1. TITLE PAGE

Oxytocin receptor activation rescues opioid-induced respiratory depression by systemic fentanyl in the rat

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3. ABSTRACT

Opioid overdose intervention by naloxone, a high affinity receptor antagonist, reverses opioid-induced respiratory depression (OIRD) and analgesia by displacing opioids. Systemic naloxone stimulates release of the hypothalamic neuropeptide oxytocin, which has analgesic properties and participates in cardiorespiratory homeostasis. To test the hypothesis that oxytocin can reverse OIRD, we assessed the rescue potential of graded doses (0, 0.1, 2, 5, 10, 50 nmol/kg, i.v) of oxytocin to counter fentanyl (60 nmol/kg, i.v.-) induced depression of neural inspiration indexed by recording phrenic nerve activity (PNA) in anesthetized (urethane/α-chloralose), vagotomized, and artificially ventilated rats. Oxytocin dose-dependently rescued fentanyl OIRD by almost immediately reversing PNA burst arrest \( (P=0.0057) \) and restoring baseline burst frequency \( (P=0.0016) \) and amplitude \( (P=0.0025) \) at low, but not high doses, resulting in inverted bell-shaped dose-response curves. Oxytocin receptor antagonism (40 nmol/kg, i.v.) prevented oxytocin reversal of OIRD \( (\text{Arrest: } P=0.0066, \text{Frequency: } P=0.0207, \text{Amplitude: } P=0.0022) \). Vasopressin 1A receptor (V1aR) antagonism restored high-dose oxytocin efficacy to rescue OIRD \( (P=0.0170 – P<0.0001) \), resulting in classic sigmoidal dose-response curves, and prevented \( (P=0.0135) \) transient hypertension from V1aR cross-activation \( (P=0.0275) \). Alone, vasopressin (5 nmol/kg, i.v.) failed to reverse fentanyl respiratory arrest \( (P=0.6184) \). The non-peptide oxytocin receptor agonist WAY-267464 (75 nmol/kg, i.v.), which has V1aR antagonist properties, quickly reversed fentanyl OIRD \( (P<0.0001) \), with rapid recovery of PNA frequency \( (P=0.0011) \) and amplitude \( (P=0.0044) \) without adverse hemodynamic consequences \( (P=0.9991) \). Findings indicate that peptide and non-peptide agonist activation of oxytocin receptors without V1aR cross-activation rescues fentanyl OIRD. Oxytocin receptor agonists could be lifesaving resuscitation agents that enhance rather than interrupt opioid analgesia.
4. SIGNIFICANCE STATEMENT

Oxytocin receptor activation produces analgesia. Here, we demonstrate that activation by the FDA-approved agonist oxytocin and the non-peptide partial agonist WAY-267464 can each reverse fentanyl cardiorespiratory depression. Selective targeting of oxytocin receptors for resuscitation from opioid overdose, alone or in combination with an opioid antagonist, could eliminate or attenuate negative side effects associated with traditional opioid receptor antagonism.
6. INTRODUCTION

Opioid overdose is characterized by respiratory depression, which underlies the lethality that drives the opioid epidemic (Webster et al., 2011). Emergency reversal of opioid-induced respiratory depression (OIRD) involves systemic administration of high affinity opioid receptor antagonists that compete with opioids for receptor binding (Handal et al., 1983). Although highly effective at reversing respiratory, and associated cardiovascular, depression by exogenously administered opiates, opioid receptor antagonism also reverses analgesia and, for chronic opiate users, induces symptoms of opiate withdrawal (Handal et al., 1983). Thus, there is need for overdose resuscitation without antagonism of opioid receptors.

Opioid antagonist experiments have demonstrated that hypothalamic neurons that release oxytocin, in contrast to those that release vasopressin, are under strong opioid inhibition (Bicknell et al., 1988). Notably, systemic naloxone increases intrinsic excitability of oxytocin neurons (Brown et al., 2005) and induces oxytocin release into the bloodstream and cerebrospinal fluid. The latter is primarily mediated by neurons of the hypothalamic paraventricular nucleus (Coombes et al., 1991). Although never reported in the context of opioids or OIRD, a number of studies have connected oxytocin to enhanced respiratory function. Oxytocin delivered by iontophoresis or microinjection, for example, excites multiple pools of cardiorespiratory neurons and enhances ventilatory output (Henry and Sessle, 1989; Mack et al., 2002; Mack et al., 2007). In humans, oxytocin safely and effectively attenuates non-opioid respiratory depression (Jain et al., 2020) in patients at increased risk for OIRD (Subramani et al., 2017). In addition to its potential to reverse respiratory depression, oxytocin induces potent analgesia (Eliava et al., 2016) and can mitigate opioid-seeking behavior (Ibragimov et al., 1987).

Here, we hypothesized that cardiorespiratory depression by opioids can be effectively reversed by oxytocin receptor activation. Fentanyl, the clinically prescribed opioid most
associated with OIRD (Zedler et al., 2018), was used to induce hypotension as well as prolonged apnea, identified as the absence of inspiratory motor output determined from recordings of phrenic nerve activity (PNA), in anesthetized, vagotomized and artificially ventilated rats. Rescue potential of oxytocin receptor activation was examined by measuring effects of graded systemic doses of oxytocin post-fentanyl, as well as effects of the non-peptide oxytocin receptor agonist WAY-267464. Results indicate that cardiorespiratory depression by fentanyl was rescued by oxytocin receptor activation when devoid of vasopressin type 1A receptor (V1aR) cross-activation. The latter accounted for a transient elevation of blood pressure at the highest administered dose of oxytocin. Combining this newly discovered resuscitation potential of oxytocin with its known capacity to counteract symptoms of withdrawal, including nociception and opioid-seeking, makes oxytocin receptor activation a promising therapeutic approach to better combat the opioid epidemic.
7. MATERIALS & METHODS

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 300-500 g were group housed in temperature controlled rooms (22-23°C) maintained on a 14:10 h light-dark cycle (lights on at 07:00 h) and with ad libitum access to food and water. Experimental procedures were conducted in accordance with the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and with approval of the Animal Care and Use Committee of the University of Texas Health San Antonio.

