with naloxone-atipamezole blocked both central and peripheral hypoxia induced by the fentanyl-xylazine mixture. In humans, however, the overdose rescue treatment is given following exposure to fentanyl-xylazine mixtures. Therefore, we next tested whether naloxone + atipamezole can reverse brain and peripheral hypoxia when given at the time of the peak hypoxia induced by fentanyl-xylazine mixture (~3 min under our experimental conditions).

Naloxone + atipamezole

As shown in Fig. 4A, the second injection of the fentanyl-xylazine mixture induced a rapid decrease in brain oxygen levels, which was like that induced by the first drug injection. When naloxone + atipamezole were injected 3 min after the second injection of the drug mixture, oxygen levels rapidly increased from their nadir to levels significantly exceeding those seen after the first drug

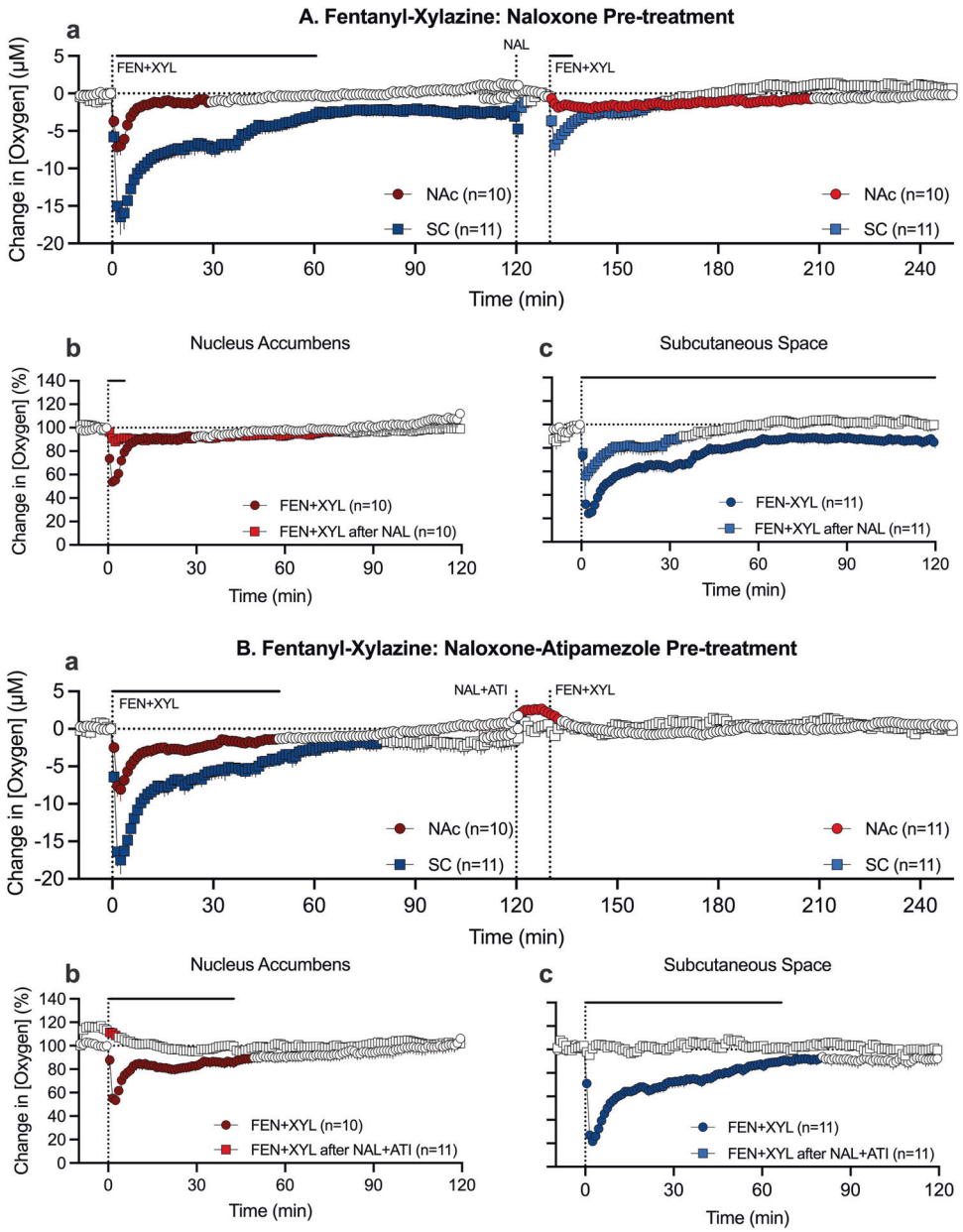

Fig. 3 The effects of naloxone or naloxone + atipamezole pretreatment on oxygen responses induced by fentanyl + xylazine in male and female rats. Mean (±SEM) changes in oxygen levels in the NAc and SC space induced by fentanyl-xylazine mixture (20 μg/kg + 1.0 mg/kg, IV) before and after pretreatment with naloxone (**A** 0.2 mg/kg, IV) and naloxone-atipamezole (**B** 0.2 mg/kg + 0.25 mg/kg, IV). In both panels, **a** = changes for the entire test session calibrated in μM, **b** and **c** = changes for the 1st and 2nd drug injection shown as percent (baseline = 100%). Filled symbols show values significantly different from pre-injection baseline ($p < 0.05$. n = numbers of averaged responses obtained in 5 male and 5 female rats. Bold horizontal lines show time intervals during which mean oxygen values between the 1st and 2nd drug injection are significant ($p < 0.05$).

