5-HT<sub>1A</sub> Receptor Agonist Befiradol Reduces Fentanyl-induced Respiratory Depression, Analgesia, and Sedation in Rats

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ABSTRACT

Background: There is an unmet clinical need to develop a pharmacological therapy to counter opioid-induced respiratory depression without interfering with analgesia or behavior. Several studies have demonstrated that 5-HT<sub>1A</sub> receptor agonists alleviate opioid-induced respiratory depression in rodent models. However, there are conflicting reports regarding their effects on analgesia due in part to varied receptor selectivity and presence of anesthesia. Therefore the authors performed a study in rats with befiradol (F13640 and NLX-112), a highly selective 5-HT<sub>1A</sub> receptor agonist without anesthesia.

Methods: Respiratory neural discharge was measured using in vitro preparations. Plethysmographic recording, nociception testing, and righting reflex were used to examine respiratory ventilation, analgesia, and sedation, respectively.

Results: Befiradol (0.2 mg/kg, n = 6) reduced fentanyl-induced respiratory depression (53.7 ± 5.7% of control minute ventilation 4 min after befiradol vs. saline 18.7 ± 2.2% of control, n = 9; P < 0.001), duration of analgesia (90.4 ± 11.6 min vs. saline 130.5 ± 7.8 min; P = 0.011), duration of sedation (39.8 ± 4 min vs. saline 58 ± 4.4 min; P = 0.013); and induced baseline hyperventilation, hyperalgesia, and “behavioral syndrome” in nonsedated rats. Further, the befiradol-induced alleviation of opioid-induced respiratory depression involves sites or mechanisms not functioning in vitro brainstem–spinal cord and medullary slice preparations.

Conclusions: The reversal of opioid-induced respiratory depression and sedation by befiradol in adult rats was robust, whereas involved mechanisms are unclear. However, there were adverse concomitant decreases in fentanyl-induced analgesia and altered baseline ventilation, nociception, and behavior. (Anesthesiology 2015; 122:424-34)

S uppression of central respiratory drive by opioid analgesics is a clinical problem for which improved therapeutic treatments and safety are needed. Balancing the trade-off between analgesia and respiratory depression is one of the major challenges of anesthesiology, pain, and intensive care medicine. Current approaches to reversing opioid-induced respiratory depression, such as naxoxone, can be effective short-term, but only at the price of impaired analgesia. Thus, there is a need to develop a safe and effective pharmacological therapy to counter opiate-induced respiratory depression that does not interfere with analgesia or significantly modulate baseline cardiorespiratory parameters.

Recent advances in understanding the neurochemical control of respiration have provided the foundation for targeting specific neurotransmitter receptor systems for potential drug therapies. A major component of excitatory synaptic drive necessary for respiratory rhythmogenesis and activation of respiratory motoneurons is via the amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subclass of glutamate receptors.2–5 Those studies provided the foundation for the investigations of ampakine (positive modulators of AMPA receptors) therapy to alleviate drug-induced respiratory depression.6–10 Further conditioning of respiratory activity is provided by a diverse group of neuromodulators, including 5-HT released from the raphé nuclei that potently alters the excitability of respiratory motoneurons, the preBötzinger complex (preBötC) and other brainstem respiratory nuclei. Several studies have demonstrated that activation of 5-HT<sub>1A</sub> receptors (5-HT<sub>1A</sub>R) with the agonists 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), buspirone or repinotan alleviates opioid-induced respiratory depression in rodent models.11–15 However, there are conflicting reports regarding the effects of these agents on analgesia, sedation, baseline nociception, and cardiorespiratory parameters.12–24 Contradictory findings in those previous reports likely reflect varied drug receptor specificity,

Submitted for publication June 24, 2014. Accepted for publication September 4, 2014. From the Department of Physiology, Neuroscience, and Mental Health Institute, University of Alberta, Edmonton, Alberta, Canada (J.R., X.D., J.J.G.); and the Alberta Innovates Health Sciences Foundation, Edmonton, Alberta, Canada (J.J.G.).

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route of administration, dose range, and critically, the absence, presence, and type of anesthesia. In this study, we performed a systematic analysis of the effects on ventilation and analgesia of a novel agonist, 4-piperidinemethanamine, 1-((3-chloro-4-fluorobenzoyl)-4-fluor-N-(5-methyl-2-pyridinyl))methyl,(2E)-2-butenedioate (befiradol, also known as F13640 and NLX-112), that has high selectivity and agonist efficacy at 5-HT1aR located at both presynaptic and postsynaptic sites,23,25,26 and has shown efficacy in rodent models of neuropathic, inflammatory, and surgical pain.27 We performed these studies without the confounding effects of anesthesia that may interfere with detection of drug-induced changes in important baseline parameters related to ventilation, pain sensitivity, and arousal.

