A comparison of breathing stimulants for reversal of synthetic opioid-induced respiratory depression in conscious rats

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Running Title Page

Stimulant reversal of opioid-induced respiratory depression

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Abstract

Potent synthetic opioids are an important cause of death in the United States' opioid epidemic, and a breathing stimulant may have utility in treating opioid overdose. We hypothesized that sufentanil-induced respiratory depression may be reversed by breathing stimulant administration.

METHODS: Using nose-only plethysmography and arterial blood analysis, we compared effects of several breathing stimulants in reversing sufentanil-induced respiratory depression in conscious rats. We studied taltirelin (1 mg/kg IV), PKTHPP (5 mg/kg IV), CX717 (30 mg/kg IV), BIMU8 (1 mg/kg IV), A85380 (30 mcg/kg IV), 8-OH-DPAT (150 mcg/kg IV/IM), and used sufentanil (10 mcg/kg IV).

RESULTS: By plethysmography (in % baseline, mean±SEM), taltirelin restored ventilation in sufentanil-treated rats (from 50±5 to 102±8%) by increased breathing rates (from 80±4 to 160±12%). By arterial blood analysis, however, taltirelin did not correct hypoxia, decreased hypercarbia only after 45 min, and worsened metabolic acidosis (base excess from +0±1 to -7±1 mEq/L). Additionally, taltirelin increased exhaled carbon dioxide, an estimate of oxygen consumption, by up to 64%. PKTHPP, CX717, BIMU8, and A85380 failed to significantly change ventilation or arterial blood values in sufentanil-treated rats. 8-OH-DPAT, however, improved ventilation (from 54±8 to 92±10%), reversed hypercarbia (from 64±6 to 47±2 mmHg) and shortened time-to-righting from 43±4 to 15±1 min in sufentanil-treated rats placed supine.

CONCLUSION: Taltirelin has limited therapeutic potential as its ventilatory effects are offset by metabolic acidosis, possibly from increased oxygen consumption. At the doses studied, PKTHPP, CX717, BIMU8, and A85380 have limited effects in reversing sufentanil-induce respiratory depression; 8-OH-DPAT, however, warrants further study.
Significance Statement

Respiratory depression is an important cause of death following potent synthetic opioid overdose. 8-OH-DPAT or related compounds may be useful in treating respiratory depression as caused by potent synthetic opioids.
Introduction

Opioids are highly-effective drugs used in pain management, however they impose a range of undesired and sometimes lethal side-effects including sedation, respiratory depression, skeletal muscle rigidity, and impaired coordination (Dahan et al., 2010). Because they cause euphoria, opioids are also addictive and a common drug-of-abuse (Volkow et al., 2016).

There are three general opioid classes: 1) natural opiates found in opium (e.g., morphine and codeine); 2) semi-synthetic, chemical derivatives of opiates (e.g., heroin, hydromorphone); and 3) fully synthetic (e.g., fentanyl and its many derivatives). Fentanyl and several derivatives (e.g., sufentanil, alfentanil, and remifentanil) are widely used in anesthesia and pain management and are appreciated for their rapid onset and transcutaneous and transmucosal bioavailability. However, because of their marked clinical potency and ease of chemical synthesis and derivatization, fentanyl and its many derivatives have become endemic in the illicit drug supply fueling the ongoing United States opioid epidemic health crisis and are a major contributor to overdose deaths (Wilson et al., 2020). Potent synthetic opioids are also recognized as a chemical threat that, through weaponization (e.g., aerosolization) or accidental release, might cause mass casualty (Tsou et al., 1989; Schiermeier, 2002; Doucette, 2017; Shafer, 2019; Yeung et al., 2020).

Naloxone, an opioid antagonist drug, provides rapid and reliable reversal of opioid effects, such as respiratory depression, when administered via multiple routes (e.g., intravenous, nasal, and intramuscular). Due to its mechanism of action and short duration of action, however, large and repeat naloxone dosing followed by continuous administration is sometimes required to revive an individual suffering potent synthetic opioid overdose, which can complicate medical management and stress healthcare resources (Sutter et al., 2017; Uddayasankar et al., 2018).
Because respiratory depression is the primary cause of opioid overdose death, when naloxone therapy is insufficient, breathing stimulant drugs may potentially reverse opioid-induced respiratory depression, by themselves or in tandem with naloxone, to preserve life and to mitigate a need for endotracheal intubation and mechanical ventilation, an invasive and resource intensive therapy undertaken by skilled providers.

Our overall goal is identification of an easily administered (e.g., IM or nasal) rescue drug that will provide rapid and prolonged reversal of potent synthetic opioid-induced respiratory depression. In prior work, we had determined that intravenous- and intratracheally-administered thyrotropin releasing hormone (TRH) and its long-acting, serum-stable analog, taltirelin, were effective in reversing morphine-induced respiratory depression in isoflurane-anesthetized rats (Boghosian et al., 2018). TRH is a neurostimulant tripeptide hormone known classically for its role in thyroid hormone homeostasis and its effects in decreasing anesthesia "sleep time" in rodents. In the current project, we tested the hypothesis that taltirelin would provide similar effect in reversing morphine- and sufentanil-induced respiratory depression in restrained, conscious rats as measured by nose-only plethysmography and arterial blood gas analysis; sufentanil has a clinical potency and a mu opioid receptor binding affinity ~1000-fold and ~10-fold greater than morphine, respectively (Volpe et al., 2011). Unfortunately, taltirelin beneficial effects were limited by worsening metabolic acidosis, perhaps due to increased oxygen consumption. For this reason, we studied in a similar fashion PKTHPP, a potassium channel antagonist (Cotten, 2013; Cotten, 2016), and additional breathing stimulants identified by others for their effects in reversing sufentanil-induced respiratory depression including 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetratin), a 5-HT$_{1A}$ and 5-HT$_7$ serotonin receptor agonist (Sahibzada et al., 2000; Meyer et al., 2006), CX717, an ampakine glutamate receptor positive...
modulator (Ren et al., 2009), BIMU8, a 5-HT₄ receptor agonist (Manzke et al., 2003), and A85380, an α4β2 nicotinic acetylcholine receptor agonist (Ren et al., 2019). We also studied the effect of prazosin co-administration, an α-1 adrenergic receptor antagonist, known to reverse opioid-induced skeletal muscle rigidity (Tsou et al., 1989).