injection (Fig. 4Ab). In contrast to slow return to baseline after the first fentanyl-xylazine injection, brain oxygen levels after naloxone + atipamezole injection rapidly increased above baseline. A similar pattern of drug interaction was seen in the SC space: oxygen levels rapidly increased to baseline, showing a weak rebound-like increase (Fig. 4Ac).

Naloxone alone

Naloxone injected 3-min after the injection of the fentanyl-xylazine mixture rapidly increased NAc oxygen levels but failed to prevent the tonic decrease in oxygen levels that followed its rapid decrease (Fig. 4Ba, b). Oxygen changes in the SC oxygen were similar: rapid blockade of phasic hypoxia and no effect on the subsequent tonic decrease (Fig. 4Bc).

The differences in the blocking effects of naloxone alone versus naloxone + atipamezole are shown with a higher resolution in Fig. 4C. Naloxone and naloxone + atipamezole were equally effective in reversing acute drug-induced brain oxygen decreases. However, naloxone + atipamezole but not naloxone tonically increased brain oxygen above baseline (Fig. 4Ca). Similar differences between naloxone versus naloxone + atipamezole were observed in the SC space (Fig. 4Cb).

Together, injections of naloxone + atipamezole during peak hypoxia had similar acute reversal effects to that of naloxone