Materials and Methods

Brainstem–Spinal Cord and Medullary Slice Neonatal Preparations

All experimental procedures were approved by the Faculty of Medicine and Dentistry Animal Welfare Committee at the University of Alberta (Edmonton, Alberta, Canada). Neonatal (3 to 4 days after birth) Sprague–Dawley rats were anesthetized with metofane, decerebrated and the brainstem–spinal cord (BSSC) dissected as previously reported.28 The neuraxis was continuously perfused at 27°C ± 1°C (perfusion rate, 5 ml/min; chamber volume, 3 ml) with modified Kreb's solution that contained 128 mM NaCl, 3.0 mM KCl, 1.5 mM CaCl2, 1.0 mM MgSO4, 24 mM NaHCO3, 0.5 mM NaH2PO4 and 30 mM D-glucose equilibrated with 95% O2–5% CO2 (pH 7.4). The μ-opioid receptor agonist ∆^6-Aga2, N-MePhe4, Gly-ol-enkephalin (DAMGO, 300 nM; Sigma Canada, Markham, Ontario, Canada) was added to the bathing medium to induce respiratory depression in vitro. BSSC preparations isolated from newborn rats were pinned down, ventral surface upward, on a paraffin coated block.3 The block was mounted in the vise of a vibratory microtome (VT1000 S; Leica Microsystems, Wetzlar, Germany). The brainstem was sectioned serially in the transverse plane, starting from the rostral medulla to within approximately 150 μm of the rostral boundary of the preBötC, as judged by the appearance of the inferior olive. A single transverse slice containing the preBötC and more caudal reticular formation regions was then cut (600 μm thick), transferred to a recording chamber, and pinned down onto Sylgard elastomer (Dow Corning, Midland, MI). The medullary slice was continuously perfused with a bathing solution identical to that used for BSSC preparation with the exception that the KCl concentration was increased to 9 mM to facilitate long-term generation of stable rhythm by these preparations.3 Recordings from the fourth ventral cervical nerve roots of BSSC or hypoglossal nerve roots of medullary slice preparations were amplified, rectified, lowpass filtered, and recorded to a computer, using an analog–digital converter (Axon Instruments Digidata 1200; Molecular Devices, Sunnyvale, CA) and data acquisition software (Axon Instruments AxoScope; Molecular Devices).

Whole Body Plethysmographic Recordings

Measurements from unrestrained newborn and adult Sprague–Dawley male rats were performed in whole body, plexiglass plethysmographs that had inflow and outflow ports for the continuous delivery of fresh room air and removal of expired carbon dioxide. The plethysmograph volumes were 50 and 2,000 ml for measures of respiratory parameters of neonatal (3 to 4 days after birth) and adult (290 to 360 g) male rats with a flow rate of 50 ml/min and 1 l/min, respectively. For newborns, the plethysmograph was contained within an infant incubator (Isolette, model C-86; Air-Shields/Dräger Medical, Hatboro, PA) to maintain the ambient temperature at the approximate nest temperature of 32°C. For newborn experiments, fentanyl citrate (35 μg/kg; Sandoz, Boucherville, QC, Canada) was injected into subcutaneously the neck fat pad. For adult infusion experiments, adult rats were anesthetized with 3% isoflurane in an induction chamber and maintained with 2% isoflurane anesthesia during tail vein cannulation (P10 size tubing, with both vein cannulated). The chamber had an additional port to allow exteriorization of the tail for iv drug infusion via an infusion pump (KD Scientific, Holliston, MA). With the infusion approach, all drug deliveries can be performed with continuous monitoring of plethysmographic recordings without physical handling of the animal. Pressure changes were detected with a pressure transducer (model DP 103; Validyne, Northridge, CA), signal conditioner (CD-15; Validyne), analog-digital board (Digidata 1322A), and data acquisition (Axoscope) and analysis (Clampfit) software (Axon Inst., Molecular Devices, Sunnyvale, CA). A pulse oximeter (Norin 8600V, Plymouth, MN) was placed on the tail to monitor oxygen saturation (SaO2) levels and heart rate in adult rats. Body temperature was measured using a rectal probe (Dual thermometer; Fisher Scientific, Ottawa, ON, Canada).

It should be noted that our plethysmograph is effective for studying respiratory frequency (fR) and detection of apneas. It is not designed for precise quantification of tidal volume (Vt, ml/g). The physical principle underlying whole-body plethysmography is the detection of pressure changes in the chamber resulting from the heating and humidification of inspired gas. However, tidal volume measurements may also be influenced by gas compression effects related to the airway resistance. Because of these limitations, our whole-body plethysmographic system only provided semiquantitative measurements of Vt from which we report changes relative to the control state. As a result, our measurements of Vt (minute ventilation: ml min⁻¹ g⁻¹), which equates to fR × Vt, are also semiquantitative and only reported relative to control. An apnea is defined as the absence of airflow for a period equivalent or greater than two complete respiratory cycles.

