Opioid-Induced Respiratory Depression in C57BL/6J Mice: The Role of Prefrontal Cortex Cholinergic Transmission in the Wakefulness Stimulus for Breathing

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To my parents, Zhonge and Zhongjie, and my sisters, Mary and Eleanor, thank you all for paving the way and for sticking with me through thick and thin. Your love and support have motivated me to get to this position today and to be the best version of myself. I hope to continue making you all proud.

ABSTRACT:

Opioid-induced respiratory depression (OIRD) is the primary cause of death from opioid overdose. Opioids depress breathing by diminishing the wakefulness stimulus for breathing in humans and mice. Cholinergic transmission in the prefrontal cortex (PFC) promotes cortical EEG activation, increases wakefulness, and stimulates breathing. However, no previous studies have tested whether increasing cholinergic transmission in the PFC can mitigate respiratory depression caused by systemically administered fentanyl in mice. The series of studies comprising this honors thesis is split into two phases. The first phase included a concentration response study evaluating the effects of fentanyl on breathing in C57BL/6J mice breathing room air. Adult male mice (n = 12) received intraperitoneal (IP) injections of saline and fentanyl in increasing half log unit doses. The second phase tested the hypothesis that microinjection of the acetylcholinesterase inhibitor neostigmine into the PFC attenuates OIRD caused by systemic fentanyl administration in male B6 mice. Adult male mice (n = 4) were surgically implanted with microinjection guide tubes aimed at the medial prefrontal cortex. Subjects were given a systemic injection of either saline or fentanyl, followed by a microinjection of saline or neostigmine. Preliminary data show that neostigmine mitigated the effects of fentanyl on breathing and significantly slowed the onset of OIRD. Histology pending, these results support the interpretation that cholinergic transmission in the PFC contributes to the wakefulness stimulus for breathing, extend previous data showing that breathing is stimulated by PFC neostigmine, and demonstrate that enhancing PFC cholinergic transmission offsets OIRD caused by systemically administered fentanyl.

INTRODUCTION:

Opioids are analgesics commonly prescribed for chronic and post-operative pain treatment but bring a multitude of unwanted side effects, namely respiratory depression. Opioid-induced respiratory depression (OIRD) is the primary cause of death from opioid overdose and limits the clinical efficacy of opioids (K. T. Pattinson, 2008). Deaths involving synthetic opioids have increased by 1040% from 2013 to 2019 (Mattson et al., 2021), contributing to the ongoing opioid epidemic. In the clinical setting, patients with OIRD place a great financial burden on hospitals (Khanna et al., 2021). These findings illustrate an urgent need for research seeking to develop safer use of opioids. The National Institutes of Health (NIH) has launched a comprehensive research initiative to promote evidence-based approaches to treat Opioid Use Disorder, opioid overdose, and chronic pain (Volkow & Collins, 2017). The studies in my honors thesis align with these interests of the NIH.

The subject of OIRD is relevant for a psychology honors thesis in two major ways. First, opioids depress breathing by acting on mu-opioid receptors (A. Dahan et al., 2001) located in the brainstem respiratory network (Fig. 1). Recent studies have focused on the preBötzinger complex (preBöt) of the ventrolateral medulla and the parabrachial nucleus (PBN) of the dorsolateral pons as the brainstem nuclei mediating OIRD (Levitt, Abdala, Paton, Bissonnette, & Williams, 2015; Montandon et al., 2011; Montandon et al., 2016). The preBöt generates the respiratory rhythm in mammals, (J. C. Smith, Ellenberger, Ballanyi, Richter, & Feldman, 1991), drives the inspiratory phase of breathing (J. C. Smith, Abdala, Borgmann, Rybak, & Paton, 2013), and expresses mu-opioid receptors (Gray, Rekling, Bocchiaro, & Feldman, 1999). In the PBN, the Kölliker-Fuse (KF) nucleus mediates the transition from the inspiratory to the expiratory phase of breathing and is crucial for eupneic, or normal, unlabored, breathing (Cohen, 1971; Dutschmann & Herbert,

2006; Lumsden, 1923). Morphine and fentanyl injections into the preBöt and PBN/KF nuclei result in increased inspiratory time (Ti), decreased inspiratory airflow, and decreased respiratory rate (Bachmutsky, Wei, Kish, & Yackle, 2020). These studies emphasize the viewpoint that breathing is a behavior generated by the brain and can be understood in terms of neurophysiological mechanisms.

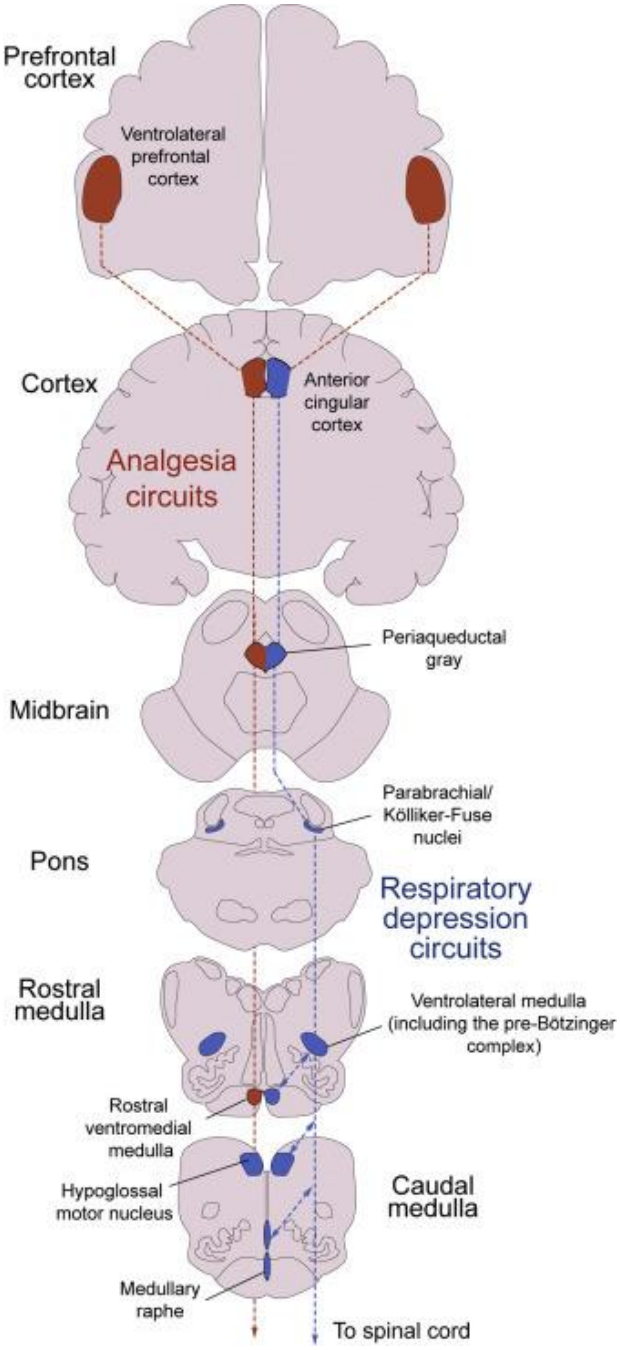


Figure 1. Schematic of the brainstem respiratory nuclei implicated in OIRD, including the preBötzinger complex (preBöt) at the ventrolateral medulla and the Kölliker-Fuse nuclei at the parabrachial nucleus in the pons. Respiratory depression from opioids is caused by activation of mu-opioid receptors in these nuclei, which then send efferent connections to the spinal cord. Figure from (Montandon & Slutsky, 2019).

Second, studies of OIRD and breathing are relevant to psychology because changes in breathing are often dependent on behavioral states. Compared to states of wakefulness, ventilation decreases in slow-wave (NREM) sleep states as well as states of altered consciousness caused by anesthetics (Hedenstierna & Edmark, 2015), benzodiazepines (Guilleminault, 1990), alcohol (Block, Hellard, & Slayton, 1986), and opioids. Although the altered states of consciousness caused by opioids are distinct from states of wakefulness, NREM sleep, or REM sleep (Montandon & Horner, 2019; O'Brien et al., 2021), the relationship between behavioral state and slowed breathing suggests that opioids depress breathing by diminishing the wakefulness stimulus for breathing. The wakefulness stimulus for breathing refers to the arousal-promoting neurons in the cortex that project to the brainstem respiratory center and modulate breathing. The wakefulness stimulus is vital for behaviors encompassing the volitional control of breathing such as breath-holding and speech, and it is relevant in the context of OIRD because it may explain individual differences in susceptibility to OIRD. For example, having an awareness of respiration or the urge to breathe allows for appropriate compensatory responses that may mitigate OIRD. A recent study found that opioids can inhibit these cortical regions implicated in the urge to breathe and awareness (K. T. S. Pattinson et al., 2009). In this study, human subjects were given instructions for breath-holding and rated on a numerical scale their urge to breathe, and functional magnetic resonance imaging measured cortical activity in response to breath-holding. Following the administration of remifentanil, urge to breathe scores and blood-oxygen-level-dependent (BOLD) levels in the cortical regions associated with breath-holding control were lower relative to the control condition. Lower BOLD levels indicate lower activation of brain regions, and the associated cortical regions include the PFC, periaqueductal gray (PAG), and the anterior cingulate cortex (ACC). However, the neuronal networks underlying this

wakefulness stimulus remain poorly understood. These findings support the interpretation that opioids diminish the wakefulness stimulus for breathing and validate the psychological relevance of the present studies.