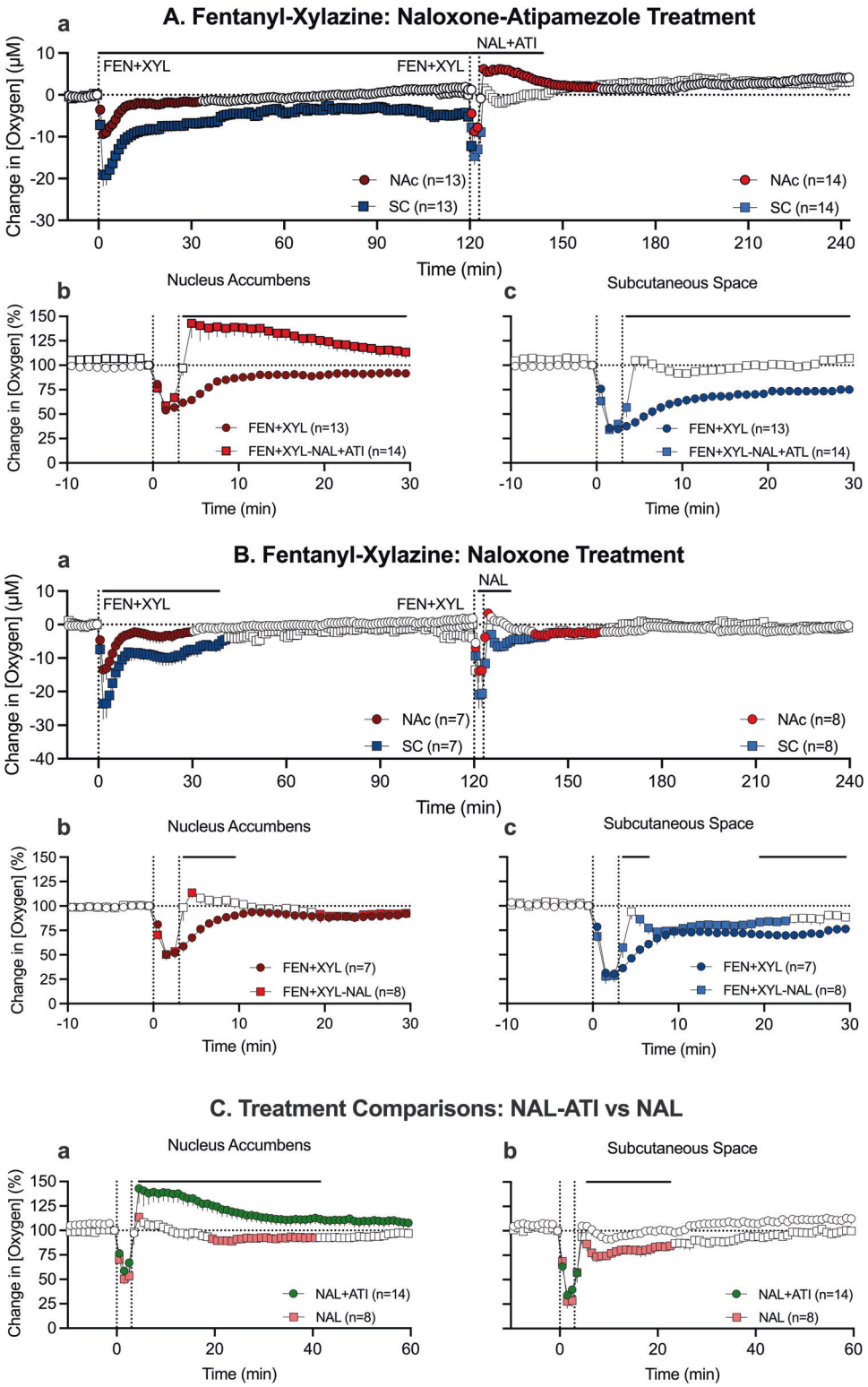

Fig. 4 The effects of naloxone + atipamezole or naloxone alone on oxygen responses induced by fentanyl-xylazine mixture in male and female rats. Effects of naloxone + atipamezole (**A**) and naloxone alone (**B**) on mean (±SEM) changes in oxygen levels in the NAc and SC space induced by fentanyl-xylazine mixture (20 μg/kg + 1.0 mg/kg, IV). Antagonists were injected 3 min after injections of agonists. **a** = changes for the entire test session calibrated in μM, **b** and **c** = changes for the 1st and 2nd drug injection shown as percent vs. baseline = 100%. **C** shows differences between the effects of naloxone and naloxone + atipamezole in NAc (**a**) and SC space (**b**). Filled symbols show values significantly different from the pre-injection baseline. *n* = numbers of averaged responses obtained from 5 male and 5 female rats. Bold horizontal lines show time intervals during which mean oxygen values between the 1st and 2nd drug injection are significant ($p < 0.05$).

alone but the treatment combination was more effective than naloxone in reversing the prolonged tonic hypoxic effects of the fentanyl-xylazine mixture in both the brain and periphery.