Surgical Procedures

Rats were anesthetized with an α-chloralose (80 mg/kg)-urethane (800 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) cocktail delivered intraperitoneally. Core body temperature was recorded with a rectal probe interfaced with a TH-5 sending unit (Physitemp, Inc., Clifton, NJ) and maintained throughout the experiment at 37±1°C using a ventrally located circulating water pad. To measure arterial blood pressure and administer drugs, PE-50 catheters were introduced into the left femoral artery and vein, respectively. Pulsatile arterial pressure was acquired through a strain gauge transducer and bridge amplifier (Coulbourn Instruments, Lehigh Valley, PA, USA), with mean arterial pressure (MAP) determined as a moving average (τ=1 s) of the pulsatile pressure waveform. Heart rate was determined from R-R intervals obtained from a lead II ECG acquired with an AC-coupled amplifier (Mdl P511, Grass Instruments). ECG signals were amplified (5 kX), passed through a 60 Hz notch filter and half-amplitude frequency filters set to a band pass of 0.1-3.0 kHz as previously described (Brackley et al., 2020). To avoid OIRD lethality, a tracheostomy was performed and a tracheal cannula fabricated from 14 G hypodermic stainless steel tubing was inserted for artificial ventilation. Rats were ventilated with 100% O₂ to maintain arterial O₂ saturation at >95% during surgery and experimental protocols. End-tidal CO₂ was monitored from a sealed opening of the tracheal cannula with a
Capstar-100 CO₂ analyzer (CWE, Inc., Ardmore, PA, USA) and maintained between 5.0-5.5% prior to each fentanyl injection by adjusting tidal volume (1.8-2.7 mL) and ventilation rate (85-95 breaths/min) using a pressure controlled ventilator (Kent Scientific, Torrington, CT, USA). Cervical vagus nerves were transected bilaterally to interrupt pulmonary stretch receptor inputs that can otherwise entrain phrenic bursts to the rate of mechanical ventilation (MacDonald et al., 2007), potentially confounding quantification of basal phrenic burst frequency and its recovery following fentanyl. Bilateral vagotomy isolated the central OIRD response to mu opioid receptor activation from peripheral respiratory depression mediated by the vagus nerve (Willette and Sapru, 1982). To prevent fentanyl-induced thoracic rigidity, a peripheral contribution to OIRD known as “wooden chest syndrome,” paralysis was induced by infusion of the neuromuscular blocking agent gallamine triethiodide (25 mg/kg bolus followed by 5 mg/kg/h infusion, i.v.), which also prevented breathing-related movements that can produce electrical artifacts in PNA recordings. Anesthesia was maintained throughout surgery by intravenous (i.v.) supplements (10% initial dose) as indicated by a noxious plantar pinch that evoked arterial blood pressure instability with an acute pressor response (>10 mmHg) and/or neural tachypnea (>10% decrease in neural expiratory phase).

**Recording Phrenic Nerve Activity (PNA)**

Rats were prepared for PNA recordings as previously described (Holbein and Toney, 2015; Brackley et al., 2020). Briefly, skin and muscle overlying the left scapula were incised and the phrenic nerve near the brachial plexus was isolated and transected. The central cut end of the nerve was placed on a bipolar silver wire electrode and embedded in silicon-based elastomer (Kwik-Sil, World Precision Instruments, Inc., Sarasota, FL, USA) to prevent desiccation and to insulate recordings from body fluids. Nerve signals obtained with a high impedance (10¹⁰ Ohm) probe connected to an AC amplifier (Mdl P511, Grass Instruments) equipped with a 60 Hz notch filter and half-amplitude frequency filters (band pass 1-1000 Hz).
Signals were amplified at 20 kX, digitized at 1.5 kHz, full-wave rectified and smoothed as a moving average (τ=30 ms).

**Experimental Protocols**

All experimental drugs were given as a bolus (120-200 µL) and chased by an equal volume of saline over a period of 10 s. OIRD was initiated following 5 min of stable baseline recording by treating rats with systemic (i.v.) fentanyl (Sigma-Aldrich, No. F-013, LOT: FE03222001) to silence PNA, as previously published with sample size appropriate to determine significance (Brackley et al., 2020). Following an established timeline, at 1 min post-fentanyl, when peak hypotension was observed, either the peptide oxytocin (oxytocin acetate salt hydrate, Sigma-Aldrich, No. O6379) or vasopressin ([Arg⁸]-vasopressin acetate salt, Sigma-Aldrich, No. V9879) or the non-peptide oxytocin receptor agonist WAY-267464 (Sigma-Aldrich, No. SML2223) was delivered systemically (i.v.) and recording continued for at least 1 h. For experiments involving antagonism of the oxytocin receptor (atosiban, Sigma-Aldrich, No. A3480) or the V1aR (V1aRX; (Phenylac⁴, D-Tyr(Et)², Lys⁶, Arg⁸, des-Gly⁹)-Vasopressin trifluoroacetate, BaChem, Torrance, CA, USA, No. H-3186.0001), the above protocol was followed except that each antagonist was given 5 min before fentanyl. To verify complete receptor antagonism, some subjects received additional agonist treatments before and after antagonist administration. Experiments testing time-dependent effects of peptide treatments were carried out by recording PNA for a minimum of 20 min without inducing OIRD. Non-peptide WAY-267464 was prepared in 2.5% dimethyl sulfoxide (DMSO) and saline and its vehicle contained DMSO and saline in the same (v/v) ratio. All other test compounds were prepared in sterile saline, which served as a vehicle control. At the end of experiments, an isoelectric ECG was used to confirm death following an overdose of α-chloralose/urethane (40/400 mg/kg, i.v.) anesthetic.

**Data Analysis and Statistics**
PNA, ECG and blood pressure signals were analyzed using Spike2 software (v10, Cambridge Electronic Design, Cambridge, UK). After systemic administration of peptide and non-peptide test compounds, latency to PNA burst reappearance post-fentanyl was quantified. Recovery of PNA burst frequency and amplitude post-fentanyl were quantified as the time required for each parameter to return to its average baseline value. PNA burst amplitude at baseline was quantified as the average of 100 bursts immediately preceding fentanyl administration. In the rare instance that PNA burst frequency or amplitude did not fully recover to baseline, the point at which each signal recovered to a steady plateau was used. For each treatment group, the baseline value of mean arterial pressure (MAP) and heart rate was compared to the corresponding nadir post-fentanyl and to values 15 s post-rescue treatment. To further assess hemodynamic actions of rescue treatments, the rate of recovery of MAP and heart rate was calculated as the average slope from 0 to 10 s after each treatment. For non-OIRD experiments, the maximum pressor response, quantified as the peak MAP increase from baseline, was correlated with the longest interval between consecutive PNA bursts following peptide administration.