For the determination of baseline breathing parameters in freely moving adult rats in experiments that did not require
in vitro drug administration and experimental protocols

Fentanyl citrate (Sandoz, Boucherville, QC, Canada) was infused intravenously into one tail vein and a bolus of befradol or saline was injected intravenously into the other tail vein. The overall fentanyl experimental protocol is depicted in figure 1. Fentanyl (60 μg/kg per 20 min) infusion commenced approximately 5 min after the plethysmographic chamber was flushed with room air to remove the residual ambient isoflurane. Righting reflex testing started approximately 4 min after fentanyl administration and continued until the animal awoke from fentanyl-induced sedation (note that this is not from residual isoflurane). Saline or befradol (provided by Dr. Mark Varney, Ph.D., Neurolixis Inc., San Diego, CA, dissolved in a 0.9% saline, 0.1 to 0.6 mg/kg, iv, bolus) was administered at approximately 6 min after fentanyl infusion. The concentration of befradol administered in vitro and in vivo in our study was based on the demonstration that befradol activates 5-HT1AR with an EC50 in the nM range.23,25,26 Tail clamping started from approximately 11 min after fentanyl infusion until a positive response was observed. Tail clamping was only tested in some animals (six of nine vehicle groups and five of six 0.2 mg/kg befradol group, but not in other dosage of befradol groups). When the animal regained righting reflex, it was taken out from the recording chamber for thermal nociceptive testing. Thermal nociception testing was repeated every 2 to 5 min until a positive paw withdrawal response of less than 8 s (upper-limit of response time on baseline) was observed on two consecutive tests. The time from the beginning of fentanyl administration to the positive paw withdrawal of less than 8 s was arbitrarily defined as the duration of analgesia, since we were unable to measure the onset of analgesia by thermal nociception testing in this study. For experiments measuring baseline parameters, the nociception testing and behavioral observation started 10 min before, and 10 min postinjection of drugs (subcutaneous) and was repeated every 3 min for a total of three times outside of Buxco chamber, followed by 30 min of measurement of breathing parameters via plethysmography in the Buxco system.

Statistical Analysis

Data are expressed as mean ± SEM (Sigmaplot 11 Systat Software Inc., San Jose, CA). Sample sizes were used based on previous experience. Randomization methods were used to assign units to experimental condition. Blind testing is used where one person administered the drug, and second person observed, rated the behavior, and analyzed the data without knowledge of drug administration. There was no missing for the data used for statistical analysis. Respiratory parameters were calculated by an average of 2-min, 1-min continuous recording data in vitro, in vivo, respectively. The respiratory parameters fR, VT, and VE were reported as means relative to control values (before fentanyl administration in vivo, DAMGO in vitro, control values as 1). For in vitro experiments, the significance of changes in respiratory parameters before and after befradol administration to the

behavioral observations

We measured four types of behavior: head weaving, forepaw treading, flat body posture, and lower lip retraction. Behaviors were visually scored during observation periods of 45 s on a 4-point ranked intensity scale (0 = absent, 1 = equivocal, 2 = definite, and 3 = intense).29 Scores for each behavior were averaged over three observation periods in the 10 min before drug administration and then three more starting 10 min after drug administration (neck subcutaneously). Only those animals in which the scores across all behaviors averaged greater than 2 were defined as displaying “behavioral syndrome.”
bathing medium was compared in the absence or presence of DAMGO with paired *t* test, or one-way repeated-measures ANOVA (T ukey method; fig. 2), respectively. The nature of the hypothesis testing is two tailed. For *in vivo* fentanyl experiments, the significance of changes in $f_R$, $V_T$, $V_E$, and $\text{SaO}_2$ after treatment was compared with two-way repeated-measures ANOVA (dose × time; Holm–Sidak method; figs. 3 and 4). The significance of changes in the duration of fentanyl-induced analgesia (fig. 5) or sedation (fig. 6) was compared between saline- and befiradol-treated groups with *t* test. To examine if the effects of befiradol on fentanyl-induced respiratory depression (or sedation) were correlated with its effects on analgesia, we used the Pearson product moment test (figs. 5 and 6). For experiments examining the effects of befiradol on baseline parameters, the significance of changes in $V_E$ or latency of paw withdrawal response to thermal nociceptive stimulus was compared between saline- and befiradol-treated group with *t* test (fig. 7). *P* valueless than 0.05 or critical level was taken as significant difference for two comparison (*t* test, paired *t* test, and Pearson product moment test), multiple comparisons (ANOVA), respectively.