The PFC contains cholinergic receptors that mediate excitatory neurotransmission to the brain. Given the role of PFC cholinergic transmission in promoting wakefulness, (Baghdoyan & Lydic, 2012), it is hypothesized that acetylcholine (ACh) in this region contributes to the wakefulness stimulus for breathing. Activation of muscarinic cholinergic receptors in the PFC causes behavioral arousal, as quantified by a decrease in slow-wave EEG power and increased ACh release (Douglas, Baghdoyan, & Lydic, 2002a, 2002b). Modulation of cholinergic transmission in the PFC restores consciousness in rodents from an anesthetized state, characterized by increased respiratory frequency (Pal et al., 2018; Parkar et al., 2020). Additionally, microinjection of the cholinesterase inhibitor neostigmine into the PFC of awake, freely behaving mice stimulates breathing (Glovak, O'Brien, Sun, Baghdoyan, & Lydic, 2021). Cholinergic stimulation in the parietal cortex, and noradrenergic stimulation in the PFC did not result in behavioral arousal (Pal et al., 2018), signifying that the arousal-promoting effects of ACh are specific to the PFC. The relationship between ACh in the PFC and opioids is highlighted in the finding that systemically delivered morphine inhibits ACh release in the PFC (Osman, Baghdoyan, & Lydic, 2005), suggesting that enhancing cholinergic transmission in the PFC following opioid administration may provide the excitatory neurotransmission needed to mitigate respiratory depression. Additionally, the evidence presented in these studies supports the use of EEG as a validated biomarker of behavioral arousal.

The only current available treatment for OIRD, naloxone, has significant limitations that necessitate efforts to develop new therapeutics. Naloxone was approved by the Food and Drug

Administration for treatment of opioid overdose in 1971 and is available in intranasal form under brand names such as NARCAN®. It is an opioid antagonist with high affinity for the mu-opioid receptor (Trescot, Datta, Lee, & Hansen, 2008), preventing opioid molecules from binding. However, naloxone has a very short biological half-life of only 65 minutes (Moss & Carlo, 2019). For comparison, the biological half-life of morphine and fentanyl are 2-3 hours and 8-10 hours, respectively (Berkowitz, 1976; Kharasch, 2015). Thus, the short duration of activity of naloxone also means a short-lived reversal of overdose that may require continuous infusion of naloxone or risk of relapse of OIRD (Handal, Schauben, & Salamone, 1983). Additionally, because naloxone is an opioid receptor antagonist, it also antagonizes all opioid effects, including analgesia. This limitation necessitates patients to re-administer opioids to achieve analgesia, subsequently increasing the risk for addiction. A novel treatment that can minimize the risk of or mitigate respiratory depression without compromising analgesia would eliminate these limitations and advance chronic pain management.

Preclinical and clinical data support the use of cholinergic drugs as a potential countermeasure for OIRD. The first known study to successfully reverse OIRD was conducted in 1980, where intravenous administration of the cholinesterase inhibitor physostigmine, but not neostigmine, antagonized morphine-induced respiratory depression in rabbits and anesthetized dogs (Weinstock, Roll, Erez, & Bahar, 1980). The differential effects may be attributed to the properties of neostigmine, which does not cross the blood-brain barrier when delivered systemically. Opioids also increase the apneic threshold, and this threshold is decreased by physostigmine (Berkenbosch, Olievier, Wolsink, DeGoede, & Rupreht, 1994). Apneic threshold refers to the minimum level of carbon dioxide required to maintain normal breathing, and decreasing the apneic threshold reduces the risk of apnea. Additional studies have found that

systemic administration of donepezil, which increases endogenous ACh, can rescue respiratory depression in anesthetized rabbits caused by morphine (Tsujita et al., 2007) and buprenorphine (Sakuraba et al., 2009). Cholinergic agonists stimulate the rhythm-generating nuclei in the brainstem in vitro and increase minute ventilation in vivo (Ren, Ding, & Greer, 2019). Although this honors thesis does not investigate nociception data, the use of cholinergic drugs to prevent OIRD has the potential to be more versatile than naloxone because it does not antagonize opioid-induced analgesia and may also be beneficial in treating sedation and addiction. Clinical data show the efficacy of cholinergic drugs in treating opioid-use disorder (Jensen, DeVito, Yip, Carroll, & Sofuoglu, 2018) and in treating daytime sleepiness and improving nighttime sleep in patients taking morphine without compromising analgesia (Slatkin, Rhiner, & Bolton, 2001). Given the relationship between addiction, sedation, and the arousal-promoting effects of the PFC, these findings strongly suggest that the PFC may be implicated in OIRD. To the best of our knowledge, there have been no studies testing the effect of cholinergic transmission in the PFC on OIRD in awake, unrestrained mice.

METHODS:

All procedures in these studies adhere to the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the & Use of Laboratory, 2011) and were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC) protocol #2555. The approach to this undergraduate honors thesis was comprised of two phases. The studies began by deriving direct evidence for the dose-dependent effects of fentanyl on breathing, providing essential data on the doses of systemic fentanyl to use in the next phase of experiments. The second phase of experiments tested whether respiratory depression caused by these systemic doses of fentanyl can be

prevented with PFC microinjection of neostigmine. All studies use adult male C57BL/6J (B6) mice purchased from the Jackson Laboratory (Bar Harbor, ME) at 7 weeks of age. Mice were kept in a 12 hour/12 hour light-dark cycle housing with ad libitum access to food (Tekland 8640 Rodent Diet) and water. Subject health was monitored daily by laboratory personnel.

Measures of breathing (frequency, tidal volume per gram body weight, minute ventilation per gram body weight, and duty cycle) were quantified by placing mice into whole-body plethysmography (WBP) chambers (Data Sciences International, USA) at 22◦ C. The WBP system permits the collection of data in unrestrained, freely moving mice and minimizes the effect of animal handling on breathing. Pressure differences are detected by Buxco FinePointe software and digitalized into the desired respiratory parameters. These techniques used in my honors thesis are an extension of my independent studies work and involvement in other respiratory studies in our lab (Glovak et al., 2021).

The DSI PhysioTel^{TM} HD-X02 telemetry system enables the recording of cortical electroencephalogram EEG and electromyogram EMG signals from mice. Recording electrodes and the telemeter are implanted in subjects and allow for the collection of data in unrestrained, freely moving mice. The telemeter sends signal recordings to the data acquisition computer via DSI receiver pads placed on the bottom of the WBP chambers to permit the simultaneous recording of breathing and EEG signals. EEG data were collected by DSI Ponemah software and analyzed using DSI NeuroScore software.

Fentanyl Dose-response Study

These experiments used a within-subjects design in which each mouse (n=12) received a 0.3 mL intraperitoneal (IP) injection of saline and eight doses of fentanyl in increasing concentrations, separated by one-half log unit (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 mg/kg).

This design allowed each subject to serve as its own control. These doses have been shown by preliminary data from our lab to not harm or cause apnea in mice. Injection times occurred during the same time of day for a maximum of two injection days per week, each day separated by at least 24 hours to allow subjects to recover. Fentanyl citrate ($C_{28}H_{36}N_2O_8$; 528.6 g/mol) (Sigma-Aldrich, USA) was dissolved in 0.9% saline to yield the desired doses of fentanyl for this study. Fentanyl does not have an active metabolite (H. S. Smith, 2009), reducing the likelihood of residual effects of opioid injections from previous days confounding breathing parameters. All subjects were conditioned to being handled and placed into the WBP chambers for at least two weeks before data collection. During experiments, subjects were allowed to habituate to the chambers for at least 30 minutes before an injection was given. The subject was restrained by hand, the skin over the injection site was cleaned with an alcohol prep pad, and the injection was given near the intraperitoneal cavity using a 25-gauge luer-lock needle. Breathing data were collected for 60 minutes post-injection.