Prism (v8) software (GraphPad, Inc., La Holla, CA, USA) was used for statistical analysis. Statistical analyses were performed on raw data. Data were tested for Gaussian distribution and statistical significance was determined by paired or unpaired two-tailed t-tests or by one-, two-, or three-way ANOVA with Sidak’s post-hoc tests - corrected for multiplicity error and performed with repeated measures, as appropriate. For analysis of dose-response curves, data were fitted using inverted bell-shaped dose-response curves and sigmoidal curves using non-linear regression with least squared error variable slope analysis.

In an effort to reduce animal use, interpolated curves incorporating V1aR blockade include low-dose oxytocin data points without concurrent V1aR antagonism since these doses were devoid of V1aR cross-activation. In summary dose-response curves, data are plotted as mean ± SEM for clarity. Corresponding data in the results section are expressed as mean ±
SD. In before-after dot plots of MAP and heart rate, the mean is represented by a horizontal bar and 95% confidence intervals are provided for differences before vs. after treatment. Regardless of statistical test used, $P<0.05$ was considered statistically significant.
8. RESULTS

Oxytocin rescues respiratory depression by fentanyl

As previously reported (Brackley et al., 2020), systemic fentanyl (60 nmol/kg, i.v.) silenced PNA burst discharge in anesthetized rats artificially ventilated to avoid OIRD lethality. To evaluate oxytocin rescue of fentanyl OIRD, graded doses (0, 0.1, 2, 5, 10, 50 nmol/kg, i.v.) were delivered 1 min after the onset of fentanyl-induced PNA burst arrest (Fig. 1A). As expected, vehicle did not rescue PNA, which remained silent for 15.7 ± 1.6 min after administration. Time to recovery of PNA burst frequency and amplitude post-fentanyl averaged 32.4 ± 3.8 and 27.1 ± 4.8 min, respectively. Summary data revealed that the average time required for the first PNA burst to reappear post-oxytocin was dose dependent (F(5,25)=4.327, P=0.0057, Fig. 1B), with latency for burst reappearance averaging 0.5 ± 0.1 min at an oxytocin dose of 5 nmol/kg (P=0.0212). Time to recovery of PNA burst frequency and amplitude post-oxytocin was also dose-dependent (Frequency: F(5,25)=5.459, P=0.0016, Fig. 1C; Amplitude: F(5,25)=5.021, P=0.0025, Fig. 1D). Compared to vehicle, prompt frequency recovery occurred following oxytocin doses of 5 (P=0.0011) and 10 (P=0.0066) nmol/kg and amplitude recovery was evident at the 5 nmol/kg dose (P=0.0369). Frequency recovery by 5 and 10 nmol/kg doses of oxytocin occurred within 2.8 ±1.0 and 7.8 ± 4.2 min, respectively. Amplitude recovery to the 5 nmol/kg dose of oxytocin occurred after 4.7 ± 1.9 min. Interestingly, oxytocin dose-PNA response curves were inverted bell-shaped with similar ED$_{50}$ values for duration of PNA burst arrest (2.2 nmol/kg) and time to recovery of frequency (2.3 nmol/kg) and amplitude (2.2 nmol/kg). Overall, data in Figure 1 indicate that oxytocin can dose-dependently rescue fentanyl OIRD in anesthetized artificially ventilated rats.

Oxytocin rescues cardiodepression by fentanyl

Clinical studies show that high doses of fentanyl cause cardiodepression that can require support with vasoactive drugs (Wynands et al., 1983). To assess oxytocin rescue of fentanyl cardiodepression, arterial blood pressure and heart rate responses to graded doses of
systemic oxytocin were determined post-fentanyl (Fig. 2A). Consistent with clinical observations, fentanyl reduced MAP (Fig. 2B) by an average of 30% (t(60)=8.067, P<0.0001), which was dose-dependently reversed by oxytocin (F(5,25)=10.76, P<0.0001) (Fig. 2C). Although dose-dependence was observed for reversal of the fentanyl-induced depressor response by low doses of oxytocin, higher doses increased MAP significantly above baseline (10 nmol/kg: P=0.091; 50 nmol/kg: P=0.0006). The rate of MAP recovery by oxytocin (Fig. 2D) was also dose-dependent (F(5,25)=17.07, P<0.0001) with rapid reversal of fentanyl cardiodepression following doses of 5 (P=0.0181), 10 (P<0.0001), and 50 (P<0.0001) nmol/kg. Dose-response curves for MAP were sigmoid-shaped and had similar ED$_{50}$ values for MAP (4.2 nmol/kg) and rate of MAP recovery (4.1 nmol/kg) post-oxytocin. Consistent with literature evidence (Gurkan et al., 2005), fentanyl also caused a small but consistent reduction of heart rate (Fig. 2E) that averaged 9% relative to baseline (t(60)=4.694, P<0.0001). Unlike MAP, no dose of oxytocin changed the magnitude (P=0.8933, Fig. 2F) or rate (P=0.2716, Fig. 2G) of heart rate recovery post-fentanyl.

