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Fentanyl Effects on Breath Generation in C57BL/6J and A/J Mouse Strains

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Abstract

We examined the effect of fentanyl on chemoresponsiveness in mouse strains divergent in the expression of spontaneous and post-hypoxic pauses. Frequency and tidal volume were recorded with plethysmography in A/J and C57BL/6J (B6) male mice. Mice selected at random received an intraperitoneal (IP) injection of either saline, low dose fentanyl (LDF=0.04mg/kg), or high dose fentanyl (HDF=0.4mg/kg) under hypoxia (8% O₂) or hyperoxia (100%O₂). LDF produced a decrease in frequency during hypoxia in B6, but not A/J, mice. HDF significantly decreased frequency and tidal volume in both strains under hypoxia and hyperoxia (p<0.01); naloxone, an opioid antagonist, reversed this response. The acute administration of fentanyl at any dose did not promote apneas in strains of mice exhibiting regular or irregular respiratory patterns. However, higher doses depressed respiratory frequency in both strains. The B6 mice responded with a depressive response to hypoxia that did not recover with reoxygenation, but did recover with time or naloxone.

Keywords

ventilatory control; hypoxia; apnea; genetic background

1.1 Introduction

Opioids are an effective analgesic after major trauma or surgery, and fentanyl in particular is often the drug of choice. Adverse events related to opioid use, primarily opioid-induced respiratory depression, are potentially fatal complications in both adult and pediatric populations (Barletta, 2012; Boom et al., 2012; Golder et al., 2013; Jarzyna et al., 2011; Jungquist et al., 2011; Niesters et al., 2013; Panagiotou and Mystakidou, 2012; Whittaker, 2013). Respiratory side effects are increased in those with pre-existing cardiovascular and

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respiratory conditions and with normal circadian dips in ventilation (Mortola, 2004; Mortola and Seifert, 2002). Monitoring for risk of opioid-induced respiratory depression improves clinical outcomes (Sadhasivam and Chidambaran, 2012). While the side effects of depression occur at many physiologic levels, it is the activation of opioid receptors on the respiratory pattern generator that appears to have a substantial influence in inhibiting inspiration (Bodnar, 2013; Boom et al., 2012; Fong et al., 2009). There is however a variation among patients in the degrees of respiratory depression, apnea, and hypoxemia with routine clinical use. Certainly dose does matter in terms of pain relief, but there are known differences among unrelated individuals, an effect also modelled in animals (Liu and Wang, 2012). Therefore, genetic background could also influence the expression of opioid respiratory effects in clinical practice.

Strain studies in respiratory control show ventilatory differences to the response to hypoxia or hypercapnia, but also in the response to reoxygenation after hypoxia (Yamauchi et al., 2010). For instance, the C57BL/6J (B6) shows post-hypoxic pauses while a strain like the A/J does not (Han et al., 2001; Han et al., 2002). Both *in vivo* and *in situ* preparations in rats and mice consistently demonstrate central influences of opioids (Fong et al., 2009; Mellen et al., 2003). Several studies indicate that morphine and enkephalin analogs produce strain- and dose-dependent decreases in respiration and increases in motor activity that vary among strains, including the A/J and B6, correlating with mu-opioid receptor subtype, and/or mRNA expression (Muraki and Kato, 1985, 1986; Muraki et al., 1988). The A/J strain, for example, has a steeper dose-dependent anti-nociceptive response to the mu-opioid receptor agonist DAMGO than the CBA/J strain, as well as a significantly greater number of inhibitory OFF-like neurons in its rostral ventromedial medulla (Sugino et al., 2012). Comparing locomotor, sensitization, and reward response to heroin, the B6 mouse exhibits twice as much mu-opioid receptor stimulation in the nucleus accumbens (NA) and caudate at baseline than the DBA/2J; heroin decreases the density of mu-opioid receptors and increases density of dopamine transporters in the NA and caudate in the B6, while the opposite is seen in the DBA/2J (Bailey et al., 2010).