Dual Injection Study

All mice (n=4) were surgically implanted with a PlasticsOne 26-gauge intracranial guide tube to enable in-vivo drug delivery to the PFC and a DSI HD-X02 telemeter for EEG and neck muscle electromyogram (EMG) recording. Protocols for stereotaxic surgery closely follow the procedures outlines in previous studies in our lab (O'Brien et al., 2021; Zhang et al., 2020) to ensure the humane treatment of all subjects during surgery. All surgeries were performed on a stereotaxic frame (David Kopf Instruments, USA). Isoflurane was the inhaled anesthetic and was delivered at 2.0% with 100% oxygen at a flow rate of 1 L/min. Loss of righting reflex and paw withdrawal latency were used as biomarkers for antinociception and the loss of consciousness. Subject vital signs were continuously monitored throughout the surgery. Isoflurane delivery was

measured by Datex-Ohmeda Cardiocap5 spectrophotometry system, and concentration incrementally reduced throughout the surgery to maintain subject respiratory rate above 60 breaths/min. Core body temperature was maintained at 36-37.5°C using Gaymar water pumps and heating pads. No abnormalities occurred during the surgeries.

An incision was made at the scalp to expose the mouse skull and implant the guide tube. The guide tube was aimed at the infralimbic cortex (IL) of the PFC at stereotaxic coordinates A/P = 1.94; M/L = 0.3; D/V = 2.5 relative to bregma (Franklin, 2008). A 1.0 mm craniotomy was made at this aim point using a 1.0 mm Stoelting drill bit and Foredom K1070 micromotor. Dental acrylic was applied around the skull and guide tube to keep the guide tube in place and form a headcap. Additionally, 4 pilot holes were made on the skull with a 0.45 mm Stoelting drill bit to insert 4 custom Antrin miniature screws. These screws provide an anchor for the guide tube and headcap.

The HD-X02 telemeter transmitter and accompanying EEG and EMG electrodes were also implanted during the surgery. The transmitter was implanted near the mouse's flank by lengthening the original incision towards the flank and making a subcutaneous pocket. The EMG electrodes were implanted at the dorsal region of the neck and secured with dental acrylic and sutures. To implant the EEG electrodes, two 1.0 mm craniotomies were made at stereotaxic coordinates A/P = 1.0; M/L = -1.0 and A/P = -3; M/L =3.0 relative to bregma. The dental acrylic from the guide tube head cap kept the electrodes in place. After the surgery, all implanted subjects were housed singly and given at least seven days to recover from the surgery and adjust to new housing.

Subjects were conditioned to all experimental procedures for at least 2 weeks before the start of data collection. These series of experiments used a within-subjects design in which each

mouse received all three treatment conditions: a saline IP injection followed by a saline microinjection, a fentanyl IP injection followed by a saline microinjection, and a 0.1 mg/kg fentanyl IP injection followed by a 0.016 nmol/50nL (4.8 ng/50nL) neostigmine microinjection. Fentanyl citrate and neostigmine bromide ($C_{12}H_{19}BrN_2O_2$; 302.20 g/mol) were dissolved in 0.9% saline to yield the desired injection concentrations. Before every injection, subjects were placed in the whole-body plethysmography chambers to acclimate for at least 45 minutes. The IP injection was performed following the same procedure as the previous dose-response study. The microinjection apparatus consisted of a micro syringe and a manual drive holding a 1-microliter Hamilton syringe filled with injection solution, plastic tubing, and a PlasticsOne injector cannula. To deliver a microinjection, the subject was restrained by its headcap, and the obturator was removed to place the injector into the guide tube. Injection volume (50 nL) was delivered evenly over 60 seconds, and the injector was left in the guide tube for 30 seconds post-injection before removal to prevent suction of injection solution. Due to the small volume of injection, post-injection flow was measured to confirm delivery of solution by pumping the micro syringe until fluid was present. The nL until flow was then then recorded as the post-injection flow. Breathing and EEG were recorded for 60-minutes post-injection. Subjects were monitored during this recording period and were kept awake by an investigator to prevent any breathing changes caused by sleep.

Pilot Study: Effects of carbachol microinjected into the prefrontal cortex on breathing and EEG

Given that neostigmine is an acetylcholinesterase inhibitor that can stimulate breathing when microinjected into the PFC of awake mice (Glovak et al., 2021), I performed a pilot study to test whether the same would hold true for a cholinergic drug with a different mechanism of

action. Carbachol, a mixed cholinergic receptor agonist, was chosen for this purpose. These experiments used a within subject design in which 3 mice received a 50 nL intracerebral microinjection of 1mM (9.13 nmol/50nL; 33.36 mg/mL) carbamylcholine chloride ($NH_2COOCH_2CH_2N(Cl)(CH_3)_3$; 182.65 g/mol) and 2 mice received an additional microinjection of 10 mM (91.3 nmol/50nL; 333.6 mg/mL) carbachol. Subjects used in this study were mice remaining from the dual injection study. Thus, all microinjection sites and experimental procedures followed that of previous studies.

Statistical Analysis

All data were analyzed with descriptive, parametric, and nonparametric statistics using GraphPad Prism 9.2.0. For studies evaluating the dose-dependent effects of fentanyl on breathing, measures of breathing were expressed as means across 12 subjects over the entire 60-minute post-injection recording period. Given that centrally administered drugs rapidly diffuse away from a microinjection site, measures of breathing for studies evaluating the effect of PFC neostigmine on fentanyl-induced respiratory depression were expressed as means across 4 subjects during the first 15 minutes post-injection. Analyzing the first 15 minutes provides the greatest likelihood of inferring the effect of PFC cholinergic transmission on OIRD. For microinjection studies, raw data are expressed as vertical scatterplots with median and interquartile range (IQR) bars in accordance with the NIH guidelines for enhancing rigor and reproducibility in research (https://grants.nih.gov/policy/reproducibility/index.htm). For all studies, differences in mean across treatment groups were compared using percent change calculations, and my hypotheses were tested using a repeated measures one-way Analysis of Variance (ANOVA) with Dunnett's post-hoc comparisons. The Cohen's *d* statistic was calculated to quantify the relative magnitude of treatment effect. Breathing variability is a

clinically important biomarker of normal compensatory respiratory response that is compromised by opioids (Angel et al., 2018). Thus, I also used Poincaré analysis to compare the effects of fentanyl on breathing variability with and without PFC neostigmine. Finally, regression analyses were performed to quantify the effects of PFC neostigmine over time.

RESULTS:

Fentanyl Caused Respiratory Depression in B6 Mice in a Dose-dependent Manner

Behavioral observations of the mice were made following administration of each treatment. All subjects remained awake for the duration of the 60-minute post-injection recording period. Typical behaviors in the WBP chambers included pacing around the circular perimeter of the chamber and grooming activity. Measures of frequency and minute ventilation tended to decrease during the first 20 minutes post-injection, including the saline condition. No significant phenotypic changes were observed for doses smaller than the 0.3 mg/kg dose. Beginning with the 0.3 mg/kg dose, mice exhibited the Straub Tail Reaction (STR) effect, which became more pronounced in the 1.0 mg/kg and 3.0 mg/kg dose. This behavior is characterized as the tail becoming rigid, erect, and dorsiflexed perpendicular to the rest of the body. The onset of STR was roughly 2 minutes post-injection. The STR is a well-characterized side effect of opioids, including fentanyl (Kitanaka et al., 2012; Nath, Gupta, Patnaik, & Dhawan, 1994; O'Neill, Collins, Pettit, McNutt, & Chang, 1997). Following the administration of the 3.0 mg/kg dose, mice exhibited disoriented activity. They were unable to pace around the chamber, often running into and pressing against the plexiglass wall of the chamber, and sometimes remaining still for minutes. This behavior was not observed in any other treatment condition. Despite these behaviors, no subjects were at risk of harm to themselves, and all experiments proceeded as detailed by the protocol.