**Oxytocin rescue of OIRD, but not cardiodepression, requires oxytocin receptor activation**

Oxytocin at a dose of 5 nmol/kg rescued fentanyl OIRD (Fig. 1) and depression of MAP (Fig. 2). We next explored dependence on oxytocin receptor activation and found that oxytocin receptor blockade with atosiban (40 nmol/kg, i.v.) prevented PNA resuscitation effects of oxytocin (t(8)=2.875-4.442, P=0.0207-0.0022; Fig. 3A-C). Unexpectedly, hemodynamic effects of oxytocin became more, not less, apparent during oxytocin receptor blockade (F(1.041,4.163)=15.13, P=0.0161, Fig. 3D). When given post-fentanyl, 5 nmol/kg oxytocin effectively reversed fentanyl cardiodepression in the absence (P=0.0090) or presence (P=0.0074) of atosiban and increased MAP above baseline (P=0.0351) only when oxytocin receptors were blocked with atosiban (40 nmol/kg), an effect possibly reflecting oxytocin shunting to vasopressin V1aR (Chini and Manning, 2007). Consistent with established
evidence that the pressor potency of oxytocin is less than that of vasopressin (Altura and Altura, 1984), a 5 nmol/kg dose of oxytocin transiently increased MAP above baseline ($P=0.0148$), while an equimolar dose of vasopressin produced a prolonged pressor effect ($P<0.0001$) relative to that of oxytocin ($P=0.0034$) (F(1,8)=18.88, $P=0.0025$; Fig. S1). Collectively, data in Figure 3 and S1 indicate that, although oxytocin receptor activation is required for oxytocin rescue of OIRD, oxytocin reversal of fentanyl depression of MAP may have contributions independent of the oxytocin receptor.

**V1aR cross-activation compromises OIRD rescue by high-dose oxytocin**

Because oxytocin at higher doses can cross-activate vasopressin receptors (Chini and Manning, 2007), additional experiments were performed in the presence of the V1aR antagonist (Phenylac$^1$, D-Tyr(Et)$^2$, Lys$^6$, Arg$^8$, des-Gly$^9$)-Vasopressin trifluoroacetate (V1aRX, 0.4 nmol/kg), which fully prevented transient vasopressin (1 nmol/kg) cardiorespiratory depression (Fig. 4A). To assess possible oxytocin cross-activation of vasopressin receptors, V1aR were blocked prior to testing for oxytocin reversal of fentanyl cardiorespiratory depression. As a pre-treatment, V1aRX did not affect OIRD reversal by oxytocin at the 5 nmol/kg dose (Fig. 4B), but supported rescue by the higher 50 nmol/kg dose of oxytocin (Fig. 4C), indicating that the inability of high-dose oxytocin to rescue fentanyl OIRD (Fig. 1) reflects cross-activation of V1aR. Cross-activation of V1aR by high-dose oxytocin was responsible for the prolonged latency of PNA burst recovery (F(1,16)=7.098-38.17, $P=0.0170–P<0.0001$, Fig. 4D-F). Notably, resuscitation effects of low-dose oxytocin (5 nmol/kg) on latency to burst reappearance ($P=0.9853$) as well as time to recovery of burst frequency ($P=0.8275$) and amplitude ($P=0.6821$) were unchanged by V1aRX and yet high-dose oxytocin (50 nmol/kg), which alone was ineffective, successfully rescued OIRD during V1aRX.

Analysis of MAP confirmed that V1aRX prevented the acute hypertensive response following high-dose oxytocin (F(1,16)=5.486, $P=0.0324$; Fig. 4G) and the increased rate of MAP
recovery (F(1,16)=4.920, P=0.04114; Fig. 4H). Collectively, data in Figure 4 indicate that oxytocin reversal of fentanyl cardiorespiratory depression is independent of V1aR, and that V1aR cross-activation explains both the MAP overshoot and failure of OIRD rescue by high-dose (50 nmol/kg) oxytocin.

As noted, the dose of oxytocin (5 nmol/kg) that reversed OIRD and acute hypotension by fentanyl, failed to stimulate PNA in the absence of fentanyl (Fig. S1). By contrast, an equimolar dose of vasopressin (5 nmol/kg), which increased MAP considerably more than oxytocin, generally caused acute PNA arrest in the absence of fentanyl, which is consistent with visceral afferent suppression of breathing by arterial baroreceptor inputs (Trzebski et al., 1980). It is therefore not surprising that V1aR cross-activation compromised oxytocin rescue of fentanyl OIRD. Taken together, data indicate that oxytocin does not stimulate basal inspiratory output but rescues fentanyl OIRD at low doses that lack V1aR cross-activation and at high doses during V1aR antagonism.

**Vasopressin does not rescue fentanyl OIRD**

Although oxytocin at a 5 nmol/kg dose did not increase ongoing PNA (Fig. S1), it successfully restored inspiratory drive (Fig. 1) and MAP (Fig. 2) post-fentanyl. To determine whether an equimolar dose of vasopressin can reverse fentanyl cardio-depression and OIRD, vasopressin (5 nmol/kg, i.v.) was given post-fentanyl while recording MAP and PNA responses. Similar to vehicle (Fig. 5A), vasopressin given 1 min after fentanyl failed to rescue OIRD (Fig. 5B) with PNA remaining silent for an additional 13.7 ± 3.9 min without recovery of frequency or amplitude until 22.2 ± 7.5 and 19.9 ± 6.0 min had elapsed. Summary data reveal that fentanyl OIRD was similar following vehicle and vasopressin (t(7)=0.5211-1.293, P=0.2369-0.6184; Fig. 5C). However, significant increases of MAP (F(1,7)=58.43, P=0.0001) and its rate of recovery (t(7)=7.212, P=0.0002) were apparent (Fig. 5D). These data demonstrate that oxytocin rescue of OIRD cannot be reproduced by the closely related vasopressin peptide.
Non-peptide WAY-267464 reversal of fentanyl OIRD

Given that oxytocin receptor activation reverses OIRD in the absence of V1aR cross-activation (Fig. 4), WAY-267464, a non-peptide oxytocin receptor partial agonist and V1aR antagonist, was evaluated for its ability to rescue fentanyl OIRD. Whereas vehicle did not rescue fentanyl OIRD (Fig. 6A), systemic administration of WAY-267464 (75 nmol/kg, i.v.) promptly rescued PNA post-fentanyl (Fig. 6B), reestablishing bursts within 0.2 ± 0.03 min and promoting PNA frequency and amplitude recovery within 1.0 ± 0.2 and 9.9 ± 4.6 min, respectively. Summary data (Fig. 6C) reveal that WAY-267464 reversal of fentanyl OIRD was significant ($t(8)\, P=0.0044–P<0.0001$).