Methods

**Animal Studies.** All studies were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and used 69 male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 303 to 550 grams, with none excluded. Femoral artery catheters, tunneled and exteriorized through a dorsal incision, were implanted by the vendor. Animals were housed in the MGH Center for Comparative Medicine.

All experiments were performed on animals restrained in a custom built full-body acrylic chamber using a size-appropriate Allay neck collar (Supplemental Figure S1). Animals were acclimated to restraint on two separate sessions, at least 30 min each, on two days prior to study and again for 30 min on the study day after recovery from anesthesia and just prior to baseline breathing and/or arterial blood gas measurements. Unless noted, all study compounds were administered intravenously (IV) through a 24G lateral tail vein angiocatheter placed under brief (~3 to 5 min) inhaled isoflurane anesthesia (3 to 5%). An automated heat lamp was used to maintain isoflurane-anesthetized rat body temperature at 37°C via a rectal thermistor. Because conscious animals become agitated by the rectal thermistor and expel it, we did not measure body temperature in conscious rats and allowed them to autoregulate their body temperature.

**Breathing Studies.** For nose-only breathing studies, the restrained rat's nose and mouth, facilitated by the collar restraint, were positioned in an acrylic "nose chamber" 2.5 x 5 x 5 cm (internal dimension) via a 0.7 cm diameter laser-cut aperture in a latex diaphragm (Supplemental
Figure S1; Hygenic Dental Dam, Benco Dental, Pittston, PA). The nose chamber was continuously flushed with fresh air (1 L/min) via two Luer ports using a mass flow controller (Model GE50A with Type 247 power supply; MKS Instruments Inc., Andover, MA). When used, isoflurane was administered by directing air flow upstream of the nose chamber through a variable bypass vaporizer. Rat produced carbon dioxide in the exiting gas flow and isoflurane concentration within the nose chamber were measured using a Capstar-100 (CWE, Inc.; Ardmore, PA) and a Capnomac Ultima (GE Healthcare, Buckinghamshire, U.K.), respectively, both calibrated, daily, using a calibration gas mixture (Part 20731570-001; GE Healthcare). Gas flow exiting the nose chamber was quantified using a heated pneumotachometer (Model 8420; Hans Rudolph Inc., Shawnee, KS) and a differential pressure transducer and demodulator (Models MP45-14871 and CD15; Validyne Engineering, Northridge CA). The flow and carbon dioxide analog signals were acquired (128 Hz, 4 sec time epochs) and analyzed using LabView 2014 software (National Instruments, Austin, TX) run on an Apple computer (Cupertino, CA) interfaced with three USB-6009 data acquisition boards (National Instruments). Oscillations in the gas flow exiting the nose chamber, as imparted by rat breathing, were used to measure breathing frequency and minute ventilation and as detailed elsewhere (Boghosian et al., 2018). Minute ventilation was estimated by numerically integrating the gas flow signal after digital subtraction of the baseline 1 L/min gas flow. The system was calibrated each day by occluding the nose chamber latex diaphragm aperture and by attaching a rodent ventilator (Model 683; Harvard Apparatus; Holliston, MA) providing a known minute ventilation (150 ml/min) through a Luer port into the nose chamber. Tidal volume within each 4 sec time epoch was calculated by dividing the measured minute ventilation by the frequency.
**Arterial Blood Gas Studies.** For talitirelin, arterial blood gas analysis and nose-only plethysmography breathing studies were conducted in separate sessions. However, for other study compounds, 8-OH-DPAT, prazosin, BIMU8, A85380, CX717, arterial blood gas samples were collected during (i.e., simultaneous with) nose-only plethysmography breathing studies. The arterial catheter was accessed through an opening in the plexiglass restraint (Supplemental Figure S1). Arterial blood samples (approximately 0.3 ml, heparinized at ~3 units/ml after withdrawal), taken at baseline and at several time points following study compound administrations, were analyzed immediately using a Vetscan iStat 1 (Abaxis, Union City, CA) blood gas analyzer loaded and CG4+ cartridges (Abbott Laboratories, Princeton, NJ).

**Loss of Righting Reflex Studies.** After tail vein catheterization and after 30 min anesthesia recovery time, sufentanil was administered intravenously, a stopwatch was started, and the rat was placed supine with all four paws "in the air". Five minutes after sufentanil injection, 8-OH-DPAT or saline (total volume 1 ml/kg) were administered intramuscularly into the right gastrocnemius muscle. When the animal righted with all four paws "on the ground", the stopwatch was stopped and the time recorded.

**Drugs and Study Compounds (see also Supplemental Figure S2).** Taltirelin (MedChem Express; Monmouth Junction, NJ), 8-OH-DPAT (Sigma-Aldrich, St. Louis, MO), prazosin (Sigma-Aldrich), BIMU8 (Santa Cruz Biotechnology, Inc.; Dallas, TX), A85380 (R&D Systems; Minneapolis, MN), and naloxone (Sigma-Aldrich) were all solubilized in sterile 0.9% saline prior to administration. CX717 (custom synthesized by Aberjona Laboratories; Woburn, MA) was solubilized in 10% 2-hydroxypropyl-beta-cyclodextrin (Sigma-Aldrich) in sterile 0.9% saline. PKTHPP (custom synthesized by Aberjona Laboratories; Woburn, MA) was solubilized in DMSO. Isoflurane was purchased from Patterson Veterinary (Greeley, CO), and morphine
and sufentanil from McKesson Medical-Surgical (San Francisco, CA). Excluding PKTHPP, all study drugs/compounds were administered in a final total volume of 1 ml. In PKTHPP studies, all animals received 1 ml/kg of DMSO total, which we know from prior work to be well-tolerated. All study compounds were flushed into the vein using 0.5 ml of sterile 0.9% saline. For each breathing stimulant compound, we used a higher or a slightly higher dose than used in published studies involving opioid-induced respiratory depression (Table 1).