Fentanyl caused a significant dose-dependent depression of breathing relative to the saline condition. Figure 2 shows that frequency and minute ventilation progressively decreased with higher fentanyl doses. Interestingly, tidal volume and duty cycle progressively increased at higher doses. On average, the 3.0 mg/kg dose of fentanyl caused a 63.29% decrease in frequency, 29.24% increase in tidal volume, 55.80% decrease in minute ventilation, and 59.15% increase in duty cycle. Cohen's *d* values for at the 3.0 mg/kg dose signify >95% overlap between the saline and 3.0 mg/kg distributions: frequency (d = 5.7), tidal volume (d = -2.2), minute ventilation (d = 3.7), and duty cycle (d = -6.1). It should also be noted that the standard deviation for F and MvB decreased at the 1.0 mg/kg and 3.0 mg/kg dose (Fig 2), signifying a decrease in breathing variability. Repeated-measures one-way ANOVA confirmed statistically significant differences: frequency ($F_{(8,88)} = 42.75$, $P<0.0001$), tidal volume ($F_{(3.97, 43.65)} = 30.61$, $p<0.0001$), minute ventilation ($F_{(2.47, 27.12)} = 20.30$, $p<0.0001$), and duty cycle ($F_{(2.23, 24.53)} = 66.82$, $p<0.0001$). These values signify a significantly large variance across treatment conditions. Post hoc analyses revealed that the lowest fentanyl dose causing a significant decrease in frequency and minute ventilation is 0.01 mg/kg ($p=0.02555$, $p=0.0003$, respectively). The half-maximal inhibitory and effective concentrations of fentanyl (IC50 and EC50) were 0.28 mg/kg for frequency (95% CI [0.1561, 0.4941]), 0.24 mg/kg for tidal volume (95% CI [0.1096 to 0.5583]), 0.28 mg/kg for minute ventilation (95% CI [0.1078 to 0.7033]), and 1.63 mg/kg for duty cycle (95% CI [0.9708, 3.098]).

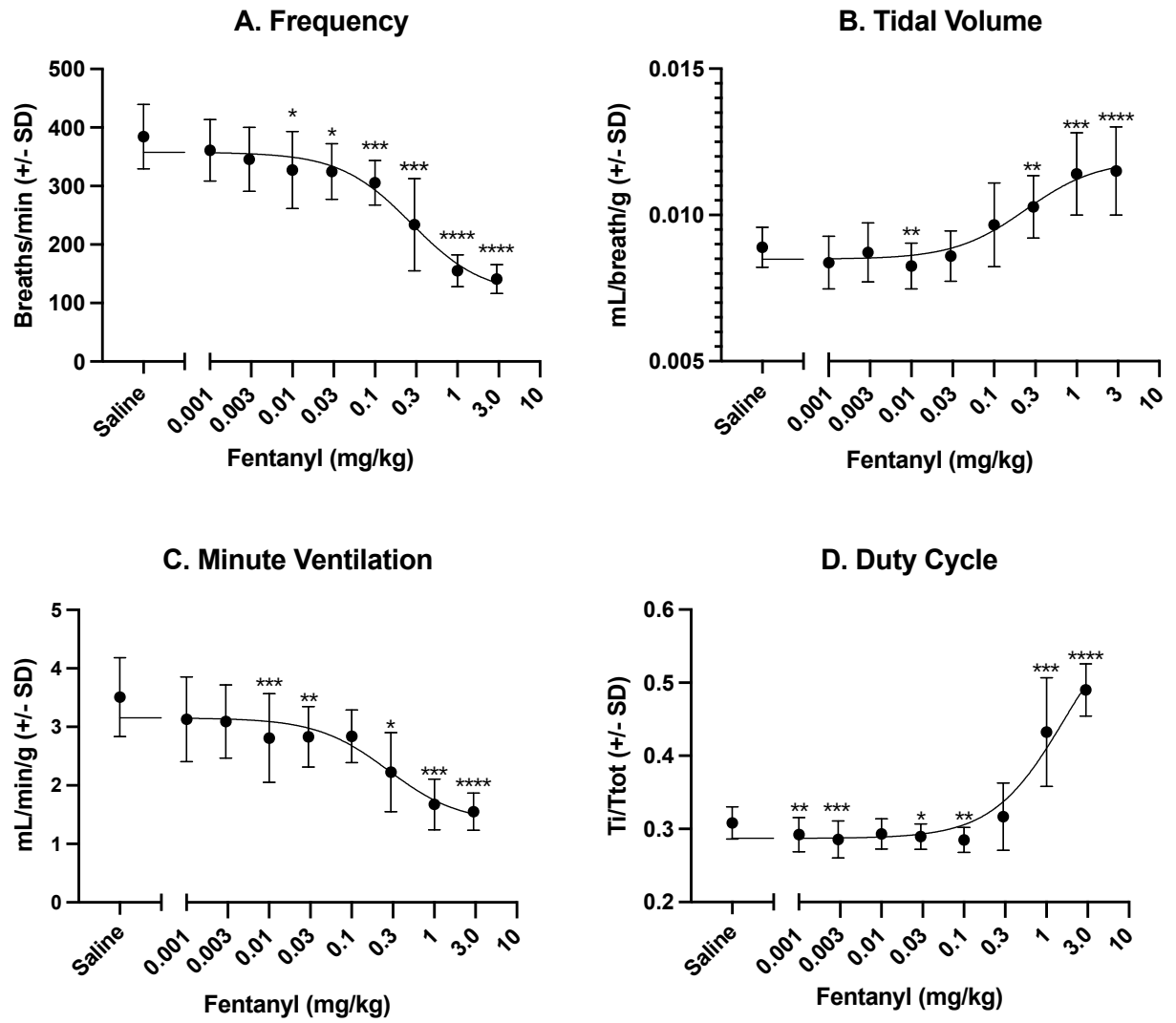


Figure 2. Fentanyl caused a dose-dependent decrease in breathing. Concentration-response curves for the effects of fentanyl on frequency (A), tidal volume (B), minute ventilation (C), and duty cycle (D). Each data point is expressed as means across all subjects for the 60-minute recording period +/- standard deviation. Data were analyzed using a repeated measures one-way ANOVA paired with Dunnett's post hoc tests. Asterisks indicate significant changes in breathing at a particular dose relative to saline: (*p <0.05, **p<0.01, ***p < 0.002 ****p<0.0001).

Regression analysis showed significant differences in the onset of OIRD between fentanyl doses. Minute ventilation was chosen for this set of analyses because it serves as the

aggregate of frequency and tidal volume and is a more comprehensive parameter of oxygenation. A second-order polynomial regression equation was fitted over time course graphs plotting every 60-second average minute ventilation data point as a function of time over the first 20 minutes post-injection (Fig 3). Comparison of all model parameters for the 0.03 mg/kg (minute ventilation = 4.976 - 0.2584(min) + 0.007535$(min)^2$), 0.3 mg/kg (minute ventilation = 4.477 – 0.3820(min) + 0.01354$(min)^2$), and 3.0 mg/kg (minute ventilation = 2.207 – 0.1345 (min) + 0.005549$(min)^2$) equations reveal that the differences in the regression equations are statistically significant: B0 ($F_{(2, 51)}$ = 99.69, p<0.0001), B1 ($F_{(2, 51)}$ = 14.57, p<0.0001), B2 ($F_{(2, 51)}$ = 7.687, p<0.0012). Comparison of r^2 values (0.03 mg/kg = 0.9231, 0.3 mg/kg = 0.9723, 3.0 mg/kg = 0.4061) show that time accounted for 92.31% and 97.23% of the variance in minute ventilation for the 0.03 mg/kg and 0.3 mg/kg doses, respectively. However, only 40.61% of the variation in minute ventilation can be accounted for by the variation in time for the 3.0 mg/kg dose. Given that this analysis focuses on the first 20 minutes post-injection, the lower r^2 for the 3.0 mg/kg dose could suggest that the onset of OIRD is less dependent on time.