**Results**

**Befiradol Has No Effects on the Fentanyl-induced Respiratory Depression or Baseline Respiratory Rhythm In Vitro**

We initiated our study using standard *in vitro* newborn rat preparations that have been used extensively to examine the neurochemical control of respiration. Similar to past studies, the respiratory frequency was markedly suppressed by the bath application of DAMGO (300 nM) to 31.9 ± 6.8% ($n = 6$, *P* < 0.001; fig. 2) of control levels observed before DAMGO. Subsequent bath application of befiradol (1 to 30 μM, n = 6 each) or medullary slice (300 nM DAMGO, 3 to 30 μM befiradol, n = 5 each). There is no significant difference in respiratory frequency after befiradol using one-way repeated-measures ANOVA (Tukey method). DAMGO = D-Ala2, N-MePhe4, Gly-ol-enkephalin.

![Fig. 2. Befiradol does not alleviate DAMGO-induced respiratory depression *in vitro*. A representative rectified and integrated recording of the respiratory discharge of fourth cervical ventral nerve roots (C4) produced by a postnatal day (P) 3 brainstem–spinal cord preparation in (A) control, (B) with bath application of DAMGO (300 nM), and (C) subsequent bath application of befiradol (10 μM) in the continued presence of DAMGO (300 nM). (D) Population data with brainstem–spinal cord (300 nM DAMGO, 1 to 30 μM befiradol, n = 6 each) or medullary slice (300 nM DAMGO, 3 to 30 μM befiradol, n = 5 each). There is no significant difference in respiratory frequency after befiradol using one-way repeated-measures ANOVA (Tukey method). DAMGO = D-Ala2, N-MePhe4, Gly-ol-enkephalin.](image-url)

The medullary slice preparation is a derivative of the BSSC preparation. Similarly to past studies, the frequency of rhythmic respiratory discharge in medullary slice preparations was markedly suppressed by the bath application of DAMGO (300 nM, n = 5) to 41.1 ± 5.4% of control levels before DAMGO. Subsequent bath application of befiradol (1 to 30 μM) in the presence of DAMGO did not alleviate the suppression of respiratory frequency (31.1 ± 7.1% of control, *P* = 0.99, n = 6; fig. 2). The amplitude of motor nerve discharge generated by BSSC preparations was not affected by DAMGO nor subsequent application of befiradol (n = 6). The administration of befiradol on its own did not significantly alter the control values of respiratory frequency or amplitude of motor nerve discharge generated by BSSC preparations (1 to 30 μM, n = 5). This is consistent with previous studies of the 5-HT 1AR agonist, 8-OH-DPAT, in rat and mouse *in vitro* preparations.

The medullary slice preparation is a derivative of the BSSC preparation. Similarly to past studies, the frequency of rhythmic respiratory discharge in medullary slice preparations was markedly suppressed by the bath application of DAMGO (300 nM, n = 5) to 41.1 ± 5.4% of control levels before DAMGO (P < 0.001), consistent with our previous study. Subsequent bath application of befiradol (3 to 30 μM, 38.8 ± 6.3% of control before DAMGO, n = 5) in the presence of DAMGO did not alleviate the respiratory depression (P = 0.96, fig. 2D). The amplitude of inspiratory motor nerve discharge generated by medullary slice preparations was not affected by DAMGO and subsequent application of
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The administration of befiradol on its own did not significantly alter the control values of respiratory frequency or amplitude of motor nerve discharge generated by medullary slice preparations (3 to 30 μM, n = 5).

**Befiradol Alleviates the Fentanyl-induced Respiratory Depression in In Vivo Neonatal Rats**

The next stage of the study was to examine befiradol in vivo. Toward correlating with the in vitro studies, we first examined the effects of befiradol (0.6 mg/kg, fig. 3) on respiratory depression induced by fentanyl (35 μg/kg, n = 5) in neonatal rats in vivo. This induced a marked suppression of \( f_R \) (31 ± 2% of control), \( V_T \) (55.4 ± 6.3% of control), and \( V_E \) (17.2 ± 2.1% of control) approximately 5 min postadministration. The fentanyl-induced respiratory depression lasted for over 35 min after subsequent saline administration. Administration of befiradol (0.6 mg/kg, at -5 min postfentanyl) alleviated the fentanyl-induced respiratory depression over 20 min (fig. 3). Specifically, alleviation of fentanyl-induced respiratory depression by befiradol was observed within 5 min after befiradol, as evidenced by increased \( f_R \) (39.2 ± 3.3% of control vs. saline: 24.2 ± 3.4% of control, \( P = 0.014 \)), \( V_T \) (76.1 ± 6.2% of control vs. saline: 52.2 ± 7.5% of control, \( P = 0.016 \)), and \( V_E \) (29.3 ± 3.1% of control vs. saline: 12.4 ± 2.8% of control, \( P = 0.009 \)).