These multi-strain studies have focused more on analgesia than on the effects of fentanyl on either the response to hypoxia and reoxygenation or the incidence of post-hypoxic pauses. Chronic opioid use has a potential to cause central apneas, and is considered in case series to be a major contributor to nocturnal hypoxemia and sleep-disordered breathing in general (Zutler and Holty, 2011). Given the widespread use both in the postoperative and out-patient settings, it is important to consider the mechanisms of action and their potential to cause adverse effects on breathing and apneas.

We suspected a fentanyl-by-strain effect based on differences in the responses to other drugs examined for ventilatory behavior among strains (Yamauchi et al., 2010). Specifically, the B6 responds to inhibition of neural nitric oxide not only by increasing the length of post-hypoxic pauses but also increasing pattern variation and the incidence of pauses during resting breathing; in contrast the A/J does not exhibit pauses but only manifests a slowing of stable breathing in response to the same agent (Price et al., 2003). As there is a synergy between opioid and NOS actions in a number of CNS regions including an augmentation of the anti-nociceptive response to morphine (Makuch et al., 2013; Rodriguez-Munoz and

Garzon, 2013), we reasoned that there might be a strain-dependent effect of opioid actions in the production of respiratory depression and in the expression of apneas. We used an animal model where age, environment, nutrition, health status, and other factors potentially confounding studies in humans are relatively well controlled, and addressed dose-by-genetic-background effects. The hypothesis was that fentanyl would produce apneas and/or modify responses to hypoxia and reoxygenation in mouse strains divergent in the expression of spontaneous and post-hypoxic pauses. The results indicate a strain-by-drug effect with complex interactions but without expression of increased pauses.

1.2 Methods

1.2.1 Animals

Experiments were performed in male B6 and A/J mice (total for two protocols of 18 C57BL/6J (B6) and 12 A/J mice; source: Jackson Laboratory, Bar Harbor, ME). Animals were housed in the Louis Stokes Department of Veterans Affairs Medical Center (LSDVAMC) Animal Research Facility for at least 3 weeks before investigation (food and water ad libitum; with a 7AM to 7 PM and 7 PM to 7 AM light-dark cycle). Mice were studied between 8–14 weeks of age. Experiments were completed over a 2 week period. The experimental protocols were approved by the LSDVAMC Animal Care and Use Committee and were in agreement with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

1.2.2 Testing

Measurements of ventilatory behavior were simultaneously recorded in six 600 mL Lucite cylindrical plethysmography chambers, as described previously (Yamauchi et al., 2008). Briefly, breathing data were measured using the pressure change across a pressure transducer (Validyne DP45, Validyne Engineering, Northridge, CA, 91324 USA), between the testing chamber to a comparably sized reference chamber. The variations of pressure in the chamber were expressed as voltage swings by the transducer output, and were recorded using Lab View 7.1 (National Instruments, Austin, TX, 78710 USA). Filtered gas mixtures of compressed air, 8% O₂/92% N₂ (hypoxia) and 100% O₂ (hyperoxia) were introduced into and at appropriate times flushed the chamber. The transducer output was analyzed and scored using a program custom written for the lab (Breath Detect, Cleveland OH). This program identifies each individual breath according to the crest and trough of the transducer's voltage output, but permits visual artifact identification and manual corrections of breath timing. Frequency (f) is determined by the cycle length from the onset of inspiration to the next. Frequency histograms were used to identify at distributions and appearance of extremes (sniffing and slow breathing). An estimate of relative tidal volume (V_T) was determined from the amplitude of the voltage change in arbitrary units (a.u.) relative to a physical calibration before and after the recording period performed to ensure integrity of plethysmographic seal.

1.2.3 Experimental Protocols

For each set of experiments, on any testing day 3 mice of each strain were used to ensure comparable room environmental conditions for testing among strains. Bedding from the

home cage of each mouse was used in clean testing chambers to reduce the influence of novel environment. Measurements were performed daily between 10:00 AM to 2:00 PM to limit the effects of circadian changes (Mortola and Lanthier, 2004). On the day before testing, the animals acclimated in the chambers for 90 minutes at normoxia. On the next (testing) day, the same acclimatization protocol was used. Animals were weighed and placed in the same chambers for 45 minutes prior to the initiation of the designated experiment.