Unlike high-dose oxytocin, which had hemodynamic consequences due to cross-activation of V1aR (Fig. 4G,H), WAY-267464 did not induce acute hypertension ($F(1,8)=0.7894, \, P=0.4002$), but did accelerate MAP recovery ($t(8)=2.451, \, P=0.0399$) post-fentanyl (Fig. 6D). Taken together, these data confirm that a non-peptide oxytocin receptor agonist that lacks V1aR agonist affinity successfully reverses fentanyl OIRD without adverse hemodynamic consequences.
9. DISCUSSION

This study found that oxytocin receptor activation in rats dose-dependently reverses cardiorespiratory depression by fentanyl. At high doses, oxytocin cross-activated V1aR causing transient rebound hypertension and prolonged neural inspiratory arrest post-fentanyl. V1aR antagonism restored high-dose oxytocin rescue of OIRD and averted undesirable hemodynamic consequences. WAY-267464, a non-peptide oxytocin receptor partial agonist and V1aR antagonist, robustly rescued fentanyl cardiorespiratory depression.

Caveats and Limitations

This study used reference grade fentanyl dissolved in methanol and diluted in saline to induce OIRD. Although we previously reported that the same quantity of methanol in saline delivered as a vehicle control had no effect on basal PNA (Brackley et al., 2020), there remains a possibility that results might have been influenced by methanol interactions with fentanyl and/or oxytocin. Our search of the literature revealed no direct evidence for such interactions. Indirect evidence relies on studies of ethanol, which might not be functionally relevant. One study reported that a sustained blood alcohol (ethanol) concentration (BAC) of 0.18% can exacerbate buprenorphine-induced respiratory depression (Cohier et al., 2017). However, the blood methanol concentration in the present study would have only reached a transient spike of ~0.03% based on calculated blood volume (Lee and Blaufox, 1985). In mice, OIRD induced by acute morphine was unaltered by low-dose ethanol (Hill et al., 2016). The above findings combined with evidence that a sustained BAC of 0.03% did not exacerbate fentanyl-induced cognitive deficits in healthy humans (Schneider et al., 1999) suggest it is unlikely that methanol in the present study would have significantly impacted fentanyl actions. Perhaps most importantly, any enhancement of fentanyl OIRD by methanol would indicate that oxytocin receptor agonists have an even greater capacity to rescue cardiorespiratory depression under real-life circumstances when fentanyl is given in the absence of methanol.
Like studies evaluating central oxytocin-induced stimulation to respiration (Henry and Sessle, 1989; Mack et al., 2002), we used anesthetized, paralyzed, vagotomized, and artificially ventilated rats. Therefore, additional studies are needed to establish the translational potential of systemic oxytocin to treat OIRD induced by opioids other than fentanyl and whether oxytocin rescue of OIRD is preserved in the intact, conscious and spontaneously breathing rat. Here, we used phrenic nerve activity to index neural inspiration. It is presently unknown how effectively systemic oxytocin will reverse fentanyl suppression of other respiratory motor outputs.

To avoid OIRD lethality, rats were artificially ventilated with 100% O₂. This unavoidably suppresses central and peripheral chemoreflex activation during fentanyl-induced PNA arrest. We, therefore, do not know if oxytocin reversal of fentanyl OIRD would be affected by concurrent chemoreflex activation. It would seem mostly likely, however, that oxytocin would be even more effective when acting together with major respiratory defense reflexes.

Although systemic oxytocin can successfully treat sleep apnea-related respiratory depression (Jain et al., 2020), which is accompanied by elevated endogenous opioids (Gislason et al., 1989), whether oxytocin can rescue OIRD in individuals with elevated central opioids brought on by chronic opioids use is unknown. Another effect of fentanyl is “wooden chest syndrome”. Although oxytocin’s effect on fentanyl-induced respiratory rigidity and laryngospasm is unknown, oxytocin receptor activation can increase genioglossus activity (Mack et al., 2007), which is expected to improve upper airway patency (Fleury Curado et al., 2017). Evidence indicates that high doses of oxytocin do not alter muscle tone (Yaksh et al., 2014), but it is unknown if oxytocin receptor activation alters fentanyl-induced respiratory muscle rigidity.

**OIRD Resuscitation by Oxytocin Receptor Activation**

Clinically, reversal of OIRD is achieved by antagonizing opioid receptors (Handal et al., 1983), but loss of analgesia and painful withdrawal effects limit the utility of this approach (Dahan et al., 2010). Respiratory stimulants are an alternative approach that might preserve
opioid analgesia (Imam et al., 2020). Here, we provide evidence that peptide (Fig. 1, 3) and non-peptide (Fig. 6) oxytocin receptor agonists effectively reverse cardiorespiratory depression by systemic fentanyl in the rat. Literature evidence indicates that endogenous opioids tonically inhibit oxytocin release from the posterior pituitary (Coombes et al., 1991), which raises the possibility that naloxone rescue of OIRD could, in part, involve increased release of oxytocin.

Central Sites of Oxytocin Action

Oxytocin rescue of fentanyl OIRD is consistent with reports that oxytocin can stimulate respiratory activity when delivered locally into specific nodes of the extended respiratory network, including the nucleus tractus solitarius (Henry and Sessle, 1989) and pre-Bötzinger complex (Mack et al., 2002). Likewise, neurons in the hypothalamic paraventricular nucleus can stimulate breathing by a mechanism dependent on oxytocin receptors (Mack et al., 2007). Oxytocin receptors are widely distributed throughout these and other regions comprising the extended respiratory network, including the parabrachial and Kölliker-Fuse nuclei (Yoshida et al., 2009) where studies have yet to directly assess oxytocin effects on breathing. Consistent with a previous study (Elorza-Avila et al., 2017), the dose of systemic oxytocin that consistently rescued fentanyl OIRD in the present study (5 nmol/kg) failed to stimulate basal PNA (Fig. S1). It is worth noting that systemic naloxone stimulates hypothalamic paraventricular nucleus oxytocin release and does so far more robustly in the presence of exogenous opioids (Coombes et al., 1991). These observations raise the possibility that full OIRD rescue by opioid antagonists might involve paraventricular nucleus release of oxytocin.