**Statistical Analysis.** All data are presented as mean ± S.E.M. Statistical analysis was performed using Prism 8.0 for Mac OS X software (GraphPad Software, Inc., La Jolla, CA). Comparisons were conducted using one-way ANOVA analysis followed by Sidak’s multiple comparisons post-test. P-values less than 0.05 indicate statistical significance.

**Results**

**Taltirelin Effects on Morphine- and Sufentanil-induced Respiratory Depression.**

After collecting 15 min of baseline breathing data, rats were injected intravenously with opioid, and after 5 min, with taltirelin or vehicle; breathing data were collected for an additional 40 min (Figures 1 and 2). Morphine (10 mg/kg IV; Figure 1) and sufentanil (10 mcg/kg IV; Figure 2) caused a maximum reduction in minute ventilation to 47±4% (n = 7) and 15±4% (n=6) of baseline, respectively. Sufentanil, immediately upon administration, caused a period of apnea (~4 to 8 seconds) followed by a brief period of shallow, irregular breathing (Supplemental Figure S1). Taltirelin (1 mg/kg IV) restored minute ventilation in both morphine- and sufentanil-treated rats by 45 min, primarily through effects on breathing rates up to 166±14% of baseline at 54 min (n =8) and up to 163±10% at 54 min (n =6), respectively. Rats co-administered opioid and taltirelin displayed tail quivering and, on occasion, profuse oral secretions, although these effects
were not quantified. By arterial blood gas analysis (Figure 3), morphine and sufentanil both caused respiratory acidosis (i.e., decreased arterial pH and increased carbon dioxide pressure; Figure 3A and 3B) and hypoxia (i.e., a decreased arterial oxygen pressure; Figure 3C). Taltirelin treatment decreased arterial carbon dioxide pressure, but only by 45 min following administration, and failed to correct hypoxia. Additionally, in morphine- and in sufentanil-treated rats, taltirelin caused a significant lactic metabolic acidosis as indicated by increases in arterial lactate and decreases in base excess relative to opioid-only treated rats (Figure 3D and 3E).

In prior studies of isoflurane-anesthetized, morphine-treated rats -- and in contrast to our current results in conscious rats (Figure 3) -- taltirelin caused normalization of both arterial oxygen and carbon dioxide pressure levels within 15 min of administration (Boghosian et al., 2018). We hypothesized that isoflurane and taltirelin effects on oxygen consumption might contribute to this discrepancy. We therefore measured exhaled, steady-state carbon dioxide levels as an estimate of oxygen consumption in both conscious and isoflurane-anesthetize, morphine-treated rats. We used morphine because sufentanil bolus administration to isoflurane-anesthetized rats is consistently lethal. Isoflurane, despite maintenance of body temperature at 37°C, caused a significant decrease in basal carbon dioxide production by greater than 65% from 42.6 ± 7.4 to 15.4 ± 0.9 ml/kg/min, respectively (Figure 1D; n = 16 and 6, respectively). Relative to opioid-only treated rats, taltirelin administration increased carbon dioxide production by up to 64% (Figures 1D and 2D).

**PKTHPP, 8-OH-DPAT, CX717, BIMU8, and A85380 Effects on Sufentanil-Induced Respiratory Depression.** Because opioid-plus-taltirelin-treated rats fared poorly, both subjectively (i.e., quivering tail and profuse oral secretions) and objectively (i.e., persistent
hypoxia and metabolic acidosis), we studied and compared the effects of additional breathing stimulants in reversing sufentanil-induced respiratory depression. 8-OH-DPAT improved minute ventilation, breathing rate, pH, and PaCO2 (Figures 4 through 6). Co-administration of prazosin, an antihypertensive with muscle relaxant properties, with 8-OH-DPAT improved tidal volume (at the expense of minute ventilation and rate) (Figure 5), which led to improved oxygenation (Figure 6C). However, other than an increase in tidal volume at one time point for BIMU8, none of the agents, PKTHPP, CX717, BIMU8, and A85380, caused significant changes in breathing or arterial blood gas and chemistry measurements (Figures 5 and 6 and Supplemental Figures S3 through S6).

Intramuscular 8-OH-DPAT Effects on Sufentanil-Induced Respiratory Depression, Sedation and Immobility. Because bioavailability and route of administration are important considerations in a rescue agent, we studied 8-OH-DPAT when administered by intramuscular injection. Intramuscular 8-OH-DPAT caused normalization of minute ventilation within 10 mins of administration (Figure 7) to sufentanil-treated rats. 8-OH-DPAT did increase exhaled carbon dioxide, which will need to be explored in future studies (Figure 7D).

To determine if 8-OH-DPAT reverses sufentanil-induced sedation and immobility, we placed rats supine immediately following sufentanil (10 mcg/kg IV) administration. Intramuscular injection with 8-OH-DPAT shortened time to righting from 43 +/- 4 to 15 +/- 1 min (n = 8 and 9; P<0.001; unpaired Student's t test). Sufentanil-saline-injected rats had a slow, continuous transition to righting, sometimes laying on their side for a period; sufentanil-8-OH-DPAT-injected rats, in contrast, tended to flip abruptly from supine to prone shortly after first signs of behavioral arousal. After righting, sufentanil-saline-injected rats remained immobile,
whereas sufentanil-8-OH-DPAT injected rats commenced exploring, despite some jerkiness in their movement.