1.2.2.1 Protocol 1 (n= 12 mice in each strain)—On the day of testing, after 45 minutes in the chamber, 3 mice of one strain randomly selected from the tested cohort received an IP injection of saline as a control and 3 mice of the alternate strain received either low dose fentanyl (LDF) or high dose fentanyl (HDF). The LDF concentration was 0.04 mg/kg and HDF concentration, ten-fold higher (0.4mg/kg). The saline volume used as a control matched either the LDF or HDF volume. Total IP volume was 0.12 ml or 0.31, for LDF and HDF, respectively. Baseline pre-injection resting measures with normoxic room air was performed during the acclimatization period; and, as fentanyl has a short duration of action, baseline post-injection breathing with normoxic room air began ~5 minutes after injection of drug or vehicle, respectively, and was completed within 5 minutes. Chambers were then flushed for 10 seconds with the hypoxic mixture (8% O₂- 92% N₂) before continuing a sustained 5 minute exposure. Then the hypoxic gas challenge was flushed out of the chamber for 10 seconds with 100% oxygen with measurements for another 5 minutes (hyperoxia). Upon completion of this exposure, mice were returned to air recovery for 30 minutes. Animals then returned to the Animal Research Facility. All animals survived the protocols and recovered fully.

1.2.2.2 Protocol 2: (6 mice in each strain)—Six A/J and 6 B6 mice were used and were tested in groups of 3 on each testing day. As in Experiment 1, mice were allowed to acclimate for 90 minutes to the plethysmography chamber on the day prior to testing and for 45 minutes on the testing day. Breathing with room air was measured before and after injection with either HDF or a corresponding volume of saline, and then measured once more after an IP injection with naloxone, an antagonist of fentanyl. The dose for IP injection of naloxone was 1 mg/kg. The hypoxic and hyperoxic sequence were identical to that described above in Protocol 1.

1.2.4. Data Analysis

Breath frequency (f: breath per minute) and relative tidal volume were measured in the pre-injection, the post-injection resting phase, the first and fifth minute of hypoxic challenge, and the first two minutes of the 100% oxygen administration. As in prior studies, frequency is the metric that is most reliable in whole-body plethysmography testing, and tidal volume especially in a protocol where respiratory drive is being reduced. Sighs, sniffs, and artifacts produced by grooming were excluded from the analysis.

Pauses (apneas) were defined as absence of breathing activity lasting longer than two breathing cycles, even when breathing frequency slowed as an effect of the fentanyl. Average breath frequencies and tidal volumes at different gas challenges are correlated, leading to a consideration of appropriate analysis. Because the data design was a balanced

one, multivariate regression analysis was performed to examine relationships between drug treatments and the outcome variables of breath frequency and tidal volume at different gas challenges. The analysis was then adjusted for the effects of strain. The overall regression model was examined for statistically significant effects of HDF on frequency, and for drug, mouse strain, and its interactions with the outcomes of average breath frequencies at different gas challenges. Baseline breath frequency was the breathing frequency for each mouse prior to any treatments or gas challenges. Collected for each test group, these baselines were used as the reference value for changes induced by the drugs and by gas challenges.

Slowing of frequency without pauses is a consistent feature of opioids (Jungquist et al., 2011) and was identified by use of Poincaré plots (Gonsenhausner et al., 2004) and analysis of frequency histograms in each condition. Poincaré plots indicate the distribution of breath frequency over time by a plot of frequency vs. frequency + 1 breath, with a greater dispersion around the line of identity showing greater variability. Distribution plots of frequency were examined and the value of $\frac{1}{2}$ the frequency of the mean for the time period of a given gas challenge chosen to represent this feature of slowed breathing.