DISCUSSION

The primary goal of our study was to test the treatment potential of the alpha-2 adrenoceptor antagonist atipamezole as a supplement to naloxone treatment for reversal of hypoxia induced by exposure to a fentanyl-xylazine mixture. We first found that, as expected, naloxone pretreatment reversed fentanyl-induced brain and peripheral hypoxia and that atipamezole pretreatment reversed xylazine-induced hypoxia. Next, we found that pretreatment with naloxone + atipamezole but not naloxone alone fully reversed hypoxia induced by the fentanyl-xylazine mixture. We also found that injections of naloxone + atipamezole during peak brain hypoxia induced by the fentanyl-xylazine mixture had similar acute reversal effects to that of naloxone. However, the treatment combination was more effective than naloxone alone in reversing the prolonged tonic hypoxic effects in both the brain and periphery. Finally, there were no sex differences in the peripheral and central hypoxic effects of fentanyl, xylazine, and the fentanyl-xylazine combination or the anti-hypoxic effects of naloxone, atipamezole, or naloxone + atipamezole. These results suggest that common sex-independent mechanisms underlie the effects of opioid and noradrenergic drugs on central and peripheral hypoxia.

Oxygen responses induced by fentanyl, xylazine and a fentanyl-xylazine mixture

Consistent with our previous study [12], fentanyl induced a biphasic brain oxygen response, with rapid and strong decrease followed by weaker and more prolonged increase. Oxygen levels in the SC space also rapidly decreased but this effect was monophasic, stronger, and more prolonged than in the brain. While respiratory depression followed by decreased blood oxygen levels is the major factor for oxygen decreases in both locations, a stronger response in the SC space is likely due to additional contribution of fentanyl-induced skin vasoconstriction [18, 33]. The subsequent brain oxygen increase induced by fentanyl may result from cerebral vasodilation and increased global cerebral blood flow due to the post-hypoxic accumulation of CO2, a powerful vasodilator [34–36], and redistribution of arterial blood from periphery to brain due to peripheral vasoconstriction [37].

Xylazine also decreased brain oxygen levels, but this effect was monophasic, much weaker, less rapid, but more prolonged than that induced by fentanyl. The oxygen response in the SC space was also monophasic, more rapid, stronger, and more prolonged than in the brain. While weak brain hypoxia may result from weak respiratory depression due to xylazine-induced decreased sympathetic activity [14, 16, 38], drug-induced cerebral vasoconstriction [39], which diminishes oxygen entry to the brain from arterial blood, may be another contributing factor.

The fentanyl-xylazine mixture also decreased oxygen levels in the brain and periphery with several distinct differences. While the initial brain oxygen decrease was somewhat like that induced by fentanyl alone, the response was monophasic, with no hyperoxic phase and a more prolonged duration of hypoxia. Like with fentanyl, hypoxia was much stronger in the periphery than in the brain and between-site differences were larger than those seen with fentanyl. We speculate that cerebral vasoconstriction induced by xylazine [39] could be responsible for disappearance of hyperoxic phase of fentanyl response and prolongation of brain hypoxia.

Effect of pretreatment with naloxone and naloxone + atipamezole on hypoxia induced by a fentanyl-xylazine mixture

As expected, the opioid receptor antagonist naloxone blocked the hypoxic effects of fentanyl in both the brain and periphery. As in our previous studies [12, 31], naloxone had a minimal effect on basal oxygen levels. Similarly, the hypoxic effects of xylazine, an α2-adrenoceptor agonist, were blocked by atipamezole, a selective α2-adrenoceptor antagonist [31, 40, 41]. In both the brain and periphery, the effect of atipamezole was seen at a low dose (0.25 mg/kg), which is ~1:120 of the drug's LD50 [29]. Unlike naloxone, atipamezole significantly increased oxygen levels in the brain with no effect in the periphery.

As a first stage to compare the blocking effects of naloxone and naloxone + atipamezole, we used a traditional pharmacological approach where the effect of the agonist drugs (fentanyl-xylazine mixture) was evaluated after pretreatment with an antagonist drug (naloxone or naloxone + atipamezole). Using this approach, we found that naloxone, which fully blocked fentanyl-induced oxygen responses, was only partially effective in reversing the initial phasic hypoxic responses induced by the fentanyl-xylazine mixture. The reversal effect was minimal in the SC space, where the fentanyl-xylazine mixture continued to produce prolonged hypoxia. In contrast, atipamezole + naloxone fully blocked oxygen responses in both the brain and SC space.