Wherever the central sites of opioid-oxytocin interactions might be, they have potential to provide a link between elevated endogenous opioids (Gislason et al., 1989) and greater risk for OIRD in at-risk patients such as those with sleep apnea (Subramani et al., 2017). Indeed, opioid antagonism can improve sleep apnea symptoms (Atkinson et al., 1985), which might reflect oxytocin neuron disinhibition following displacement of endogenous opioids from their
receptors (Heijning et al., 1991). Sleep apnea-related respiratory depression is safely treated with oxytocin (Jain et al., 2020), which might be similarly effective against OIRD in patient populations with elevated endogenous or exogenous opioids, including individuals with opioid use disorder.

As noted previously, we found that systemic oxytocin at the dose most effective at reversing fentanyl OIRD did not stimulate basal inspiratory motor activity (Fig. S1). This suggests that oxytocin selectively interfered with mechanisms of opioid depression of neural inspiration. Indeed, hyperventilation is not a side effect of systemic oxytocin in humans (Page et al., 2017). A possible explanation for differential effects on breathing by systemic versus brain site specific injections of oxytocin (see above) may be explained by pharmacokinetic limitations reflecting slow crossing of the blood-brain-barrier. Indeed, although oxytocin penetrance of the blood-brain barrier has been debated (Leng, 2000), recent evidence indicates that oxytocin does indeed gain access to brain (Yamamoto et al., 2019) such that systemically administered oxytocin is detectable in cerebrospinal fluid (Lee et al., 2018). Alternatively, the inability of systemic oxytocin to stimulate basal breathing could have a pharmacodynamic explanation involving competing actions at multiple peripheral sites. It is possible that we may have seen a left-shift in our oxytocin dose-PNA response curves if oxytocin was delivered intranasally, since this route of administration allows passage through both the blood-brain and blood-cerebrospinal fluid barriers to reach the brain at relatively high concentration (Lee et al., 2020).

**V1aR cross-activation compromises high-dose oxytocin reversal of OIRD**

Systemic vasopressin activation of V1aR increases MAP and depresses ventilation (Zera et al., 2018) (Fig. 4). Oxytocin cross-activation of V1aR has been reported (Chini and Manning, 2007) and can depress ventilation, offsetting oxytocin-induced respiratory stimulation (Fig. 4). Here, we observed that a dose of V1aR antagonist sufficient to prevent vasopressin elevation of blood pressure and suppression of PNA, abrogated high-dose oxytocin-V1aR interactions. In doing so, V1aR blockade restored high-dose oxytocin efficacy to rescue OIRD.
Low-dose oxytocin robustly rescued OIRD in the absence and presence of V1aR blockade, indicating that lower doses of oxytocin do not cross-activate V1aR to a level sufficient to compromise OIRD rescue.

Opioids suppress pituitary release of oxytocin, but enhance release of vasopressin (Hartman et al., 1986). It is therefore tempting to speculate that vasopressin could exacerbate OIRD. Although the present study did not assess fentanyl OIRD during selective V1aR blockade, OIRD recovery post-vasopressin was similar to vehicle (Fig. 5), which indicates that fentanyl did not trigger appreciable pituitary release of vasopressin. These findings suggest that significant vasopressin release might occur only with chronically elevated opioids. Notably, vasopressin is a relatively weak pressor agent in healthy humans (Graybiel and Glendy, 1941), but becomes critical when coping with hemodynamic challenges associated with hemorrhage or dehydration (Landry et al., 1997). In humans, vasopressin has been reported to transiently reduce then stimulate cardiorespiratory function, whereas oxytocin can increase $O_2$ consumption with negligible hemodynamic effects (Grollman and Geiling, 1932). Therefore, in humans, oxytocin-V1aR interactions might be functionally muted compared to rats, such that high-dose oxytocin, even without concurrent V1aR blockade, might be effective at reversing OIRD.

**Advantages of non-peptide WAY-267464**

Here, we showed that WAY-267464 can reverse fentanyl OIRD (Fig.6). WAY-267464 binds to human and rodent oxytocin receptors (Ring et al., 2010) and acts as a weak V1aR antagonist (Hicks et al., 2012). Functional studies report that pretreatment with WAY-267464 attenuate oxytocin-V1aR interactions (Hicks et al., 2014), consistent with WAY-267464 circumventing loss of OIRD rescue by high-dose oxytocin (Fig. 4). Because WAY-267464 is a non-peptide compound, it is not recognized by endogenous peptidases (Evans et al., 1992), which prolongs its half-life and allows for oral bioavailability. WAY-267464 readily crosses the blood-brain barrier and has oxytocin receptor actions greater than oxytocin (Ring et al., 2010).
Although we identified no studies using the i.v. route of administration for WAY-267464 in awake, freely moving animals, a dose of oxytocin that exceeds the maximum dose tested in this study was reported to have no adverse side effects in the rat (Klockars et al., 2017). Additionally, i.v. oxytocin bolus at a dose similar to the ED$_{50}$ for reversal of fentanyl OIRD in rats has been clinically used with opioid-containing epidurals without prominent side effects (Adnan et al., 2018).

Other oxytocin receptor agonists might also reverse fentanyl OIRD. Carbetocin is a relatively heat-stable peptide with a longer half-life than oxytocin (Manning et al., 2012). The long-acting analog oxytocin-Gly could be especially effective as it has greater potency compared to WAY-267494 (Snider et al., 2019). Like oxytocin, high doses of these compounds might require V1aR antagonism to avoid confounding effects of oxytocin-V1aR interactions. Since oxytocin is used clinically to induce parturition, targeting oxytocin receptors in late pregnancy for OIRD rescue might stimulate contractions. Indeed, naloxone use is largely contraindicated in pregnancy to avoid preterm labor (The ASAM National Practice Guideline for the Treatment of Opioid Use Disorder, 2020), which might reflect naloxone-induced oxytocin release.