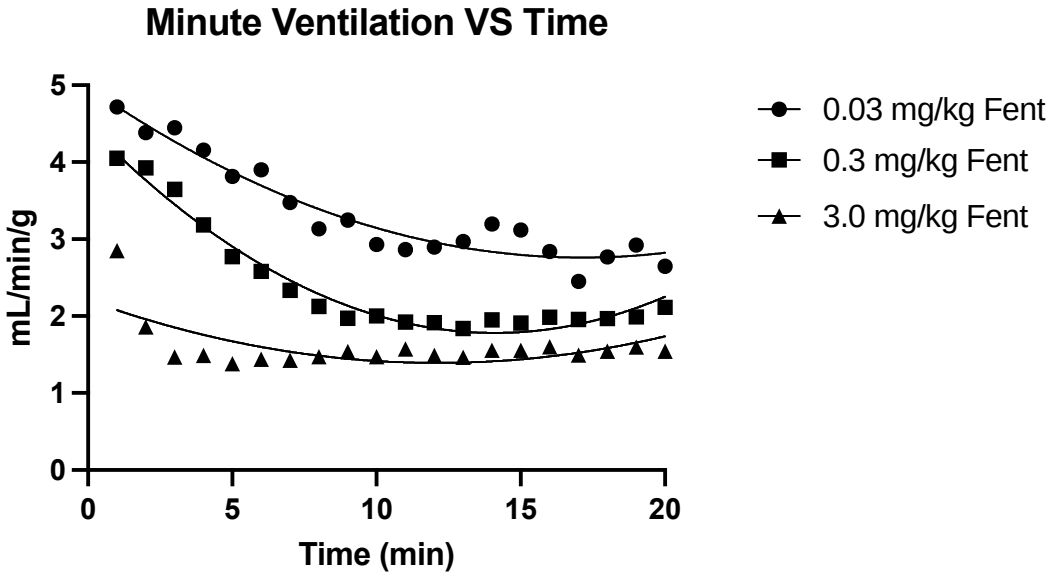


Figure 3. Fentanyl caused a dose-dependent differences in onset of respiratory depression. Second-order regression equations visualizing the effect of fentanyl on minute ventilation for the 0.03 mg/kg, 0.3 mg/kg, and 3.0 mg/kg dose of fentanyl plotted as a function of time. Each data point represents the average minute ventilation for n=12 subjects over a particular 60-second recording period for the first 20 minutes post-injection. All parameters B0, B1, and B2 were significantly different between the regression lines, suggesting differences in the onset of OIRD as a function of dose.

PFC Neostigmine Administration Mitigated OIRD

Behavioral observations of mice were made following administration of each treatment condition. Mice behaviors in the chamber were largely identical to the descriptions noted from the concentration-response study. Observations made following the PFC saline control conditions support the interpretation that the intracerebral microinjection procedure itself does not cause any behavioral confounds. Behavioral changes noted in mice following microinjection of neostigmine, however, suggest a potential cholinergic drug effect. All mice exhibited heightened activity in the chamber, characterized by a faster pacing around the chamber and increased twitch-like movements. Although this effect waned over the course of the post-injection recording period, one mouse was observed to exhibit the same hyper motoric effect up to 8 hours post-injection. Histology pending, these differences could be explained by differences in microinjection site. However, no subjects exhibited signs of distress, discomfort, or harm, and the experiments proceeded accordingly.

In the first 15 minutes post-injection, neostigmine microinjected into the PFC attenuated respiratory depression caused by fentanyl. Figure 4 shows vertical scatterplots with median and interquartile range (IQR) for frequency, tidal volume, minute ventilation, and duty cycle across 5

conditions: 1 = (IP saline + PFC saline), 2 = (IP 0.1 mg/kg fentanyl + PFC saline), 3 = (IP 0.1 mg/kg fentanyl + PFC neostigmine), 4 = (IP 0.3 mg/kg fentanyl + PFC saline), 5 = (IP 0.3 mg/kg fentanyl + PFC neostigmine). Comparing conditions 2 and 3 showed that IP 0.1 mg/kg fentanyl + PFC neostigmine caused, on average, a 26.9% increase in mean frequency (Cohen's $d = 0.68$) and a 44.3% increase in mean VE (Cohen's $d = 0.74$) relative to IP 0.1 mg/kg fentanyl + PFC saline. The ability of PFC neostigmine to mitigate OIRD diminished with a higher dose of fentanyl. The calculated effect sizes correspond to a relative moderate effect. Comparing conditions 5 and 6 showed that IP 0.3 mg/kg fentanyl + PFC neostigmine caused, on average, a 19.6% increase in mean frequency (Cohen's $d = 0.47$) and a 7.4% increase in mean VE (Cohen's $d = 0.14$) relative to IP 0.3 mg/kg fentanyl. The calculated effect sizes correspond to a relatively small effect. Due to a non-normal distribution, nonparametric statistical tests were used to evaluate differences across conditions. However, Friedman test revealed that observed differences between treatment conditions were not significant for any breathing measures: frequency ($p = 0.4421$), tidal volume ($p = 0.1435$), minute ventilation ($p = 0.4790$), and duty cycle ($p = 0.3703$), likely because of a small sample size.

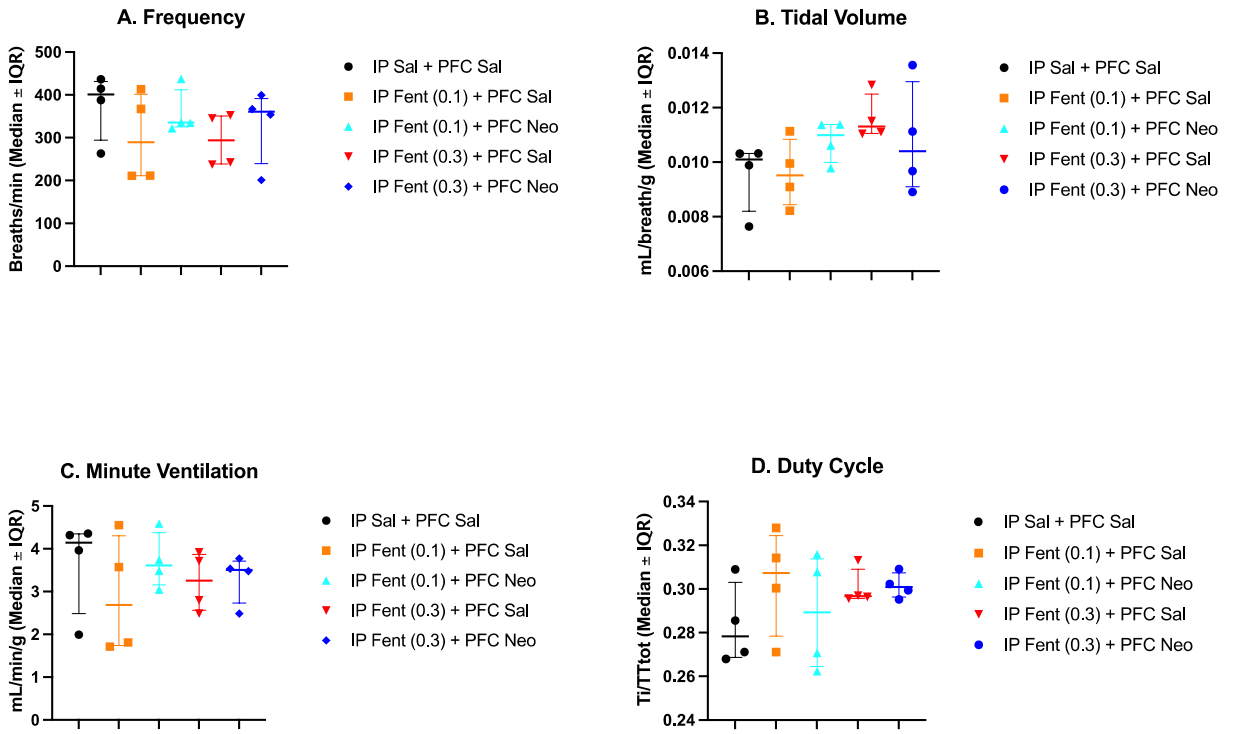


Figure 4. Vertical scatterplots with median (horizontal bar) and interquartile range (vertical bar) for frequency (A), tidal volume (B), minute ventilation (C), and duty cycle (D) across 5 treatment conditions. Each data point represents the 15-minute post-injection average value for a single subject. With a sample of n=4, Inferential statistics did not reveal a significant main effect between treatment groups.

Poincaré plots are useful for visualizing the differences in breathing variability across treatment conditions. Figure 5 shows Poincaré plots comparing minute ventilation variability between conditions: (a) IP 0.1 mg/kg fentanyl + PFC saline VS IP 0.1 mg/kg fentanyl + PFC neostigmine (b) IP 0.3 mg/kg fentanyl + PFC saline VS IP 0.3 mg/kg fentanyl + PFC neostigmine. WBP data were averaged every 1 min for the first 15 minutes. The x-coordinate of each data point represents the average minute ventilation for a particular 1-minute interval (n) averaged across all subjects, and the y-coordinate represents the average minute ventilation for the subsequent 1-minute interval (n+1). For example, if the x-coordinate of a data point is the

minute ventilation average for min 1, the y-coordinate is the minute ventilation average for min 2. The dispersion of points is an indicator of variability, where greater dispersion of points is associated with greater variability. In figure 5a, the distribution of points appears to be greater for the 0.1 Fent + Neo condition, suggesting that neostigmine increased breathing variability. Figure 5b shows that the cluster of points for the 0.3 Fent + Neo and 0.3 Fent + Sal condition overlap, and that neostigmine did not increase breathing variability for the 0.3 mg/kg fentanyl condition as was observed in the 0.1 mg/kg condition. These observations are consistent with the raw data, where the ability of neostigmine to attenuate OIRD was diminished for higher doses of fentanyl.