**Befiradol Alleviates the Fentanyl-induced Respiratory Depression in In Vivo Adult Rats**

The next series of experiments was performed in in vivo adult rats to determine the dose-dependent efficacy of the befiradol, to counter the fentanyl-mediated respiratory depression. Fentanyl (60 μg/kg, iv) was delivered to adult rats over a 20-min infusion period (fig. 4). This induced a marked suppression of \( f_R \) (44.8 ± 5.3% of control), \( V_T \) (49.8 ± 4.2% of control), \( V_E \) (22.6 ± 3.2% of control), and \( SaO_2 \) (51.6 ± 5.3% of control) at approximately 6 min after fentanyl administration (n = 9). Subsequent injection of saline vehicle (fig. 4A) did not change the course of fentanyl action (fig. 4C–F). In contrast, subsequent injection of befiradol (0.1 to 0.4 mg/kg, iv, n = 16, fig. 4B) caused a dose-dependent increase in \( f_R \), \( V_T \), and \( V_E \). Whereas a low dose of befiradol (0.1 mg/kg, n = 5) alleviated the fentanyl-induced respiratory depression for less than 10 min, the effects of high doses of befiradol (0.2 mg/kg, n = 6 and 0.4 mg/kg, n = 5) lasted beyond the duration of the fentanyl infusion (fig. 4C–F). Specifically, 4 min after befiradol, \( V_E \) was 37.4 ± 4.8% of control (0.1 mg/kg,
Fig. 4. Effects of befiradol on fentanyl-induced respiratory depression in adult rats. (A–B) Representative continuous whole body plethysmographic recordings from two adult rats. A 20-min infusion of fentanyl (60 μg/kg, iv) caused a significant depression of respiratory frequency (f_R) and tidal volume (V_T). (A) Saline or (B) befiradol (0.2 mg/kg, iv, bolus) was injected approximately 6 min after administration of fentanyl. Respiratory variables and oxygen saturation were measured before fentanyl, and 5 to 20 min after administration of fentanyl. Nociceptive testing via tail clamping was performed starting approximately 11 min after fentanyl administration. (C–F) Dose-dependence data showing the relative respiratory frequency (f_R, C), tidal volume (V_T, D), minute ventilation (V_E, E), and oxygen saturation (SaO_2, F) after injection of vehicle or increasing doses of befiradol approximately 6 min after fentanyl infusion. Respiratory variables (f_R, V_T, and V_E) are presented as % relative to control (i.e., prefentanyl administration). Each animal tested only once with vehicle or one dose of befiradol. *Significant difference, compared with vehicle control group; #compared between two doses of befiradol-treatment groups; and using two way repeated measures ANOVA (Holm–Sidak methods).

n = 5, P < 0.001), 53.7 ± 5.7% of control (0.2 mg/kg, n = 6, P < 0.001), and 65.8 ± 5.1% of control (0.4 mg/kg, n = 5, P < 0.001), significantly increased from 18.7 ± 2.2% of control in the saline group (n = 9). Four minutes after befiradol, SaO_2 was 59.5 ± 3.5% (0.1 mg/kg, n = 5, P = 0.013), 75.4 ± 3.7% (0.2 mg/kg, n = 6, P < 0.001), and 78.5 ± 3.1% (0.4 mg/kg, n = 5, P < 0.001), significantly increased from 45.7 ± 4.8% in the saline group (n = 9). Note the decrease of body temperature at 4 min after befiradol treatment (0.2 to 0.4 mg/kg, −0.48°C ± 0.07°C, n = 5) was not significantly different (P = 0.14) from saline treatment (−0.65°C ± 0.06°C, n = 4).

**Befiradol Decreases the Fentanyl-induced Analgesia**

We then determined whether befiradol, at the doses of alleviating the fentanyl-induced respiratory depression, affected fentanyl-induced analgesia. Figure 4 shows measures of analgesia by examining responses to tail clamping with forceps at 3 to 5 min intervals starting at approximately 11 min after
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Fig. 5. Effects of befiradol on fentanyl-induced analgesia in adult rats. (A) The duration (min) of analgesia—caused by fentanyl (60 μg/kg for 20-min iv infusion) was measured from the beginning of fentanyl administration to the time when a positive response to nociceptive stimulus occurred. A positive response to pinching the tail with forceps was based on observing obvious changes in at least two of following: heart rate, oxygen saturation, respiratory frequency, and body movement. Positive paw withdrawal response to thermal nociceptive stimulus is defined as a withdrawal in less than 8 s. Saline or befiradol (0.2 mg/kg, iv, bolus) was given approximately 6 min after fentanyl. *P < 0.05, significant difference between two groups using t test. Each data point from left to right bars is from 6, 5, 9, and 6 animals, respectively. (B) Correlation between fentanyl-induced analgesia and respiratory depression: duration of fentanyl-induced analgesia plotted against the fentanyl-induced suppression of minute ventilation (V̇e, at ~10 min after fentanyl, relative to control, and before fentanyl) in saline-treated (n = 9) or befiradol-treated (0.1 to 0.6 mg/kg, iv, bolus, n = 12) groups. Correlation coefficients were 0.67, P = 0.048 and 0.74, P = 0.006 for saline and befiradol groups, respectively.