Effects of treatment with naloxone and naloxone + atipamezole during peak hypoxia induced by a fentanyl-xylazine mixture

The results of the pretreatment experiment show that the combined treatment of naloxone + atipamezole had a stronger effect than naloxone alone on brain hypoxia induced by the fentanyl-xylazine mixture. However, in humans, the overdose treatment is given after exposure to fentanyl-xylazine mixtures and appearance of life-threatening symptoms. Thus, treatment efficacy should be determined under these conditions. In real life, the timing from appearance of opioid-induced hypoxia and naloxone treatment greatly varies. This timing issue is especially important for fentanyl because its hypoxic effects in the brain are rapid and strong but relatively short. We previously found that when naloxone was injected 10 min after 20 μg/kg fentanyl, it had minimal effects on oxygen levels because brain hypoxia was no longer observed [12]. At a higher dose (60 μg/kg), the hypoxic effect of fentanyl in the brain was larger in amplitude but only slightly more prolonged (~11 min vs. 8 min). In this case, naloxone decreased the hypoxic effects of fentanyl in the periphery but only had a weak and transient effect in NAc. These findings underscore the critical importance of timing of naloxone injection after fentanyl exposure.

In the present study, we modified our protocol and injected naloxone or naloxone + atipamezole 3 min after exposure to the fentanyl-xylazine mixture, the time of peak hypoxia. In this case, both naloxone and naloxone + atipamezole rapidly reversed the hypoxic effects of fentanyl-xylazine mixture. The effect was exceptionally rapid (30–40 s) and correlated with awakening and increase in locomotor activity (data not shown). Despite similar initial effects, there were subsequent differences between the two treatment conditions. After naloxone injections, brain oxygen levels rapidly returned to the pre-injection baseline, but then they modestly decreased again. Similar delayed effects were also seen in the SC space. In contrast, after naloxone + atipamezole injections, the peripheral hypoxia was fully reversed and the treatment also induced a strong post-hypoxic oxygen increase in the brain, which was maintained for ~30–40 min post-treatment.

Together, naloxone, which was highly effective in reversing the initial hypoxic effects of the fentanyl-xylazine mixture, only partially reversed the subsequent prolonged oxygen decreases, especially in the periphery. In contrast, naloxone + atipamezole fully reversed hypoxia in both the brain and periphery and induced relatively strong and prolong post-hypoxic oxygen increase. Thus, atipamezole, by blocking central vascular effects of xylazine, revealed a hyperoxic effect of fentanyl, thus opposing brain hypoxia resulting from respiratory depression. This adaptive response was inhibited

after exposure to the fentanyl-xylazine mixture and was not reversed by treatment with naloxone alone. Therefore, naloxone + atipamezole mixture, by providing better brain oxygenation, is more effective than naloxone alone in reversing the more severe hypoxic effects of fentanyl-xylazine mixtures.

Limitations of the study

Despite the advantages of electrochemical assessment of brain oxygenation, our study has several limitations. First, the complexity of the electrochemical technique places limits on the numbers of subjects and drug doses that can be assessed. In this regard, one potential limitation of our study is the use of a single drug dose for the four drugs we used. However, the choice of the single dose for each drug was based on the literature and our previous studies [12, 18, 23]. Thus, it is unlikely that additional doses, either lower or higher than the chosen doses, will change the main conclusion of the study: that naloxone + atipamezole combination is more effective than naloxone alone in preventing the hypoxic effect of fentanyl-xylazine mixtures. Nevertheless, it will be important in future studies to test a range of doses of each drug and their combinations, injected acutely or repeatedly, to determine the optimal conditions for the protective effects of naloxone + atipamezole.