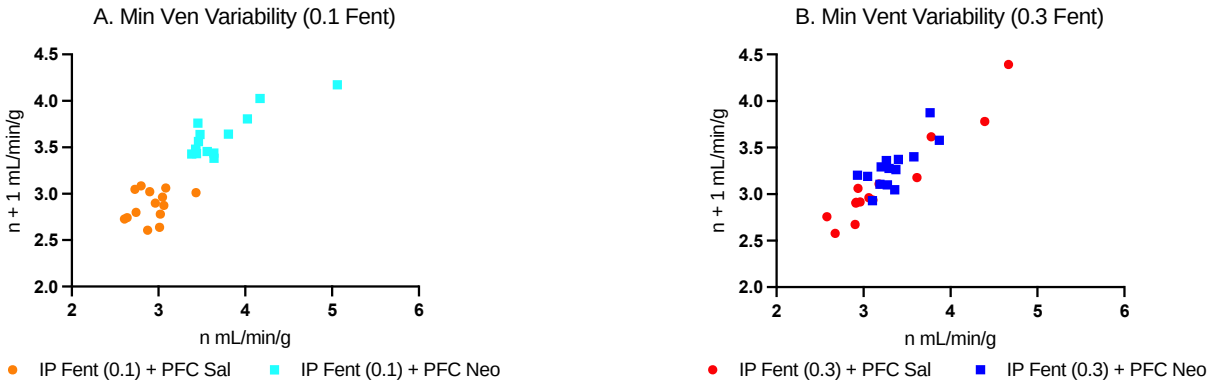


Figure 5. Poincaré plots visualizing breathing variability across treatment conditions for min 1-15 post-injection. Each data point represents the average minute ventilation across all subjects for a particular 60-second period (x coordinate), plotted against the average minute ventilation of the subsequent 60-second period (y coordinate). For the 0.1 mg/kg dose of fentanyl, PFC neostigmine appears to increase breathing variability (A), as seen by a greater distribution of data points. For the 0.3 mg/kg dose of fentanyl, PFC neostigmine does not appear to increase breathing variability (B).

Results from Poincaré analyses show that breathing variability is differentially altered across treatment conditions. Breathing variability was quantified by short term variability, known as standard deviation 1 (SD1), and long-term variability, known as standard deviation 2 (SD2). SD1 calculates the average variability in Mvb between each 60-second period, and SD2 calculates Mvb variability across the entire 15-minute period. Figure 6 shows vertical scatterplots with median and IQR bars to characterize SD1 and SD2 between each treatment condition. Following administration of the 0.1 mg/kg dose of fentanyl, PFC neostigmine caused, on average, a 29.5% increase in SD1 for minute ventilation (Cohen's $d = 1.2$) and a 9.45% increase in SD2 for minute ventilation (Cohen's $d = 0.7$) compared to the saline condition. The Cohen's *d* statistics correspond to a large effect for SD1 and medium effect for SD2. Neostigmine did not increase SD1 or SD2 for minute ventilation following administration of the 0.3 mg/kg dose of fentanyl, consistent with previous observations. Nonparametric statistics via the Friedmann test revealed a significant main effect of treatment condition on minute ventilation SD1 ($p = 0.0022$) but not minute ventilation SD2 ($p = 0.8946$). Despite a significant main effect, Tukey's post-hoc comparisons were inconclusive on which treatment conditions were significantly different in minute ventilation SD1.

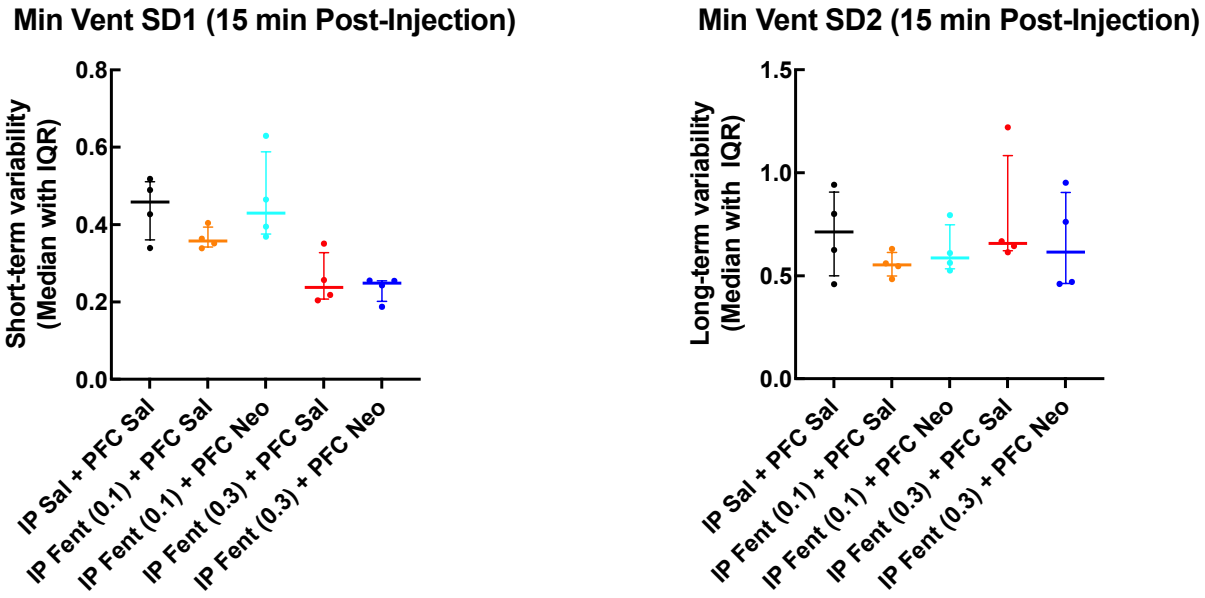


Figure 6. Comparison of minute ventilation short-term variability (SD1) and long-term variability (SD2) across treatment condition, with horizontal bar representing median and vertical bar representing IQR. Each data point represents the average SD1 or SD2 across all subjects for each treatment condition. Although nonparametric statistics found significant differences between treatment groups in minute ventilation SD1, post-hoc comparisons were inconclusive. No significant differences between treatment groups were found for minute ventilation SD2.

Regression analyses revealed that 4.8 ng of PFC Neo slowed the onset of OIRD caused by 0.1 mg/kg fentanyl. Minute ventilation was quantified during the initial 15 minutes after PFC Neo microinjection, and a simple linear regression was used to model the min 1-5, min 6-10, and min 11-15 post-injection separately, as well as the entire 15 min (Fig 7). Given that PFC neo exerted little to no effect in the 0.3 mg/kg fentanyl conditions, analyses focused on 0.1 mg/kg fentanyl conditions. During min 1-5 after PFC injection (Fig 7a), comparison of the slopes for PFC Sal (minute ventilation = -0.1537(min) + 3.38; r^2 = 0.5972) and PFC Neo (minute

ventilation = -0.3207(min) + 5.1; r^2 = 0.8408) equations show that there are no significant differences in slopes of the regression equations for the first 5 minutes ($F_{(1,6)}$ = 2.363, p = 0.1752). For PFC Neo, variance in time accounted for 84% of the variance in minute ventilation, whereas time accounted for 60% of the variance in minute ventilation after PFC saline. Comparison of the slopes for PFC Sal (minute ventilation = -0.1165(min) + 3.803; r^2 = 0.7920) and PFC Neo (minute ventilation = 0.03205(min) + 3.217; r^2 = 0.2689) found significant differences between slope during min 6-10 after PFC injection ($F_{(1,6)}$ = 2.363, p = 0.0180) (Fig 7b). During the 6-10 min after PFC neo injection, time only accounted for 27% of the variance in minute ventilation, likely due to drug effect and neostigmine diffusing away from the PFC. For min 11-15 after injection, the slopes of the regression lines remained significantly different between PFC Sal (minute ventilation = -0.04723(min) + 3.556; r^2 = 0.4906) and PFC Neo (minute ventilation = 0.06466(min) + 2.694; r^2 = 0.5711) ($F_{(1,6)}$ = 6.885, = 0.0394). Finally, analysis of regression equations for the first 15 minutes found significant differences in slope between PFC Sal (minute ventilation = -0.009748(min) + 2.991) and PFC Neo (minute ventilation = 0.06211(min) + 4.213) ($F_{(1,26)}$ = 2.363 p = 0.0434). These analyses support the interpretation that PFC neostigmine diminishes the effect of fentanyl on breathing by altering the onset and time course trajectory of respiratory depression.