Fig. 6. Effects of befiradol on fentanyl-induced sedation in adult rats. (A) The duration (min) of sedation caused by fentanyl (60 μg/kg for 20 min iv infusion) was measured from the beginning of fentanyl administration to the time when the animal regained a righting reflex from the prone to supine position. Saline (n = 9) or befiradol (0.2 mg/kg, iv, bolus, n = 6) was administered approximately 6 min after fentanyl. *P < 0.05, significant difference between two groups using t test. (B) Correlation between fentanyl-induced analgesia and sedation: duration of fentanyl-induced analgesia plotted against the duration of fentanyl-induced sedation in saline-treated (n = 9) or befiradol-treated (0.1 to 0.6 mg/kg, iv, bolus, n = 12) groups. Correlation coefficients were 0.75, P = 0.019 and 0.76, P = 0.004 for saline and befiradol groups, respectively.

Fig. 7. Effects of befiradol on baseline breathing and nociception in normal adult rats. Minute ventilation (V̇e) and nociception (latency of paw withdrawal response to thermal nociceptive stimulus) relative to control (i.e., before saline or befiradol 0.2 mg/kg) were measured. *P < 0.05, significant difference between two groups, with t test. Each data point is six animals.

fentanyl administration. No positive response to tail clamping was observed during fentanyl administration in salinetreated rats (fig. 4A, n = 6). However, a positive response to tail clamping was observed during fentanyl administration in two of five befiradol (0.2 mg/kg, fig. 3B)-treated rats. The onset of a positive response to tail clamping was significantly shortened by befiradol (0.2 mg/kg, 28.4 ± 6.8 min, n = 5 vs. saline 52.4 ± 5.2 min, n = 6; P = 0.019 fig. 5A). The duration of analgesia based on paw withdrawal data was also significantly shortened by befiradol (0.2 mg/kg, 90.4 ± 11.6 min, n = 6 vs. saline 130.5 ± 7.8 min, n = 9; P = 0.011, fig. 5A). To establish whether there is a correlation between fentanyl-induced respiratory depression and analgesia, we plotted suppression of V̇e against duration of analgesia in vehicle groups (fig. 5B). The correlation coefficient was 0.67 (n = 9, P = 0.048, Pearson product moment test), suggesting that fentanyl-induced respiratory depression and analgesia increase together. We then plotted the same relationship based on data obtained in the presence of befiradol to determine if 5HT1AR antagonism changes the correlation (fig. 5B). The correlation coefficient was 0.74 for befiradol (0.1 to 0.6 mg/kg, n = 12, P = 0.006, Pearson product moment test). All data demonstrated that the positive correlation between fentanyl-induced respiratory depression and analgesia remains in the presence of befiradol; that is, that befiradol reduces respiratory depression but also reduces analgesia.
Befiradol Alleviates the Fentanyl-induced Sedation
We found that 0.6 mg/kg befiradol caused a rapid (within 2 min after injection, at ~6 min after fentanyl) loss of fentanyl-induced sedation in two of four rats. These two rats had full recovery of ventilation with 2 min after injection. However, the loss of sedation prevented any further data collection from those two rats due to disruption of continuous infusion of fentanyl. Fentanyl-induced duration of sedation was significantly shortened in befiradol-treated group (0.2 mg/kg, 39.8 ± 4 min, n = 6 vs. saline 58 ± 4.4 min, n = 9; P = 0.013, t test, fig. 6A). We then determined whether there is a correlation between fentanyl-induced respiratory sedation and analgesia. Figure 6B shows fentanyl-induced duration of sedation plotted against duration of analgesia (measured with paw withdrawal to thermal stimulus) in vehicle groups. The correlation coefficient was 0.75 (n = 9, P = 0.019, Pearson product moment test), suggesting that fentanyl-induced respiratory sedation and analgesia tended to increase in parallel. Further, we plotted the same relationship based on data obtained in the presence of befiradol to determine if 5HT1AR agonism changes the correlation (fig. 6B). The correlation coefficient was 0.76 for befiradol (0.1 to 0.6 mg/kg, n = 12, P = 0.004). All data demonstrated that the positive correlation between fentanyl-induced sedation and analgesia remains in the presence of befiradol; that is, that befiradol reduces sedation but also reduces analgesia.