**Discussion**

We compared the effects of several breathing stimulants in reversing sufentanil-induced respiratory depression in conscious rats. We studied taltirelin, PKTHPP, CX717, BIMU8, A85380, and 8-OH-DPAT using plethysmography and arterial blood analysis. Although taltirelin stimulated breathing, its effects were offset by metabolic acidosis, possibly from increased oxygen consumption. PKTHPP, CX717, BIMU8, and A85380 failed to provide significant breathing stimulation; however, 8-OH-DPAT did, and co-administration of prazosin improved tidal volume and oxygenation. Intramuscular 8-OH-DPAT was effective in breathing restoration and decreased time to righting in sufentanil-treated rats.

The taltirelin results were informative. We had previously determined that taltirelin 1 mg/kg IV fully-corrected the hypoxia and hypercarbia caused by 5 mg/kg IV morphine in isoflurane-anesthetized rats (Boghosian et al., 2018). In conscious rats, taltirelin worsened morphine-induced hypoxia and partially corrected hypercarbia despite ventilation normalization (Figures 1 and 3). We used a higher morphine dose (10 mg/kg IV) in the current study to provide an equivalent level of respiratory depression (~50%). As others have observed (Li et al., 2012), isoflurane caused a decrease (>65%) in steady-state carbon dioxide production (Figures 1 and 3), an estimate of oxygen consumption, which may preserve oxygenation and lessen hypercarbia. Anesthesia also prevented muscle rigidity, tail quivering, and sometimes profuse oral secretions. Finally, taltirelin caused a decrease in arterial blood base excess and an increase in lactate levels in sufentanil-treated rats (Figure 3). Lactic metabolic acidosis implies increased
anaerobic metabolism from oxygen supply-demand mismatch. Taltirelin stimulates oxygen consumption (Puissant et al., 2015), and increased carbon dioxide production. Other contributors to metabolic acidosis might be inadequate oxygen uptake, delivery, and/or impaired utilization. Consideration of oxygen consumption is very important in evaluating the therapeutic potential of a breathing stimulant and has not been previously reported upon in the context of opioid reversal; any drug/compound that enhances oxygen consumption will stimulate breathing, but may have less therapeutic potential as it could promote hypoxia, hypercarbia, and acidosis. Therefore, our taltirelin observations identified novel, important, and frequently ignored considerations in breathing stimulant evaluation: 1) presence of anesthesia; 2) stimulant effects on breathing as well as arterial blood gas and chemistry; and 3) stimulant effects on oxygen consumption.

We studied stimulants with differing pharmacologic targets and previously shown effective in reversing opioid-induced respiratory depression (Table 1) using similar dosing. No one has previously published a study directly comparing multiple stimulants, which is essential in assessing their relative translational potential and in determining which warrant further investigation (e.g., large animal studies). Of these, 8-OH-DPAT was the only to increase minute ventilation and to decrease arterial carbon dioxide pressures relative to sufentanil-only treated rats. The other stimulants, however, did trend towards improved ventilation and decreased arterial carbon dioxide. The negative results for some may be due to: 1) opioid type, dose, and administration rate; and 2) lack of anesthesia. A high opioid dose with rapid administration likely causes a greater degree of respiratory depression, rigidity, hypoxia, and acidemia. This is illustrated by arterial blood gas and chemistry results from rats treated with an equipotent morphine and sufentanil dose (Figure 3). Finally, some prior studies of stimulants were
conducted using anesthetized animals (Table 1). Although general anesthetics do worsen respiratory depression, they decrease muscle rigidity, oral secretions (our subjective impression), and oxygen consumption. Therefore, anesthetics may facilitate stimulant effects, as we observed with taltirelin (Figures 1 and 2)(Boghosian et al., 2018).

Prior studies have demonstrated 8-OH-DPAT reverses or prevents opioid-induced respiratory depression in rats and goats (Table 1) (Sahibzada et al., 2000; Meyer et al., 2006; Dutschmann et al., 2009; Guenther et al., 2009). Because 8-OH-DPAT showed the greatest effects in our study and because it provides beneficial effects in a larger mammal, the etorphine-treated goat, we also undertook studies studies to address its potential as a first responder rescue agent. By plethysmography, intramuscular 8-OH-DPAT administration was as effective as intravenous in reversing sufentanil-induced respiratory depression (Figure 7); of note, we did not undertake arterial blood gas and chemistry analysis of rats treated intramuscularly. 8-OH-DPAT is one of several 5-HT\textsubscript{1A} G\textsubscript{ai} protein-coupled receptor agonist compounds (e.g., buspirone, bifiridol (NLX-112/F13640), NLX-101 (F15599), and repinotan (BAYx3702). 8-OH-DPAT is also a low-affinity 5-HT\textsubscript{7} agonist, but its breathing effects are through 5-HT\textsubscript{1A} as demonstrated by 5-HT\textsubscript{1A} antagonist studies (Sahibzada et al., 2000; Guenther et al., 2009). Other selective 5-HT\textsubscript{1A} agonists are effective in reversing opioid-induced respiratory depression including bifiridol (NLX-112/F13640) in fentanyl-treated, conscious rats (Ren et al., 2015) and repinotan in remifentanil- or morphine-treated, sevoflurane-anesthetized rats (Guenther et al., 2010; Guenther et al., 2012). Buspirone, a partial 5-HT\textsubscript{1A} agonist, was effective in anesthetized rats (Sahibzada et al., 2000) but, given orally, failed to prevent morphine-induced respiratory depression in humans (Oertel et al., 2007). Bifiridol (NLX-112) and NLX-101 are in development by Neurolixis (San Diego, CA) for treatment of L-DOPA-induced dyskinesia, a Parkinson’s disease
treatment side-effect, and for depressive disorders and for breathing difficulties in Rett's syndrome, respectively. Repinotan was studied in humans as a therapy for stroke (Teal et al., 2009) and brain injury (Ohman et al., 2001). There are two general 5-HT$_{1A}$ receptor populations, pre- and post-synaptic. Since 5-HT$_{1A}$ receptors are $G_{ai}$ protein-coupled, which cause neuronal inhibition through potassium channel activation, pre-synaptic 5-HT$_{1A}$ receptor activation in brainstem raphe neurons may cause breathing stimulation by disinhibition (Sahibzada et al., 2000; Oertel et al., 2007). This hypothesis can be tested since agonists "biased" for pre-synaptic (F13714) and post-synaptic (NLX-101) 5-HT$_{1A}$ receptors are available. Finally, 8-OH-DPAT may provide toxicity as "fatal circulatory failure and pulmonary edema" was observed in morphine-treated, sevoflurane-anesthetized rats (Guenther et al., 2009) at the highest dose (100 mcg/kg). We observed no overt toxicity in rats using 150 mcg/kg and none was reported in (Sahibzada et al., 2000) and (Cheng et al., 2016) at doses up to 1 mg/kg.