Second, the goal of our study was to mimic the human condition of the life-threatening effects of fentanyl that occur within a short time after consumption of fentanyl-xylazine mixtures in people with minimal or no prior opioid or xylazine experience. Thus, a question for future research is the generality of our results to opioid-dependent rats after tolerance to the hypoxic effects of opioids in the brain and periphery has developed and naloxone injections induce physical withdrawal and sympathetic activation [42, 43]. Another important future direction is the generality of our results to experimental subjects with a prior history of opioid dependence after a period of forced abstinence during which tolerance has dissipated, a putative animal model for relapse to opioid use after inpatient detoxification treatment or release from jail. Another important future direction is the study of the long-term effects of fentanyl-xylazine mixture-induced acute hypoxia on opioid reward, relapse, and cognition.

Third, the rats in our study had prior experience with a single high dose of xylazine (8 mg/kg) during surgery, which may affect their response to a low dose of xylazine (1 mg/kg) during testing. We believe that this possibility is unlikely because of the short duration of action of xylazine and the fact that several days or more separated the surgery from the testing days.

Finally, there are large differences metabolic activity and drug pharmacokinetics between rats and humans [44–46]. Thus, further research is needed to translate our data to effective dose combinations of naloxone and atipamezole to reverse hypoxia induced by fentanyl-xylazine mixtures in humans.

Clinical implications

Our results highlight the critical importance of using naloxone in most cases of human misuse of either fentanyl or fentanyl-xylazine mixtures. In our rat model, naloxone was highly effective in reversing the initial severe brain hypoxia induced by either fentanyl or the fentanyl-xylazine mixture. Since the hypoxic effects of fentanyl in the brain are very rapid, strong, but relatively short, and followed by post-hypoxic hyperoxia, our data underscore the importance of the narrow time range for initiating naloxone treatment, which is most effective immediately after overdose symptoms are detected. Perhaps more important, our data strongly support the potential use of atipamezole together with naloxone in overdose cases where patients do not immediately respond behaviorally to naloxone, suggesting the presence of xylazine. Atipamezole is highly potent but safe in rats, suggesting a similar safety profile in humans.

DATA AVAILABILITY

Raw data and the results of their primary analyses are available on request from Dr. Eugene A. Kiyatkin (NIDA-IRP, NIH; ekiyatki@intra.nida.nih.gov).