Befiradol Causes Hyperalgesia, Hyperventilation, and Behavioral Syndrome in Normal Adult Rats
The final series of experiments focused on the effects on baseline behaviors of befiradol (0.2 mg/kg, neck subcutaneously). With thermal nociceptive testing, the latency of paw withdrawal was 5.4 ± 0.5 s before saline (n = 6) treatment. When the animals were treated with saline (n = 6), there was no difference in the latency of paw withdrawal to thermal stimulus (99.1 ± 5.4% relative to that before saline, P = 0.79, paired t test). When the animals were treated with befiradol (n = 6), the latency of paw withdrawal was shortened (79.1 ± 6.8% relative to that before befiradol, P = 0.018, paired t test). There was a significant difference between the two groups after befiradol treatment versus saline treatment (P = 0.045, t test, fig. 7). With Buxco plethysmographic recording, there was no difference in \( V_E \) after saline treatment (101.7 ± 3.9% relative to that before saline, n = 6, P = 0.82, paired t test). But befiradol-treated animals had an increased \( V_E \) (n = 6; 119.3 ± 6.8% relative to that before befiradol, P = 0.018, paired t test; P = 0.048 compared with saline treatment, t test, fig. 7). All befiradol-treated animals (n = 6) had at least two aspects of “behavioral syndrome,” characterized by head weaving (two of six), forepaw treading (two of six), flat body posture (three of six), and lower lip retraction (four of six). None of six saline-treated animals displayed characteristics consistent with “behavioral syndrome.”

Discussion
The major findings of this study were that befiradol: (1) dose dependently countered fentanyl-induced respiratory depression; (2) reduced fentanyl-induced analgesia; (3) reduced fentanyl-induced sedation; and (4) induced hyperventilation, hyperalgesia, and “behavioral syndrome” in nonsedated rats. Further, the befiradol-induced alleviation of opioid-mediated respiratory depression involves sites or mechanisms not present or functional in the isolated brainstem–spinal cord and medullary slice preparations.

The role of 5-HT in modulating respiration has been extensively studied and its relative importance debated. Although the contention that 5-HT is essential for eupnea and gasping has been disproven, serotonergic modulation of ventilation is very potent and warrants consideration as a prime target for development of pharmacological therapies to counter respiratory depression. For example, the 5-HT1A R agonist F15599 stabilizes respiratory rhythm in mouse models of Rett syndrome. Early reports of the 5-HT1A R agonist countering opioid-induced respiratory depression in mouse models showed promise. However, studies with a 5-HT1A agonist suitable for clinical use, mosapride, did not show efficacy in rodent models and a human study. That line of investigation may require development of improved 5-HT1A agonists. The results with 5-HT1A R agonists, as discussed in the Introduction, have been mixed in rodent models. Further, a high dose of bisporone (60 mg, oral) was ineffective at reversing morphine-induced respiratory depression in a human study. However, with the emergence of more selective and efficacious 5-HT1A R agonists, such as befiradol and related compounds, there is an impetus for further preclinical and clinical studies.

Alleviation of fentanyl-induced respiratory depression by befiradol in vivo in neonatal rats was robust. That is why the lack of effect of befiradol on the DAMGO-induced respiratory depression observed in vitro was unexpected. Opioids act at multiple sites within the central nervous system to depress respiratory drive, including directly via μ-opiate receptors within the preBotC in vitro and in vivo. The other major class of drugs used to counter opiate-induced respiratory depression, ampakines, induces a clear reversal of DAMGO-induced respiratory depression in vitro. Data examining the interactions between 5-HT1A R agonists and opioids, which are limited to analysis of in situ working heart brainstem preparation and in vivo (often anesthetized) models, show a clear 5-HT1A R-mediated reversal of opiate-induced respiratory depression. Within the brain, 5-HT1A Rs are mainly expressed in the hippocampus, lateral septum, cortical regions, raphe nuclei, hypoglossal nuclei, solitary tract nuclei, and respiratory regions, with a higher level of 5-HT1A Rs in neonatal period (i.e., hypoglossal nuclei). The mechanisms proposed include 5-HT1A R agonist actions on post-1 or late depressive neurons via G protein–coupled inwardly rectifying potassium channels, and glycinergic inhibitory neurons within the preBotC. Why those
mechanisms might not be functional in vitro is not clear. The medullary slice contains only a subset of medullary respiratory nuclei and a limited repertoire of neuronal populations based on discharge patterns. Further, chloride-mediated conductances are likely altered in medullary slice preparations due to the elevated extracellular potassium ion concentration typically used to enhance neuronal and network excitability. In contrast, the BSSC preparation is maintained under physiological potassium ion concentration conditions. Detailed extracellular recordings of neuronal activity in the ventrolateral reticular formation, including the preBötC region, demonstrated at least five distinct classes of respiratory neurons that are active during either I or E phases. Anatomically, the BSSC preparation has the full complement of medullary nuclei. However, the extent to which neuronal populations within the medulla are functional is not clear, given the varied oxygen and pH profile within the tissue. Further studies across multiple experimental models will be necessary to clarify what mechanistic substrate of 5-HT1A receptor action is absent in vitro.