Hey plots are used to illustrate the relationship between frequency and tidal volume during changes in ventilation. An increase in ventilation can be due to variation in either frequency, tidal volume, or both. In humans, the relationship between frequency and tidal volume is similar during various stimuli, including during hypercapnia and exercise (Hey et al. 1966). In mice, however, there is less effort required to change frequency rather than tidal volume in order to maintain an adequate work of breathing (Crosfill and Widdicombe, 1961). We used Hey plots to assess rhythmogenesis in the context of relative tidal volume, as measured by plethysmography.

1.3 Results

Twelve animals of each strain were tested to determine the response to LDF and HDF in terms of ventilatory control and pause production. Shown in Table 1 are the characteristics at baseline (before injection). A/J mice were significantly older and had a greater baseline frequency and tidal volume in comparison to the B6; the B6 mice, however had significantly greater mass ($p<0.05$). By design there was a significant difference in the number of pauses between strains ($p<0.05$). Independently, 6 animals of each strain were tested to determine response to HDF and naloxone. Baseline characteristics are Table 2. Groups were comparable.

1.3.1 Protocol 1

1.3.1.1. Breathing Frequency—Multivariate analysis shows that there is no significant difference in frequency between the response of A/J mice treated with saline and those treated with LDF during hypoxia and hyperoxia ($p>0.05$) (Figure 1A). A/J mice treated with LDF display a significant increase in frequency just after injection ($p<0.05$). There is a significant decrease in frequency after injection of HDF in the A/J that persists under both hypoxic and hyperoxic conditions (Figure 1a; $p<0.01$). Hence, there are differing effects

between LDF and HDF in regard to baseline values for frequency, with HDF exhibiting immediate and sustained reductions in frequency with both hypoxia and hyperoxia.

B6 mice treated with LDF showed no difference in frequency response after injection ($p=0.25$) or during hyperoxia ($p=0.33$) (Figure 1B), but exhibited a lower mean frequency during both the 1st and 5th minute of hypoxia ($p<0.01$). Similar to the A/J, B6 mice treated with HDF displayed a decrease in frequency after injection ($p<0.05$) and during hypoxia ($p<0.01$) and hyperoxia ($p<0.01$; Figure 1b).

Multivariate analysis comparing the two strains of mice shows that there is a significant difference in response in the 1st minute of hypoxia ($p<0.01$) and during hyperoxia ($p<0.05$) between the strains; however, an interaction between strain and LDF is present during the 1st minute hypoxia that could account for this strain difference.

1.3.1.2. Breathing Pattern—An example of breathing pattern difference between the two strains is illustrated via Poincaré plots in Figure 2a–2b (A/J) and Figures 2c–2d (B6). Stable breathing is illustrated with data points that cluster; irregular breathing is illustrated by greater dispersion of data points. Figure 2a–2b show that breathing is stable in the A/J, regardless of treatment, consistent with the known trait of stable breathing. The B6, however, display this central convergence only during hypoxia; this convergence during the first minute of hypoxia is reduced with injection with HDF. Furthermore, the data points are again distributed variably with hyperoxia, correlating with the presence of pauses.

To further assess stability, the incidence of slowed breathing (SB) was assessed in the A/J and B6. A slowed breath was defined as less than half of the mean frequency for a measured period of time; this slowed breath may or may not meet the definition of a pause, which was earlier defined as a breath lasting longer than 2 breathing cycles. The A/J treated with LDF displayed significantly fewer slowed breaths after injection than at baseline; however, this did not differ from the control group. A/J treated with HDF had a significantly higher number of slowed breaths that persisted during hypoxia and hyperoxia, consistent with the finding that HDF produces frequency depression in the A/J (Figure 3a). The B6 displayed greater slowed breaths at baseline in all treatment groups than it did during the gas challenges (Figure 3b); the A/J also displayed this tendency in the LDF and LDF control groups, indicating a probable injection effect. Overall, the B6 displayed significantly more slowed breaths in all treatments under all conditions than the A/J, which supports that the B6 has greater instability of breathing. Treatment with LDF in the B6 increased the number of slowed breaths during the 5th minute of hypoxia, but there was otherwise no significant difference.