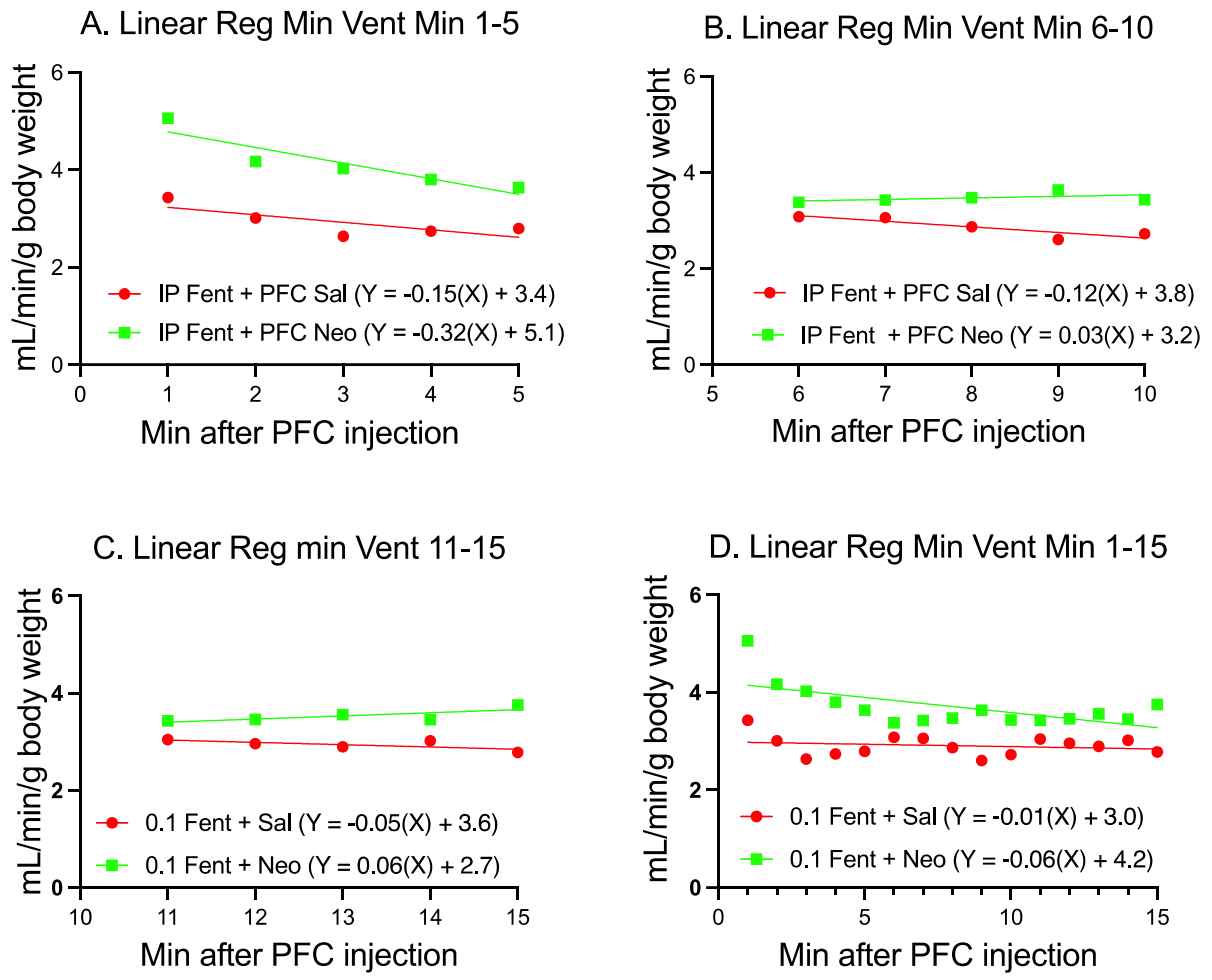


Figure 7. Regression equations comparing differences in on the onset of OIRD between the IP Fent + PFC Sal and IP Fent + PFC Neo conditions for min 1-5 (A), min 6-10 (B), min 11-15 (C), and min 1-15 (D) post-injection. Each data point represents the average minute ventilation for a particular 60-second recording period over all subjects. The slopes of the regression equations were not significantly different for min 1-5 but were significantly different for min 6-10 and min 11-15. Additionally, the slopes of the regression equations for min 1-15 were significantly different.

Effects of PFC Carbachol on Breathing

Although no abnormalities in behavior were noted in subjects following administration of the 1 mM dose of carbachol, an adverse reaction occurred in both mice following the administration of the 10 mM dose of carbachol. Initially, mice exhibited heightened ambulation that were distinct from observations in previous studies. At approximately 5-10 minutes post-injection, both mice begin to show seizure-like activity, with frequent convulsions and hyperventilation. Mice alternated between episodes of normal behavior and seizure activity. EEG waves during the seizure episodes showed abnormally high amplitude, higher than that of delta waves, and lower frequency than beta waves. One mouse was observed to exhibit a red, vessel-like discoloration that circled its entire snout. Mice were euthanized before completion of data collection for the experiment to ensure humane treatment of subjects.

Data collected from the 1.0 mM PFC carbachol experiment were analyzed. Figure 8 compares the average VE for min 1-15 post-injection across treatment conditions. Compared to the IP Sal + PFC Sal condition, 1 mM PFC Carb increased minute ventilation, on average, by 47.53% (Cohen's d = 1.0). The calculated effect size corresponds to a large effect. No inferential statistics were run due to an unequal sample size between groups. Given that the 10 mM dose of PFC carbachol was lethal with adverse effects on B6 mice, future studies using PFC carbachol on B6 mice should initially focus on the 1.0 mM dose.

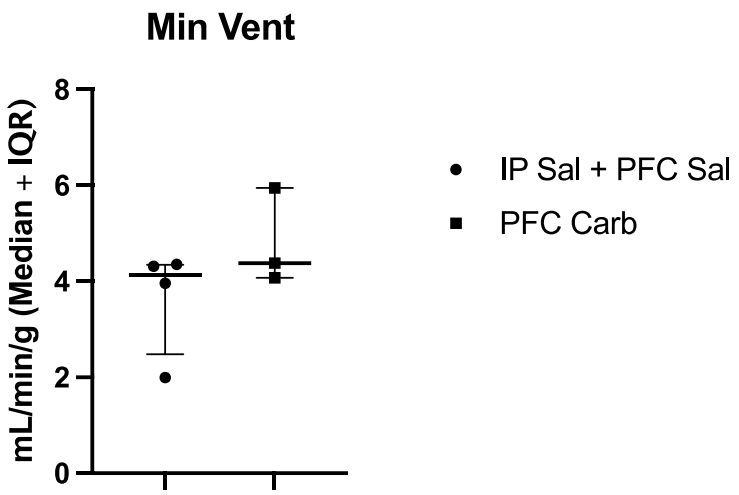


Figure 8. Results of pilot study testing effects of PFC Carb on breathing. Vertical scatterplots showing minute ventilation during min 1-15 after administration of 1mM PFC Carb compared to IP Sal + PFC Sal. Each data point represents the average minute ventilation over 15 minutes for one subject. No inferential statistics were run.

DISCUSSION

Three main findings emerged from this study. First, fentanyl significantly depressed breathing in a dose-dependent manner in unrestrained, awake B6 mice. Second, preliminary data show that PFC neostigmine has the potential to mitigate respiratory depression caused by systemically administered fentanyl. Third, regression analyses show that PFC neostigmine significantly slowed the onset of OIRD. These findings are discussed in the context of two major themes. First, the idea that cholinergic transmission in the PFC contributes to the wakeful stimulus for breathing. Second, the potential for cholinergic drugs as a countermeasure for OIRD. Finally, limitations of the studies are discussed.

Fentanyl caused respiratory depression in a dose-dependent manner

My concentration response study shows that increasing the dosage of fentanyl diminishes respiratory frequency (Fig. 2a) and minute ventilation (Fig. 2c) but increases tidal volume (Fig.

2b) and duty cycle (Fig. 2d). To the best of my knowledge, there are no previously published studies on the dose-dependent effects of fentanyl on breathing in awake, unrestrained B6 mice breathing room air. Previous dose-response studies have manipulated the concentration of CO_2 (Hill et al., 2018) or measured breathing during an anesthetic state (Sakuraba et al., 2009). Thus, this study fills a critical void left in the literature characterizing dose-dependent changes in breathing in wild-type mice under normal conditions.