**Oxytocin and the Opioid Epidemic**

Opioids can cause hyperalgesia, addiction, and lethal cardiorespiratory arrest (Zedler et al., 2018; Volkow et al., 2019). Here, we have laid critical pre-clinical groundwork for oxytocin receptor-mediated reversal of OIRD. The oxytocin receptor agonists oxytocin (Fig. 1,3) and WAY-267464 (Fig. 6) reversed cardiorespiratory depression by fentanyl. Activation of oxytocinergic neurons (Eliava et al., 2016) and systemic administration of oxytocin at doses absent of oxytocin-V1aR interaction produces analgesia in rodents (Juif and Poisbeau, 2013) and might also provide analgesia to humans (Boll et al., 2018). Oxytocin attenuates opioid-seeking behavior in animals (Ibragimov et al., 1987), but not at high doses (Moaddab et al., 2015). One recent randomized, double-blind study found that oxytocin can reduce opioid...
craving and elevations of the stress hormone cortisol related to withdrawal (Moeini et al., 2019). Oxytocin receptor activation also has the potential to benefit withdrawal-related emotional responses (Zanos et al., 2014). Targeting oxytocin receptors could reduce opioid cravings, protect against overdose, and provide analgesia that allows for opioid titration to safer efficacious levels. *In vitro*, oxytocin can act as a positive allosteric modulator of opioid receptor signaling and enhance fentanyl’s potency (Meguro et al., 2018), however, we showed here that systemic oxytocin, *in vivo*, opposes fentanyl OIRD. Positive modulation of opioid signaling by oxytocin could be cell-type specific and beneficial if restricted to peripheral tissues.

**Conclusions**

Our findings show that systemic oxytocin dose-dependently reverses fentanyl OIRD in rats. At high doses, oxytocin cross-activates V1aR, subverting reversal of respiratory arrest. V1aR blockade restores high-dose oxytocin rescue of OIRD and prevents undesirable hemodynamic effects. The non-peptide oxytocin receptor partial agonist and V1aR antagonist WAY-267464 rescues fentanyl OIRD without adverse hemodynamic consequences. We conclude that peptide and non-peptide agonist activation of oxytocin receptors rescues fentanyl cardiorespiratory depression. Selective targeting of oxytocin receptors for resuscitation from opioid overdose could eliminate or attenuate negative side effects associated with opioid receptor antagonism, including loss of analgesia, mood disturbances and painful withdrawal symptoms.
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11. AUTHORSHIP CONTRIBUTIONS

Participated in research design: Brackley and Toney.

Conducted experiments: Brackley.

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Wrote the manuscript: Brackley and Toney.
12. REFERENCES


13. FOOTNOTES
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14. FIGURE LEGENDS

Figure 1: Low-dose oxytocin rescues respiratory depression by fentanyl.

(A) Representative integrated phrenic nerve activity (PNA, top) and burst frequency (bottom) responses to vehicle (VEH) and graded systemic doses (0.1, 2, 5, 10, 50, nmol/kg) of oxytocin (OT, i.v., n=5-6/dose) (grey-black triangles) following fentanyl (FENT, 60 nmol/kg, i.v., red triangles/trace). Note, boxed panel denotes OT dose effective for reversal of FENT respiratory depression. Summary data for reversal of FENT action on PNA burst (B) arrest, (C) frequency and (D) amplitude post-OT (black circles). Black triangles on the abscissa and ordinate indicate ED_{50} and E_{\text{max}} values, respectively. Symbols denote significance vs. VEH (one-way ANOVA with Sidak posthoc test). In B: *P=0.0255. In C: †P=0.0012 for 5 nmol/kg and P=0.0076 for 10 nmol/kg. In D: *P=0.0451. Data in B, C, D are expressed as mean ± SEM for clarity. a.u. = arbitrary units.

Figure 2: Oxytocin dose-dependently rescues cardio-depression by fentanyl.

(A) Representative blood pressure (mean arterial pressure, MAP - superimposed white line) and heart rate (HR) responses to systemic fentanyl (FENT, 60 nmol/kg, i.v., red triangle/trace) followed by vehicle (VEH) or graded systemic (i.v.) doses (0.1, 2, 5, 10, 50, nmol/kg, n=5-6/dose) of oxytocin (OT) (grey-black triangles). (B) MAP values at baseline (BL, black circles) and following FENT (red X's) prior to each dose of OT with treatment difference from BL (Δ, grey circles) plotted with 95% confidence intervals (CI, right). (C) Summary of MAP responses to OT (black circles) post-FENT. (D) Summary of rate of MAP recovery during first 10 s post-OT (black circles). (E) HR values at BL (black circles) and following FENT (red X's) prior to each dose of OT with treatment difference from BL (Δ, grey circles) plotted with 95% CI (right). (F) Summary of HR responses to OT (black circles) post-FENT. (G) Summary of rate of HR recovery during first 10 s post-OT (black circles). In B-G: n=31 rats. In B,E: § denotes
P<0.0001 between BL and post-FENT (paired two-tailed t-test). In C: *, †, ‡ denote P=0.0191, P=0.0068 and P=0.0006 vs. BL (repeated measures two-way ANOVA with Sidak posthoc tests). In D: *, § denote P=0.0181 and P<0.0001 vs. VEH, respectively (one-way ANOVA with Sidak posthoc test). In C,F: grey and red horizontal lines reference MAP values at BL and post-FENT, respectively. In C,D: black triangles on the abscissa and ordinate indicate values of ED$_{50}$ and E$_{max}$, respectively. For analysis of dose-response curves (C,D,F,G), data expressed as mean ± SEM for clarity. BPM = beats per min.

**Figure 3: Oxytocin reversal of fentanyl OIRD requires oxytocin receptors.**

Representative blood pressure (mean arterial pressure, MAP - superimposed white line), phrenic nerve activity (PNA) and PNA frequency responses to oxytocin (OT, 5 nmol/kg, i.v., dark grey triangle/trace) in the (A) absence and (B) presence of OT receptor antagonist atosiban (40 nmol/kg, i.v., n=5, blue triangle/trace) post-fentanyl (FENT, 60 nmol/kg, i.v., red triangle/trace). (C) Summary data for reversal of FENT action on PNA burst arrest, frequency and amplitude in the absence (black circles) or presence (blue triangles) of atosiban. (D) Summary data for MAP response at baseline (BL, light grey) and post-atosiban (blue), - FENT (red), and -OT (dark grey) in the absence (left/circles) or presence (right/triangles) of OT receptor blockade. In C: symbols denote significance of 5 nmol/kg OT alone vs. 5 nmol/kg OT + atosiban (unpaired two-tailed t-test) for time to recovery of PNA burst arrest (*P=0.0207), frequency (†P=0.0066) and amplitude (‡P=0.0022). In D: red † denotes P=0.0090 (-Atosiban) and P=0.0074 (+Atosiban) vs. FENT, blue ‡ denotes P=0.0007 vs. +Atosiban, black * denotes P=0.0351 vs. BL, and ns = not significant (repeated measures two-way ANOVA with Sidak posthoc test). a.u. = arbitrary units.