The relative changes in breath volume at a given frequency is informative of the manner in which breathing is regulated for depth as well as rate, and is demonstrated in the Hey plot (Hey et al., 1966). The patterning of A/J and B6 mice treated with LDF overlapped with their respective control groups; however, in general the B6 mice appear to vary less in tidal volume than the A/J in both the saline and LDF groups (Figures 4a and 4c). Treated with HDF, however, there is a significant decrease in frequency when compared to their

respective control groups, with the A/J also displaying a uniform tidal volume with LDF that was not seen with saline (Figures 4b and 4d).

1.3.2 Protocol 2

1.3.2.1 Breathing Frequency—A/J mice displayed a significant decrease in frequency after treatment with HDF ($p<0.01$). No significant differences to baseline were apparent after administration of naloxone, and all changes in frequency seen were similar to those seen in A/J mice treated only with saline and naloxone (Figure 5a). B6 mice treated with HDF and naloxone displayed a similar pattern in frequency as their control group, showing no significant changes in frequency (Figure 5b).

1.3.2.2. Breathing Pattern—An example of breathing pattern difference between the two strains is illustrated again via Poincaré plots in Figure 6a (A/J) and Figure 6b (B6). Figure 5a shows that breathing is stable in the A/J, regardless of treatment, consistent with the trait of stable breathing. The B6, however, display this central convergence only during hypoxia; this convergence during the first minute of hypoxia vanishes with injection with HDF. Furthermore, the data points are again distributed variably with hyperoxia, correlating with the presences of pauses. Figure 6b demonstrates that the breathing patterns seen with HDF treatment are reversed with administration of naloxone.

Assessment of slowed breaths showed that the B6 has significantly more slowed breaths across all treatments and conditions than the A/J. The A/J had significantly more slowed breaths after treatment with HDF, but significantly fewer slowed breaths after treatment with naloxone with comparison to the control group (Figure 7a). The B6 showed a decrease in slowed breathing during hypoxia that reversed with hyperoxia in both treatment groups, but the observed trend was not significant (Figure 7b).

Hey's plots of A/J mice treated with HDF followed by naloxone show a decrease in frequency after injection with HDF that is reversed with treatment with naloxone (Figure 8a). Post-naloxone, the tidal volume and frequency ranges mirror that of the control group. B6 mice treated with saline and HDF show similar distribution and overlap over all gas challenges (Figure 8b).

1.3.3 Pauses

There were few if any pauses (<2) in the A/J strain not only at baseline but over all conditions. In contrast, B6 mice exhibited pauses at baseline in all conditions, which were reduced by an injection of either saline or fentanyl, as observation made previously. With LDF and HDF, pause rate was reduced compared to the control in all conditions (Table 3).

1.4 Discussion

This investigation confirmed the depressive effects of fentanyl on respiratory rate and depth but also uncovered effects with hypoxia and reoxygenation that had a dose dependent expression. In one strain after the higher dose, hypoxia and reoxygenation had an additive depressive effect. Moreover, the presence of fentanyl does not produce apneas in mouse strains divergent in the expression of spontaneous and post-hypoxic pauses. Differences

among strains are present at certain points in the protocol like in the first minute of hypoxia, and in the dichotomous response with HDF in which the A/J strain still appeared to exhibit to some degree a response to hypoxia and reoxygenation, while the B6 showed a marked depressive response with the same sequence. These results indicate both a fentanyl dose and a dose-by-strain effect.

Mice treated with saline had a predictable response to gas challenges: frequency increases during hypoxia and decreases to a value closer to baseline during hyperoxia. This response persists after treatment with LDF in the A/J but not with LDF in the B6. Analysis shows that the frequency responses during the first minute of hypoxia and during hyperoxia correlate with strain differences, regardless of fentanyl treatment ($p=0.002$ and $p=0.025$, respectively); a similar analysis shows that a strain difference effect is seen in the reoxygenation from hypoxia in mice treated with both HDF and naloxone ($p=0.042$). Differences in tidal volume response during the same gas challenges appear not to be related to strain differences. The A/J maintained a similar pattern of changes in frequency and tidal volume after treatment with LDF; with HDF, there was a decrease in frequency without any variation in tidal volume. The B6, however, displayed decreased variation in tidal volume with both the LDF and HDF treatments. This effect was reversible with naloxone, and might be ascribed in part to different levels of expression of mu-opioid receptors in the pneumotaxic center of the pons, which inhibits inspiratory neurons in the in the medulla (van der Schier et al., 2014).