REFERENCES

1. Butelman ER, Huang, Y, Epstein DH, Shaham Y, Goldstein RZ, Volkow ND, et al. Overdose mortality rates for opioids and stimulant drugs are substantially higher in men than in women: state-level analysis. Neuropsychopharmacology. 2023; https://doi.org/10.1038/s41386-023-01601-8.
2. Kariisa M, O'Donnel J, Kumar S, Mattson CL, Goldberger BA. Illicitly manufactured fentanyl-involved overdose death with detected xylazine – United States, January 2019-June 2022. CDC Report 72 (No 26).
3. Friedman J, Montero F, Bourgois P, Wahbi R, Dye D, et al. Xylazine spreads across the US: a growing component of the increasingly synthetic and polysubstance overdose crisis. Drug Alcohol Depend. 2022;233:109380.
4. Dahan A, Yassen A, Bijl H, Romberg R, Sarton E, Teppema L, et al. 2005. Comparison of the respiratory effects of intravenous buprenorphine and fentanyl in humans and rats. Br J Anaesth. 2005;94:825–34.
5. Yeadon M, Kitchen I. Opioids and respiration. Prog Neurobiol. 1989;33:1–16.
6. Gupta K, Prasad A, Nagappa M, Wong J, Abrahamyan L, Chung FF. Risk factors for opioid-induced respiratory depression and failure to rescue: a review. Curr Opin Anesthesiol. 2018;31:110–19.
7. MacKenzie M, Zed PJ, Ensom MHH. Opioid pharmacokinetics-pharmacodynamics: clinical implications in acute pain management in trauma. Ann Pharmacother. 2016;50:209–18.
8. Skolnick P. On the front lines of the opioid epidemic: rescue by naloxone. Eur J Pharmacol. 2018;835:147–53.
9. Skolnick P. Treatment of overdose in the synthetic opioid era. Pharmacol Ther. 2021:108019. https://doi.org/10.1016/j.pharmthera.2021.108019.
10. Lynn RR, Galinkin JL. Naloxone dosage for opioid reversal: current evidence and clinical implications. Ther Adv Drug Saf. 2018;9:63–88.
11. Moss RB, Carlo DJ. Higher doses of naloxone are needed in the synthetic opioid era. Subst Abuse Treat Prev Policy. 2019;14:6. https://doi.org/10.1186/s13011-019-0195-4.
12. Curay CM, Irwin MR, Kiyatkin EA. The pattern of brain oxygen response induced by intravenous fentanyl limits the time window of therapeutic efficacy of naloxone. Neuropharmacology. 2023;231:109507. https://doi.org/10.1016/j.neuropharm.2023.109507.
13. Compton VM, Valentino RJ, DuPont RL. Polysubstance use in the U.S. opioid crisis. Mol Psychiatry. 2021;26:41–50.
14. Greene SA, Thurmon JC. Xylazine—a review of its pharmacology and use in veterinary medicine. J Vet Pharmacol Ther. 1988;11:295–313.
15. Reyes JC, Negron JL, Colob HM, Padilla AM, Millan MY, et al. The emerging of xylazine as a new drug of abuse and its health consequences among drug users in Puerto Rico. J Urban Health. 2012;89:519–26.
16. Capraro AJ, Wiley JF, Tucker JR. Severe intoxication from xylazine inhalation. Pediatr Emerg Care. 2001;17:447–8.
17. Schwartz DD, Clark TP. Affinity of detomidine, medetomidine and xylazine for alpha-2 adrenergic receptor subtypes. J Vet Pharmacol Ther. 1998;21:107–11.
18. Choi S, Irwin MR, Kiyatkin EA. Xylazine effects on opioid-induced brain hypoxia. Psychopharmacology. 2023;240:1561–71. https://doi.org/10.1007/s00213-023-06390-y.
19. Kiyatkin EA. Physiological and drug-induced fluctuations in brain oxygen and glucose assessed by substrate-sensitive sensors coupled with high-speed amperometry. In: Wilson GS, Michael AC, editors. Compendium of in vivo monitoring in real-time molecular neuroscience. 3. Probing brain functions, disease and Injury with enhanced optical and electrochemical sensors. World Scientific; 2019. p. 219–50.
20. Badiani A, Belin D, Epstein D, Calu D, Shaham Y. Opiate versus psychostimulant addiction: the differences do matter. Nat Rev Neurosci. 2011;12:685–700.
21. Mogenson GJ, Jones DL, Yim CY. From motivation to action: functional interface between the limbic system and the motor system. Prog Neurobiol. 1980;14:69–97.
22. Wise RA, Bozarth MA. A psychomotor stimulant theory of addiction. Psychol Rev. 1987;94:469–92.
23. Solis E Jr, Cameron-Burr KT, Shaham Y, Kiyatkin EA. Fentanyl-induced brain hypoxia triggers brain hyperglycemia and biphasic changes in brain temperature. Neuropsychopharmacology. 2018;43:810–9.
24. Thomas SA, Curay CM, Kiyatkin EA. Relationships between oxygen changes in the brain and periphery following physiological activation and the action of heroin and cocaine. Sci Rep. 2021;11:6355.
25. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. San Diego: Academic Press; 1998.