Opioids cause skeletal muscle rigidity in rodents (Barnett et al., 1975) and humans (Streisand et al., 1993) and this may cause acute death associated with fentanyl overdose (Torralva and Janowsky, 2019). In rodents, rigidity causes the "Straub phenomenon", which includes a rigid, S-shaped, dorsiflexed tail and extension paralysis of the hind limbs (Bilbey et al., 1960). A rigid, "wooden chest" and laryngospasm (Bennett et al., 1997) may compromise mask ventilation in patients receiving high-dose fentanyl-based anesthesia for cardiac surgery. A non-compliant chest wall or airway limits tidal volume to impede ventilation and promote hypoxia. Oxygenation effects are important since hypoxia, as caused by hypoventilation, is what causes death. Skeletal muscle rigidity can be quantified by electromyogram (EMG) activity, mechanography, or subjective means and is diminished by co-administration of $\alpha_1$-adrenergic antagonists (e.g., prazosin)(Lui et al., 1990), $\alpha_2$-adrenergic agonists (e.g.,
dexmedetomidine) (Jerussi et al., 1987; Weinger et al., 1989), benzodiazepines (e.g., diazepam) (Sanford et al., 1994), and general anesthetics (Jerussi et al., 1987). Also, 8-OH-DPAT (300 and 1000 mcg/kg IP), itself, "abolishes" fentanyl-induced rigidity in rats (Jaros and Kolasiewicz, 1995). We observed that prazosin co-administration with 8-OH-DPAT improved both tidal volume and oxygenation (Figures 4, 5, and 6) suggesting that rigidity eradication is a worthy approach to augment stimulant effects. This novel finding suggests that opioid-induced rigidity is a "road block" to breathing stimulation and adds a new approach and new considerations in developing and testing pharmacologic strategies to reverse opioid-induced respiratory depression. We, however, can not exclude prazosin oxygenation effects may be mediated by other mechanism(s) such as improved lung ventilation-blood perfusion matching. Muscle relaxant agents such as prazosin may impose additional toxicities (e.g., hypotension, sedation, or further respiratory depression), which must be considered. Finally, we determined that intramuscular 8-OH-DPAT caused a 64% decrease in time-to-righting in sufentanil-treated rats. The righting effects imply that 8-OH-DPAT reverses both sedation and skeletal muscle rigidity, consistent with the study by (Jaros and Kolasiewicz, 1995). We speculate that 8-OH-DPAT effects in reversing opioid-induced respiratory depression may be due in part to muscle relaxation. These novel and important findings highlight the potential utility of 8-OH-DPAT as a rescue agent that, following IM administration, can reverse opioid-induced respiratory depression as well as sedation and immobility.

There are limitations to our studies. First, we studied a single dose of each drug, both opioid and stimulant. Although the synthetic opioid lethal dose in conscious rats is high (Janssen, 1982), we chose a dose that provides reliable respiratory depression and a practical recovery time. In future work, more extreme opioid doses should be studied. For the stimulants,
we chose the highest or a slightly higher dose than used in published studies (Table 1). Therefore, this was not an exhaustive comparison between stimulants. Second, we did not study hemodynamic effects (e.g., cardiac rhythm, rate, and blood pressure), an important factor in opioid recovery. Third, rats may be a less than ideal animal model as they are resistant to opioid-induced respiratory depression relative to non-human primates, which better mimic the human opioid response. However, non-human primates and other large mammals are costly and, for ethical and technical reasons, impractical to study and best reserved for validation work. Rats, given their size, cost, and wealth of published data, are a more reasonable model for pre-clinical, discovery work. Finally, nose-only plethysmography requires restraint, which might be a stressor and provide an exaggerated response to sufentanil depressant effects and its subsequent reversal. However, after two acclimatization sessions before first study, rats are more restraint tolerant. Relative to unrestrained, whole-body plethysmography, nose-only studies provide: 1) more precise and dynamic inhaled gas composition measurement and control, 2) tidal volume measurements independent of chamber and body temperature and chamber humidity estimates, 3) simplified arterial blood removal and venous drug administration, 4) simplified exhaled CO2 measurement, and 5) less artefact (e.g., sniffing or exploring).