In contrast, the dose-dependent alleviation of fentanyl-induced respiratory depression by befiradol in vivo was clear. There was also a reduction in the duration of fentanyl-induced sedation by befiradol that is similar to findings from previous studies of 5-HT1A receptor agonists and opioids (8-OH-DPAT). Some are there clinical situations where that could be clearly advantageous. However, some actions of befiradol are likely to be problematic. The analgesia induced by fentanyl was reduced by befiradol. Although not as complete as that caused by naloxone, the depression of analgesia is a counter indication. This finding is consistent with previous reports of 5-HT1A receptor agonists attenuating opioid-induced analgesia in unanesthetized rodents.

Additional challenges associated with using befiradol for the therapeutic alleviation of fentanyl-induced respiratory depression include the alteration in baseline respiration, nociception, and behavior. Befiradol induced an approximate 20% increase in baseline ventilation that would be experienced by patients if befiradol was given (1) before fentanyl to decrease the ensuing severity of respiratory depression or (2) subsequent to fentanyl to alleviate respiratory depression if the befiradol action persisted longer than those of fentanyl. The potential for the induction of “behavioral syndrome” was consistent with studies examining 5-HT1A receptor agonist 8-OH-DPAT. An additional increase in nociception observed would also be problematic. Our finding of an early pronociceptive effect of befiradol is consistent with previous studies in rat that reported an initial 2-h phase of hyperalgesia followed by hypoalgesia 8 h later. However, only the initial 2 h of hyperalgesia was related to the current study of acute effects of 5-HT1A receptor agonists on nociception in normal rats. Notably, there have been reports of an increase in baseline nociception in unanesthetized rodent models after administration of other 5-HT1A receptor agonists. The finding that befiradol induced hyperventilation and hyperalgesia in untreated animals suggests that the effect on fentanyl-treated animals may be mechanistically unrelated to the opioid effect. The lack of effect of befiradol on spinal and medullary respiratory function with in vitro preparations would seem to support this conclusion. However, these data do not allow for determination of the mechanisms underlying robust reversal of opioid-induced respiratory depression, analgesia, and sedation by befiradol.

Clinical Implications

Developing alternatives to naloxone for reversing opioid-induced respiratory depression is a clear unmet clinical need. Ampakines are effective at reducing opioid-induced respiratory depression without altering analgesia or baseline cardiorespiratory parameters in all in vitro, in situ, and in vivo rodent models studied. Further, those findings translated in a human study of alfentanily-induced respiratory depression. A limitation of ampakines is that only oral formulations are available for clinical trials and they are associated with a significant delay in ampakines reaching plasma therapeutic levels (>1 h). Although useful for a variety of clinical settings, it will be important to have injectable formulations for acute intervention. An injectable formulation of the ampakine CX1942 is under development, but it has not advanced beyond the preclinical stage of testing. The additional development of serotonergic agents for drug-induced and disease-related (e.g., Rett syndrome) is clearly warranted. There may be cases where targeting of AMPA or 5-HT receptor systems is more effective for stabilizing central respiratory drive. Data from this study of the highly selective 5-HT1A receptor agonist befiradol demonstrates its efficacy in reversing respiratory depression, but also the partial loss of analgesia. The potential problems of altered baseline respiratory function, behavior, and hyperalgesia are additional concerns that will require monitoring. Future studies of related compounds, F13714 and F15599, that preferentially bind to pre- and postsynaptically located 5-HT1A receptors, respectively, may have an enhanced therapeutic profile.

Acknowledgments

The authors thank Monica Gorassini, Ph.D. (Department of Biomedical Engineering), and Karim Fouad, Ph.D. (Faculty of Rehabilitation Medicine), University of Alberta (Edmonton, Alberta, Canada), for the loan of essential equipment. Befiradol was generously provided by Mark Varney, Ph.D. (Neurolixis Inc., San Diego, California). Supported by the Canadian Institutes of Health (Edmonton, Alberta, Canada).

Competing Interests

The authors declare no competing interests.

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References


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