26. Bolger FB, Bennett R, Lowry JP. An in vitro characterization comparing carbon paste and Pt microelectrodes for real-time detection of brain tissue oxygen. Analyst. 2011;136:4028–35.
27. Wade CL, Vendruscolo LF, Schlosburg JE, Hernandez DO, Koob GF. Compulsive-like responding for opioid analgesics in rats with extended access. Neuropsychopharmacology. 2015;40:421–48.
28. Reiner DJ, Lofaro OM, Applebey SV, Kohan H, Venniro M, et al. Role of projections between piriform cortex and orbitofrontal cortex in relapse to fentanyl seeking after palatible food choice-induced voluntary abstinence. J Neurosci. 2020;40:2485–97.
29. Claypool SM, Reiner DJ, Behdin S, Orihuel J, Batista A, Caldwell K, et al. Role of piriform cortex and its afferent projections in relapse to fentanyl seeking after food choice-induced voluntary absinence. J Neurosci. 2023;43:2597–614.
30. Xylazine hydrochloride 1999; Committee for veterinary medical products. Xylazine Hydrochloride: Summary report. The European Agency for the Evaluation of Medicinal Products. https://www.ema.europa.eu/en/documents/mrl-report/xylazine-hydrochloride-summary-report-1-committeeveterinary-medicinal-products_en.pdf.
31. Perekopskiy D, Afzal A, Jackson SN, Muller L, Woods AS, Kiyatkin EA. The role of peripheral opioid receptors in triggering heroin-induced brain hypoxia. Sci Rep. 2020;10:833.
32. Pertovaara A, Haapalinna A, Sirvio J, Virtanen R. Pharmacological properties, central nervous system effects, and potential therapeutic applications of atipamezole, a selective alpha2-adrenoceptor antagonist. CNS Drug Rev. 2005;11:273–88.
33. Bola RA, Kiyatkin EA. Brain temperature effects of intravenous heroin: state dependency, environmental modulation, and the effects of dose. Neuropharmacology. 2017;126:271–80.
34. Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature. 2010;468:232–43.
35. Battisti-Charbonney A, Fisher J, Duffin J. The cerebrovascular response to carbon dioxide in humans. J Physiol. 2011;589:3039–48.
36. Schmidt CF, Kety SS. Recent studies of cerebral blood flow and cerebral metabolism in man. Trans Assoc Am Physicians. 1947;60:52–58.
37. Kiyatkin EA. Functional role of peripheral vasoconstriction: not only thermoregulation but much more. J Integr Neurosci. 2021;20:755–64.
38. Sanford TD, Colby ED. Effect of xylazine and ketamine on blood pressure, heart rate and respiratory rate in rabbits. Lab Anim Sci. 1980;30:519–23.
39. Kanawati S, Yaksh TL, Anderson RE, Marsh RW. Effects of clonidine on cerebral blood flow and the response to arterial CO2. J Cereb Blood Flow Metab. 1986;6:358–65.
40. Drew GM. Effects of alpha-adrenergic agonists and antagonists on pre- and postsynaptically located alpha-adrenoceptors. Eur J Pharmacol. 1976;36:313–20.
41. Kobinger W. Central alpha-adrenergic systems as target for hypotensive drugs. Rev Physiol Biochem Pharmacol. 1978;81:39–100.
42. Blasig J, Herz A, Reinhold K, Zieglgansberger S. Development of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. Psychopharmacologia. 1973;33:19–38.
43. Bossert JM, Kiyatkin EA, Korah H, Hoots JK, Afzal A, et al. In a rat model of opioid maintenance, the G Protein-biased mu opioid receptor agonists TRV130 decreases relapse to oxycodone seeking and taking and prevents oxycodone-induced brain hypoxia. Biol Psychiatry. 2020;88:935–44.
44. Schmidt-Nielson K. Animal physiology: adaptation and environment. Cambridge: Cambridge University Press; 1989.
45. Trescot AM, Datta S, Lee M, Hansen H. Opioid pharmacology. Pain Physician. 2008;11:S133–53.
46. Ohtsuka H, Fujita K, Kobayashi H. Pharmacokinetics of fentanyl in male and female rats after intravenous administration. ArzneimForschDrugRes. 2007;57:260–63.

ACKNOWLEDGEMENTS

The study was supported by the Intramural Research Program of the NIH, NIDA.

AUTHOR CONTRIBUTIONS

EAK: Conceptualization, Surgery procedures, Participation in experiments, Data analyses, Writing the manuscript; SC, MRI and MN: Conceptualization, Performance of experiments, Data analyses, Graphic work, Histological work, Review and editing the manuscript. YS: Conceptualization, Writing, and editing the manuscript.

FUNDING

The study was supported by the Intramural Research Program of the NIH, NIDA (1ZIADA000566-12 [EAK]; 1ZIADA000434-17 [YS]).

COMPETING INTERESTS

YS is an Associate (Reviewing) Editor for Neuropsychopharmacology. The other authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41386-023-01782-2.

Correspondence and requests for materials should be addressed to Eugene A. Kiyatkin.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.