Potent synthetic opioids are an important cause of overdose death in the ongoing opioid health crisis and are a chemical threat to both the public and the military. In our survey of several breathing stimulants as potential therapeutics, 8-OH-DPAT was the most effective and without overt toxicity. Futures studies employing 8-OH-DPAT and other 5-HT1A agonists are warranted and might address: 1) magnitude and duration of breathing effects with larger sufentanil doses and/or with other potent synthetic opioids (e.g., carfentanil); 2) hemodynamic effects; 3) additional routes of administration (e.g., nasal or inhaled/intratracheal); 4) effects on
opioid-induced skeletal muscle rigidity, immobility, and discoordination; 5) breathing enhancement by muscle relaxant co-administration (e.g., with a α2-adrenergic agonist such as dexmedetomidine); and 6) co-administration studies with naloxone. Such studies will delineate the therapeutic potential of this compound class and clarify which, if any, warrant further development.

**Authorship Contributions**

*Participated in research design:* Dandrea and Cotten

*Conducted experiments:* Dandrea

*Contributed new reagents or analytic tools:* Dandrea and Cotten

*Performed data analysis:* Dandrea and Cotten

*Wrote or contributed to the writing of the manuscript:* Dandrea and Cotten
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Footnotes

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Figure Legends

Figure 1. Rat breathing and CO\textsubscript{2} production following morphine and taltirelin treatment, with and without isoflurane anesthesia. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO\textsubscript{2} (D) as measured by nose-only plethysmography versus time. Animals received 10 mg/kg IV morphine (M) over 5 min or 1 ml IV saline (NS) at 15 min followed by 1 mg/kg IV taltirelin (T) or 1 ml IV saline (NS) at 20 min; only one group received anesthesia with continuous 1.5% inhaled isoflurane (w/ Iso), throughout. For each animal, data were normalized in A through C to 15 min of baseline data; each data point represents an average of 1 min of data ± S.E.M.; n = 6 to 8 animals in A through C and n = 5 to 6 in D. "ns" indicates no significance (P>0.05); asterisks ( and ) indicate statistical significance (P < 0.05, P < 0.001, respectively) by one-way ANOVA test with a Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following first morphine or saline administration. For all conscious experiments, average respiratory rate, minute ventilation, tidal volume and CO\textsubscript{2} production at baseline were 121 ± 4 breaths/min, 53 ± 3 ml/min/100g, 0.44 ± 0.02 ml/100g, and 42.6 ± 7.4 ml/kg/min, respectively; n = 21 (rate, MV, and TV); n = 16 (CO\textsubscript{2} production). For isoflurane anesthetized animals, average respiratory rate, minute ventilation, tidal volume and CO\textsubscript{2} production at baseline were 71 ± 3 breaths/min, 33 ± 1 ml/min/100g, 0.46 ± 0.02 ml/100g, and 15.4 ± 0.9 ml/kg/min, respectively ; n = 6.

Figure 2. Conscious rat breathing and CO\textsubscript{2} production following sufentanil and taltirelin treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO\textsubscript{2} (D) as measured by nose-only plethysmography versus time. Animals received 10 mcg/kg
IV sufentanil (S) by bolus (over ~5-10 sec) or 1 ml IV saline (NS) at 15 min followed by 1 mg/kg IV taltirelin (T) or 1 ml IV saline (NS) at 20 min. Data were averaged and normalized similar to Figure 1 and were collected from n = 6 animals. "ns" indicates no significance (P>0.05); asterisks ( and ) indicate statistical significance (P < 0.05, P < 0.0001, respectively) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following first sufentanil or saline administration. Baseline average respiratory rate, minute ventilation, tidal volume, and CO₂ production at baseline were 126 ± 3 breathes/min, 55 ± 2 ml/min/100g, 0.44 ± 0.02 ml/100g, and 33.0 ± 1.8 ml/kg/min, respectively; n = 18.

Figure 3. Morphine, sufentanil, and taltirelin effects on arterial blood pH, carbon dioxide and oxygen partial pressures, and base excess and lactate levels in conscious, air breathing rats. Data were collected using animals different than those of Figures 1 and 2. Drug dosing and administration order and times were as in Figures 1 and 2: 10 mg/kg IV morphine (M) (over 5 mins) or 10 mcg/kg IV sufentanil (S) by bolus (over ~5-10 sec) or 1 ml IV saline (NS) at 15 min followed by 1 mg/kg IV taltirelin (T) or 1 ml IV saline (NS) at 20 min. Data indicate change (Δ) in measured value from baseline -- collected just prior to first opioid/saline administration -- and 15, 30, and 45 min after taltirelin (or saline) administration. Data are averages from 6 to 8 animals (n = 6 to 8). "ns" indicates no significance (P>0.05); asterisks and diamonds ( , , , and ) indicate statistical significance (P < 0.05, P < 0.01, and P < 0.001) by one-way ANOVA test with a Sidak’s multiple comparisons post-test relative to morphine plus saline (M+NS) or sufentanil plus saline (S+NS) animals, respectively. Baseline arterial blood
pH, $P_a CO_2$, $P_a O_2$, base excess, and lactate were $7.48 \pm 0.01$, $38.9 \pm 0.8$ mmHg, $90 \pm 2$ mmHg, $+5.0 \pm 0.3$ mEq/L, and $0.7 \pm 0.1$ mmol/L, respectively; $n = 21$.

**Figure 4.** Conscious rat breathing and CO$_2$ production following sufentanil, 8-OH-DPAT, and prazosin treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO$_2$ (D) as measured by nose-only plethysmography versus time. Animals received 10 mcg/kg IV sufentanil (S) by bolus (over ~5 to 10 sec) at 15 min followed by 150 mcg/kg IV 8-OH-DPAT (D) or 150 mcg/kg IV 8-OH-DPAT plus 250 mcg/kg IV prazosin (P) at 20 min and 1 mg/kg IV naloxone at 60 min. Data were averaged and normalized similar to Figure 1 and were collected from $n = 6$ animals. "ns" indicates no significance ($P>0.05$); asterisks (*) and (**) indicate statistical significance ($P < 0.05$, $P < 0.001$, respectively) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following sufentanil. Baseline average respiratory rate, minute ventilation, tidal volume, and CO$_2$ production for all experiments, were $127 \pm 4$ breathes/min, $59 \pm 2$ ml/min/100g, $0.47 \pm 0.02$ ml/100g, and $29.2 \pm 1.7$ ml/kg/min, respectively; $n = 18$.