Other areas in the brain and spinal cord express mu-opioid receptors, with higher densities in the pons, parabrachial nucleus, and locus coeruleus, pathways which also regulate respiratory pattern generators (Arvidsson et al., 1995; Mansour et al., 1994). Specific and selective approaches will be needed to examine strain-specific effects, and further elucidate patterning or initiation of breathing; it is quite possible that all these nuclei are involved. As for an extra-medullary mechanism, higher doses of opioids will decrease tidal volume, and some have attributed this effect (studied in one strain only) to decreased tonic inputs from opioid-sensitive chemoreceptors (Pattinson 2008). Although differences in tidal volume did not appear in this study, effects of fentanyl in B6 and A/J mice could still be as much through peripheral as central pathways.

Periods of breathing of varying rate and depth for a certain length, interspersed with periods of apnea or hypopnea, can be a property of the medullary rhythm-generating network in the pons and/or midbrain, with modulation by inputs from chemoreceptors and pulmonary stretch receptors (Fong et al., 2009). Opioids are proposed to cause respiratory depression via mu-opioid receptors in the pre-Botzinger complex of the ventrolateral medulla (Boom et al., 2012). The pre-Botzinger complex, coupled with the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), is part of the mechanism of pattern generation in respiration; the pre-Botzinger complex is active during inspiration and is sensitive to opioids whereas the RTN/pFRG is active during expiration and is insensitive to opioids. In anesthetized rats opioids cause continuous slowing of the respiratory rhythm via the I-neurons in an isolated pre-Botzinger complex; however, opioids cause 'skipped breaths' (integer multiples of normal interburst intervals) and irregular breathing patterns when transmission between the pre-Botzinger complex and the RTN/pFRG is intact (Mellen et al.,

2003). The rationale is that when the pre-Botzinger complex is depressed by opioids, the RTN/pFRG takes over pattern generation (Pattinson, 2008). Expression- and activity-based analysis of mu-opioid receptors in the A/J and B6 strains might be approaches to address strain differences seen in response to fentanyl.

Studies show a synergy between the NOS and opioid systems in the context of analgesia; opioids inhibit activation of NOS, which enhances the anti-nociceptive effects of morphine (Makuch et al., 2013). NOS inhibitors have also been found to increase behavioral responses to morphine (Karami et al., 2014; Ma et al., 2014). Evaluation of the synergistic effect of opioids and the NOS systems have not been done for respiration. However, NOS inhibition in the A/J and B6 has been found to induce post-hypoxic frequency decline, increase periodic breathing and apnea length in the B6, and cause no periodic breathing in the A/J (Price et al., 2003). At one level this could indicate that synergy between the NOS and opioid systems at the circuit level of rhythmogenesis resulted in slowing but not in the inhibition of breathing. In the absence of direct testing of the brainstem actions, we suggest that the pattern of breathing is different from breath initiation occurring in pontine structures. This contention needs to be critically addressed further.

Differences in tidal volume response during the same gas challenges appear not to be related to strain differences. In the mouse, tidal volume does not need as much of a dynamic range as does frequency, because the work of breathing is largely independent of frequency (Crosfill and Widdicombe, 1961). Larger mammals including humans have a stiffer chest wall leading to a restricted optimal frequency range. Therefore, a direct attribution to pattern differences to central brainstem networks that determine rhythmogenesis and depth can be made more easily in mice than in larger animals or humans.