**Figure 4: Vasopressin V1aR blockade restores rescuing actions of high-dose oxytocin.**

(A) Representative blood pressure (mean arterial pressure, MAP - superimposed white line), phrenic nerve activity (PNA) and burst frequency responses to vasopressin (VP, 1 nmol/kg, i.v.,
n=4, black triangle/trace) before and during VP V1a receptor antagonism (V1aRX, 0.4 nmol/kg, i.v., orange triangle/trace). Representative MAP, PNA and burst frequency responses to OT doses of (B) 5 nmol/kg (n=6, grey triangle/trace) and (C) 50 nmol/kg (n=4, black triangle/trace) following FENT in the presence of V1aRX (orange triangle/trace). Summary data for time to reversal of FENT action on PNA burst (D) arrest, (E) frequency and (F) amplitude post-OT in the absence (black circles) and presence of V1aRX (orange triangles). Summary data for (G) MAP response and (H) rate of MAP recovery post-OT in the absence (black circles) or presence of V1aRX (orange triangles). In D-F: black symbols denote significance vs. vehicle (VEH) (one-way ANOVA with Sidak posthoc test) for V1aRX + 5 nmol/kg OT (PNA burst arrest: *P=0.0221, frequency: ‡P=0.0002, amplitude: ‡P=0.0004) and V1aRX + 50 nmol/kg OT (PNA burst arrest: *P=0.0414, frequency: ‡P=0.0005, amplitude: †P=0.0011). Orange symbols denote significance (two-way ANOVA with Sidak posthoc tests) of 50 nmol/kg OT vs. V1aRX + 50 nmol/kg OT (PNA burst arrest: §P<0.0001, frequency: §P<0.0001, amplitude: †P=0.0011). In G: grey and red horizontal lines reference MAP values at baseline and post-FENT, respectively. In G,H: * denotes P=0.0275 (G) and P=0.0204 (H) comparing 50 nmol/kg OT alone vs. baseline. In G,H: orange * and † denote P=0.0135 and P=0.0013, respectively, comparing 50 nmol/kg OT alone vs. 50 nmol/kg OT with V1aRX. Data analyzed by repeated measures three-way (G) and two-way (H) ANOVA, respectively, each with Sidak posthoc tests. Red downward arrows indicate change in dose-response curve by V1aR blockade. Black triangles on the abscissa and ordinate in D-G indicate values of ED_{50} and E_{max}, respectively. Dose-response data expressed as mean ± SEM. a.u. = arbitrary units.

Figure 5: Vasopressin does not reverse fentanyl OIRD.

Representative integrated phrenic nerve activity (PNA) and PNA frequency and blood pressure (mean arterial pressure, MAP - superimposed white line) responses to (A) vehicle (VEH, i.v., n=5, grey triangle/trace) and (B) vasopressin (VP, 5 nmol/kg, i.v., n=4, black triangle/trace) post-
fentanyl (FENT, 60 nmol/kg, i.v., red triangle/trace).  (C) Summary data for time to reversal of FENT action on PNA burst arrest, frequency and amplitude post-VEH (grey circles) or VP (black squares).  (D) Summary data for MAP response and rate of MAP recovery over 10 s following VEH (grey circles) and VP (black squares) given post-FENT. Grey and red horizontal lines (left) reference baseline and post-FENT values of MAP. In C: ns = not significant (unpaired two-tailed t-test). In D: *, † (left) denote $P=0.0156$ and $P=0.0004$ vs. BL and § denotes $P<0.0001$ vs. VEH (repeated measures two-way ANOVA with Sidak posthoc test). For rate of MAP recovery (right), ‡ denotes $P=0.0002$ vs. VEH (unpaired two-tailed t-test). a.u. = arbitrary units.

Figure 6. Non-peptide WAY-267464 reversal of fentanyl OIRD.

Representative blood pressure (mean arterial pressure, MAP - *superimposed white line*), phrenic nerve activity (PNA) and PNA frequency responses to (A) vehicle (VEH, i.v., n=5, grey triangle/trace) and (B) WAY-267464 (WAY, 75 nmol/kg, i.v., n=5, black triangle/trace) post-fentanyl (FENT, 60 nmol/kg, i.v., red triangle/trace).  (C) Summary data for reversal of FENT action on PNA burst arrest, frequency, and amplitude post-VEH (grey circles) or WAY (black circles).  (D) Summary data for MAP response and rate of MAP recovery over 10 s following FENT by VEH (grey circles) or WAY (black circles). Grey and red horizontal lines (left) reference baseline and post-FENT values of MAP. In C: symbols denote significance vs. VEH (unpaired two-tailed t-test) for PNA burst arrest ($\S P<0.0001$), frequency ($\dagger P=0.0011$) and amplitude ($\dagger P=0.0044$). In D: * denotes $P=0.0399$. a.u. = arbitrary units.
Figure 1

Phrenic Nerve Activity

A

B

C

D

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A) Phrenic Nerve Activity and Blood Pressure in response to FENT (60 nmol/kg) vs VEH (Saline).

B) Phrenic Nerve Activity and Blood Pressure in response to VP (5 nmol/kg).

C) PNA Burst Recovery in response to VEH (Saline) vs VP (5 nmol/kg).

D) MAP and Rate of MAP Increase in response to VEH vs VP.

VEH (Saline) vs VP (5 nmol/kg)
Figure 6

(A) Phrenic Nerve Activity and Blood Pressure in response to FENT (60 nmol/kg) and VEH (2.5% DMSO/Saline).

(B) Phrenic Nerve Activity and Blood Pressure in response to WAY-267464 (75 nmol/kg) and FENT (60 nmol/kg).

(C) PNA Burst Recovery: ○ VEH (2.5% DMSO/Saline) ● WAY-267464 (75 nmol/kg).

(D) MAP and Rate of MAP Increase: VEH WAY.