**Fig. 5.** Conscious rat breathing and CO$_2$ production following sufentanil, 8-OH-DPAT, prazosin, CX717, BIMU8, and A85380 treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO$_2$ (D) as measured by nose-only plethysmography at three time points (30, 45, and 60 min); includes 8-OH-DPAT data shown in Figure 4. Rats received 10 mcg/kg IV sufentanil at 15 min followed by 1) 1 ml of saline, 2) 150 mcg/kg IV 8-OH-DPAT, 3) 150 mcg/kg IV 8-OH-DPAT plus 250 mcg/kg IV prazosin, 4) 30 mg/kg IV CX717, 5) 30 mcg/kg IV A85380, or 6) 1 mg/kg IV BIMU8 at 20 mins. Data were averaged and normalized
similar to Figure 1 and were collected from n = 6 animals for each compound. "ns" indicates no significance (P>0.05); asterisks (□ and □□□) indicate statistical significance (P < 0.05, P < 0.001, respectively) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points. The average baseline respiratory rate, minute ventilation, tidal volume, and CO₂ production for all experiments were 128 breathes/min, 61 ± 1 ml/min/100g, 0.47 ± 0.01 ml/100g, and 29.8 ± 1.0 ml/kg/min, respectively; n = 36.

**Figure 6.** Sufentanil, 8-OH-DPAT, prazosin, CX717, BIMU8, A85380 effects on arterial blood pH, carbon dioxide and oxygen partial pressures, and base excess and lactate levels in conscious, air breathing rats. Blood gas data were collected on the same animals and simultaneous with the breathing data of Figures 4 and 5, such that drug dosing and administration order and times are the same. Data indicate change (Δ) in measured value from baseline -- collected just prior to first opioid administration -- and 15, 30, and 45 min after study drug(s) (or saline) administration. Data are averages from 6 animals (n = 6). "ns" indicates no significance (P>0.05); asterisks and diamonds (□, □□, and □□□) indicate statistical significance (P < 0.05, P < 0.01, and P < 0.001) by one-way ANOVA test with a Sidak's multiple comparisons post-test relative to sufentanil plus saline (S+NS) animals, respectively, at the same time point. Baseline arterial blood pH, PₐCO₂, PₐO₂, base excess, and lactate were 7.44 ± 0.01, 41.0 ± 0.6 mmHg, 90 ± 1 mmHg, +3.0 ± 0.3 mEq/L, and 1.38 ± 0.1 mmol/L, respectively; n = 36.

**Figure 7.** Conscious rat breathing and CO₂ production following intravenous (IV) sufentanil and intramuscular (IM) 8-OH-DPAT treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO₂ (D) as measured by nose-only plethysmography versus time.
Animals received 10 mcg/kg IV sufentanil (S) by bolus (over ~5 to 10 sec) at 15 min followed by 150 mcg/kg IM 8-OH-DPAT (D) or 1 ml/kg IM normal saline at 20 min and 1 mg/kg IV naloxone at 60 min. Data were averaged and normalized similar to Figure 1 and were collected from n = 6 animals. "ns" indicates no significance (P>0.05); asterisks (• and ••) indicate statistical significance (P < 0.05, P < 0.01, respectively) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following sufentanil. Baseline average respiratory rate, minute ventilation, tidal volume, and CO2 production for all experiments, were 120 ± 3 breathes/min, 58 ± 1 ml/min/100g, 0.48 ± 0.01 ml/100g, and 28.9 ± 1.2 ml/kg/min, respectively; n = 24.
Figure 1
Figure 2

Normalized Respiratory Rate (%)

Normalized Tidal Volume (%)

Sufentanil or Saline
Taltirelin or Saline

S+N vs S+T: ns
S+N vs NS+T: *** *** ***

S+N vs S+T: ns
S+N vs NS+T: *

S+N vs S+T: ns
S+N vs NS+T: *

S+N vs S+T: ns
S+N vs NS+T: *

S+N vs S+T: ns
S+N vs NS+T: *

S+N vs S+T: ns
S+N vs NS+T: *
Figure 4

A) Normalized Respiratory Rate (%)

B) Normalized Minute Ventilation (%)

C) Normalized Tidal Volume (%)

D) CO₂ Production (%)

Compounds(s):
- Sufentanil
- Naloxone
- 8-OH-DPAT
- Prazosin

Sufentanil + Normal Saline (S+NS)
- S+NS vs S+D: ns
- S+D vs S+D+P: ns
- S+NS vs S+D+P: ns

Sufentanil + 8-OH-DPAT (S+D)
- S+D vs S+D+P: ns

Sufentanil + 8-OH-DPAT + Prazosin (S+D+P)
- S+D vs S+D+P: ***

S+NS vs S+D+P: ns

Time (mins):
0 15 30 45 60 70

Normalized Values:
S+NS S+D S+D+P S+NS S+D S+D+P

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Figure 5
Figure 6

A. \( \Delta \) Arterial pH

B. \( \Delta \) PaCO\(_2\) (mmHg)

C. \( \Delta \) PaO\(_2\) (mmHg)

D. \( \Delta \) Blood Base Excess (mEq/L)

E. \( \Delta \) Blood Lactate (mmol/L)
Figure 7

**Statistical Analysis:**

- NS
- **NS**
- **NS**

**Sufentanil + 8-OH-DPAT IM**

**Sufentanil + Saline IM**

Normalized Respiratory Rate (%)