My concentration response data reveal an inverse relationship between the measures of breathing. Higher doses of fentanyl reliably decreased average frequency and minute ventilation but also increased average tidal volume and duty cycle. Duty cycle is defined as the proportion of time spent in the inspiratory phase of breathing during one breath cycle. Prolonged inspiration caused by opioids can also been seen in the waveforms of respiratory traces (Hill et al., 2018) and allows for an increased tidal volume to compensate for a decrease in the number of respirations. However, Bachmutsky and colleagues identify a “pause” phase following the inspiratory phase and preceding the expiratory phase that is characterized by little to no airflow (Bachmutsky et al., 2020). This finding potentially explains why the increase in tidal volume does not fully compensate for the decrease in frequency, as evidenced by the reduction in per gram body weight minute ventilation. Increases in inspiratory duty cycle are also observed in patients with sleep-disordered breathing (SDB) (Schneider et al., 2009). These compensatory behaviors are also indicative of a loss of the volitional control of breathing and the drive to breathe, both of which are vital for psychological well-being (Zaccaro et al., 2018) and patient stability (Vaporidi et al., 2020). Thus, given the relationship between opioid use, OIRD, and SDB (Farney, Walker, Cloward, & Rhondeau, 2003; Jungquist, Card, Charchaflieh, Gali, & Yilmaz, 2018), these findings highlight the importance of sleep-wake regulation in managing

state-dependent disorders of breathing. For example, patients receiving opioids are more likely to exhibit respiratory depression (Nagappa, Weingarten, Montandon, Sprung, & Chung, 2017) or central sleep apnea (Farney et al., 2003) when they are asleep compared to when they are awake. The importance of sleep-wake mechanisms is further evidenced by findings that opioid administration is associated with EEG changes in patients (Nagappa et al., 2017) and rodents (Montandon & Horner, 2019; Osman et al., 2005). These studies show that opioids cause a dissociated state of consciousness characterized by a reduction of EEG waves associated with wakefulness and an increase in EEG waves such as delta. They also support the need for more studies quantifying the relationship between opioids, breathing, and EEG.

Fentanyl-induced respiratory depression remains unique in that it is the only opioid known to cause the "Wooden Chest Syndrome", (Hamilton & Cullen, 1953; Pergolizzi Jr, Webster, Vortsman, Ann LeQuang, & Raffa, 2021), which is characterized by laryngospasm and chest wall and diaphragm rigidity. Onset of the Wooden Chest Syndrome complicates ventilation efforts (Çoruh, Tonelli, & Park, 2013) and has been observed even in low fentanyl doses (Ackerman, Phero, & Theodore, 1990). The mechanisms of the Wooden Chest Syndrome are not fully understood. However, it is believed to be mediated within the nucleus raphe pontis and caudate nucleus (Freye & Kuschinsky, 1976; Sokoll, Hoyt, & Gergis, 1972). Several neuroanatomical studies show mutual projections between the PFC and the nucleus raphe and caudate (Celada, Puig, Casanovas, Guillazo, & Artigas, 2001; Geddes et al., 2016; Nakamura, Ozawa, & Koike, 2020; Ulrich, Keller, & Grön, 2016), highlighting the importance of identifying the neurochemical mechanisms through which the PFC modulates respiration. Additionally, the Wooden Chest Syndrome also demonstrates the importance of pharmacological

studies like this one and encourages future studies characterizing the nature of respiratory depression caused by fentanyl.

PFC neostigmine mitigated respiratory depression caused by systemic fentanyl

Although these data are preliminary, they are, to the best of my knowledge, the first to demonstrate that modulating neurotransmission in a single brain region can attenuate a systemic opioid effect in awake, unrestrained mice breathing room air. As previously discussed, fentanyl-induced respiratory depression is the most severe form of OIRD (Hill, Santhakumar, Dewey, Kelly, & Henderson, 2020), where respiratory arrest can occur in as little as 5 minutes after fentanyl administration (Topacoglu, Karcioglu, Cimrin, & Arnold, 2005). My emerging data also shows that PFC Neo significantly slowed the onset of fentanyl-induced respiratory depression (figure 7). The finding that cholinergic transmission can mitigate OIRD is particularly significant because there are no respiratory neurons in the PFC that generate the respiratory rhythm. Thus, these results suggest that the PFC modulates breathing by projecting to the brainstem respiratory network. The pre-Böt contains cholinergic receptors (Shao & Feldman, 2009), and the respiratory motoneurons also receive cholinergic input (Woolf & Butcher, 1989). Modulating cholinergic transmission in these regions had direct effects on respiratory rhythm and tonic output (Shao & Feldman, 2009), where increased cholinergic transmission stimulated respiratory output. Thus, my data are consistent with evidence documenting the association between cholinergic transmission in the PFC and opioids (Osman et al., 2005) and respiration. Additionally, they agree with studies showing that PFC dysregulation is implicated in drug addiction (Goldstein & Volkow, 2011).

The concept of a wakefulness stimulus for breathing was first characterized after the observations that breathing was slowed during slow-wave sleep, and that sleep apnea often

occurred after the loss of wakefulness (Fink, Hanks, Ngai, & Papper, 1963; John Orem, Netick, & Dement, 1977). Understanding the neuronal mechanisms underlying this wakefulness stimulus is relevant for OIRD because electrophysiological data show that antinociceptive doses of opioids alter EEG-defined states of consciousness in mice (O'Brien et al., 2021). These data suggest the interpretation that respiratory depression from opioids occur due to altered consciousness, which suppresses the wakefulness stimulus for breathing. Although this wakefulness stimulus is not fully understood, previous studies have localized this wakefulness stimulus to the activity of the reticular activating system (Horner, 2001), which influences upper airway muscle activity (J. Orem, Lovering, Dunin-Barkowski, & Vidruk, 2002). My findings not only extend previous studies from our laboratory (Glovak et al., 2021) but also demonstrate that the PFC is an important brain region contributing to this wakefulness stimulus by enhancing cholinergic transmission.

It is important to highlight the antagonistic relationship between the PFC and the pontine reticular formation (PRF), where cholinergic transmission yields opposite effects in both brain regions. Cholinergic transmission in the PFC promotes wakefulness, but cholinergic transmission in the PRF promotes REM sleep (Baghdoyan & Lydic, 2012). Acetylcholine release in the PFC can be decreased by cholinergic transmission in the PRF (Demarco, Baghdoyan, & Lydic, 2004). Enhancing acetylcholine release in the PRF is associated with respiratory depression (Lydic & Baghdoyan, 1993). These data support past studies (Lydic, 1996) and demonstrate the importance of brain mechanisms in modulating sleep/wake states and regulation of respiration. These data also support the need to better understand brain-region specific effects of neurotransmission on behavioral states.

Limitations

There are some limitations of the present studies. First, these studies had a small sample size and only used wild-type, adult, male B6 mice. Biological sex modulates the effects of opioids on breathing (Albert M. Dahan, Sarton, Teppema, & Olievier, 1998), and therefore, it is unknown if the effects of PFC Neo on OIRD is sexually dimorphic. Given that the extent to which opioids alter breathing is determined by other factors such as leptin status (Glovak et al., 2021), they encourage future studies to investigate whether the ability of PFC Neo to modulate breathing is dependent on these factors. My studies did not analyze the effects of opioids or PFC Neo on chemoreception, but it is important to acknowledge that opioids depress chemosensitivity (Kirby & McQueen, 1986; K. T. Pattinson, 2008). Additionally, the exact mechanisms and descending pathways from the PFC to the brainstem respiratory network are unclear. These studies were primarily aimed at understanding how altering neurochemistry modulates breathing, but they serve as an important first step towards identifying such mechanisms.

CONCLUSION

The studies in this psychology honors thesis are the first, to my knowledge, to characterize the dose-dependent effect of fentanyl on respiration in awake, unrestrained male B6 mice breathing room air. Preliminary data also demonstrate that cholinergic transmission in the PFC can mitigate respiratory depression caused by systemically delivered opioids. Higher doses of fentanyl significantly decreased respiratory frequency and minute ventilation, increased tidal volume and duty cycle, and resulted in a faster onset of respiratory depression. Microinjection of neostigmine into the PFC attenuates the effects of fentanyl on frequency and minute ventilation while significantly slowing the onset of OIRD in the first 15 minutes. These results extend my

previous data showing that breathing of intact, awake mice is stimulated by PFC Neo and support the interpretation that cholinergic transmission in the PFC contributes to the wakefulness stimulus for breathing.

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