Normalized Tidal Volume (%)

Normalized Minute Ventilation (%)

CO₂ Production (%)

Supplemental Figure S1. A, nose-only chamber and restraint used in collection of breathing data. B, neck collar used in restraint. C, alternate, close-up of “nose chamber” with its green, latex diaphragm and gas inflow and outflow Luer ports labeled. D, examples of flow signal generated by a conscious rat in the above chamber just before and shortly after 10 mcg/kg IV sufentanil administration. Note the shallow, irregular breathing typical on recovery from transient, sufentanil-induced apnea. Smaller, intermittent, rapid cardiac oscillations from heart beats are also apparent in the sufentanil signal.
Supplemental Figure S2. Chemical structures of study drugs and compounds. 8-OH-DPAT, (±)-8-Hydroxy-2-(dipropylamino)tetralin; A85380, 3-[(2S)-2-Azetidinylmethoxy]-pyridine; BIMU8, 2,3-Dihydro-N-[(3-endo)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl]-3-(1-methylethyl)-2-oxo-1H-benzimidazole-1-carboxamide; CX717, 2,1,3-Benzoxadiazol-5-yl(morpholin-4-yl)methanone; PKTHPP, 1-[1-(6-((1,1′-Biphenyl)-4-ylcarbonyl)-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-4-piperidinyl]-1-butano; Prazosin, 1-(4-Amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl) piperazine; and Taltirelin, N-[[4(S)-Hexahydro-1-methyl-2,6-dioxo-4-pyrimidinyl]carbonyl]-L-histidyl-L-prolinamide.
“A comparison of breathing stimulants for reversal of synthetic opioid-induced respiratory depression in conscious rats”,

Supplemental Figure S3. Conscious rat breathing and CO2 production following sufentanil and PKTHPP treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO2 (D) as measured by nose-only plethysmography versus time. Animals received 10 mcg/kg IV sufentanil by bolus (over ~5 to 10 sec) at 15 min followed by 5 mg/kg IV PKTHPP or 1 ml/kg IV DMSO at 20 min. Data were averaged and normalized similar to Figure 1 and were collected from n = 6 animals. "ns" indicates no significance (P>0.05); asterisks (*) and ***) indicate statistical significance (P<0.05 and P<0.001, respectively) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following sufentanil. Baseline average respiratory rate, minute ventilation, tidal volume, and CO2 production for all experiments, were 124±4 breathes/min, 58±3 ml/min/100g, 0.5±0.02 ml/100g, and 28.8 ml/kg/min, respectively; n = 18.
A comparison of breathing stimulants for reversal of synthetic opioid-induced respiratory depression in conscious rats,

Supplemental Figure S4. Conscious rat breathing and CO2 production following sufentanil and CX717 treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO2 (D) as measured by nose-only plethysmography versus time. Animals received 10 mcg/kg IV sufentanil by bolus (over ~5 to 10 sec) at 15 min followed by 30 mg/kg IV CX717 or 1 ml/kg IV normal saline at 20 min and 1 mg/kg IV naloxone at 60 min. Data from these studies were used in Figures 5 and 6. Data were averaged and normalized similar to Figure 1 and were collected from n = 6 animals. "ns" indicates no significance (P>0.05) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following sufentanil. Baseline average respiratory rate, minute ventilation, tidal volume, and CO2 production for all experiments, were 134±4 breathes/min, 64±2 ml/min/100g, 0.48±0.02 ml/100g, and 31.2±1.8 ml/kg/min, respectively; n = 12.
**Supplemental Figure S5.** Conscious rat breathing and CO₂ production following sufentanil and BIMU8 treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO₂ (D) as measured by nose-only plethysmography versus time. Animals received 10 mcg/kg IV sufentanil by bolus (over ~5 to 10 sec) at 15 min followed by 1 mg/kg IV BIMU8 or 1 ml/kg IV normal saline at 20 min and 1 mg/kg IV naloxone at 60 min. Data from these studies were used in Figures 5 and 6. Data were averaged and normalized similar to Figure 1 and were collected from \( n = 6 \) animals. "ns" indicates no significance (\( P>0.05 \)); an asterisk (*) indicates statistical significance (\( P < 0.05 \)) by one-way ANOVA test with Sidak's multiple comparisons post-test at data points 15, 30, or 45 mins following sufentanil. Baseline average respiratory rate, minute ventilation, tidal volume, and CO2 production for all experiments, were 127±4 breathes/min, 61±2 ml/min/100g, 0.48±0.02 ml/100g, and 32.1±1.5 ml/kg/min, respectively; \( n = 12 \).
A comparison of breathing stimulants for reversal of synthetic opioid-induced respiratory depression in conscious rats,

Supplemental Figure S6. Conscious rat breathing and CO₂ production following sufentanil and A85380 treatment. Average respiratory rate (A), minute ventilation (B), tidal volume (C), and exhaled CO₂ (D) as measured by nose-only plethysmography versus time. Animals received 10 mcg/kg IV sufentanil by bolus (over ~5 to 10 sec) at 15 min followed by 30 mcg/kg IV A85380 or 1 ml/kg IV normal saline at 20 min and 1 mg/kg IV naloxone at 60 min. Data from these studies were used in Figures 5 and 6. Data were averaged and normalized similar to Figure 1 and were collected from n = 6 animals. "ns" indicates no significance (P>0.05) by one-way ANOVA test with Sidak’s multiple comparisons post-test at data points 15, 30, or 45 mins following sufentanil. Baseline average respiratory rate, minute ventilation, tidal volume, and CO2 production for all experiments, were 131±4 breathes/min, 62±2 ml/min/100g, 0.48±0.02 ml/100g, and 29.3±1.8 ml/kg/min, respectively; n = 12.