Mechanisms for these differences was not examined in regard to peripheral chemoreceptors, primarily the carotid body, or only the brainstem in the ventrolateral medulla. Mu-opioid receptors are present in the nucleus tractus solitarius, midline medullary raphe, the pre-Botzinger complex and RTN/pFRG in the medulla, and the locus coeruleus; type I glomus cells in the carotid body are also inhibited by enkephalin and morphine (Pattinson, 2008). Hence it may be that the respiratory depression caused by opioids is multifactorial. The carotid bodies of the A/J and the B6 are similar in structure and in tyrosine hydrolase morphology (Chai et al., 2011), yet a difference between the two strains in their frequency response could result from afferent pathways affected by IP administration of a respiratory depressant like fentanyl.

There are limitations in the experimental design. Arterial blood gases, blood pressure, and metabolic rate were not measured. Equipment was unavailable to measure body temperature during the study. There may be differences in the dynamics of cardiovascular or lung mechanics, which result from genetic differences. The genetic divergences of the two strains could include epigenetic effects during early life and with development. The mice were not matched for both age or mass, in part because the strains differ in the trajectory for weight gain. Experiments were performed in unrestrained, unanesthetized mice who received IP injections; rapid breathing and movement in response to the process of delivering the injection contributed to increased breathing frequencies. Additionally, the pharmacologic

administration of peritoneal injections is more likely to mimic acute exposures rather than chronic injections or continuous intravenous exposure to fentanyl, as might be encountered in post-operative care.

In conclusion, the acute administration of fentanyl at lower or higher doses did not promote apneas in strains of mice exhibiting regular or irregular breathing patterns or pauses. Higher doses did produce in both strains a depression of frequency at rest. However, one strain—the B6—exhibited a depressive response to hypoxia that did not recover with acute reoxygenation, but did recover with time. This latter trait might be particularly relevant to intermittent hypoxemia encountered with sedatives, pain medications, or sleep. We suggest a more careful temporal understanding of the relationship of opioid administration, apneas, and hypoxemia is needed as there may be interactions among these factors operating as well as genetic risk on respiratory depression.

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Highlights

- This work addresses whether genetic background is a factor in opioid-induced respiratory depression
- Utilized a strain-by-fentanyl approach to test responses in pause-prone vs pause-resistant mouse strains
- Pauses not enhanced in pause-prone and not produced in pause-resistant

Figure 1.

a. Protocol 1: Mean frequency (breaths per minute) for A/J mice (n=12) injected with vehicle (saline; Sal_LDF and Sal_HDF indicate control groups for respective treatments), LDF, or HDF. Results were measured at baseline (before injection with fentanyl), room air (after injection with fentanyl), hypoxia minute 1 (1st minute after challenge with 8% O₂), hypoxia minute 5 (5th minute after challenge with 8% O₂), and hyperoxia (within 2 minutes after challenge with 100% O₂). Significant difference between a dose and vehicle: * p<0.05. Significant difference between strains (as compared to B6 data displayed in Fig. 1b): ## p<0.01.

b. Protocol 1: Mean frequency (breaths per minute) for B6 mice (n=12) injected with vehicle (saline), LDF, or HDF. Results were measured as described in Figure 1a.

Figure 2.

a. Poincaré plots of n vs $n+1$ for breath frequency for baseline, the 1st minute of hypoxia, and hyperoxia in a typical A/J mouse. n , plotted on the x-axis, refers to a certain breath; it is plotted against $n+1$, the subsequent breath, on the y-axis. Baseline, resting breathing in room air; hypoxia minute 1, breathing during the first minute of hypoxic exposure with 8% O₂; hyperoxia, breathing during the first two minutes after addition of 100% O₂. *Left*, vehicle (saline). *Right*: 0.04 mg/kg fentanyl (LDF). Below each plot are voltage outputs from the plethysomography chambers used to generate the data points.

b. Poincaré plots of n vs $n+1$ for breath frequency for baseline, the 1st minute of hypoxia, and hyperoxia in a typical A/J mouse. n , plotted on the x-axis, refers to a certain breath; it is plotted against $n+1$, the subsequent breath, on the y-axis. Baseline, resting breathing in room air; hypoxia minute 1, breathing during the first minute of hypoxic exposure with 8% O₂; hyperoxia, breathing during the first two minutes after addition of 100% O₂. *Left*, vehicle (saline). *Right*: 0.4 mg/kg fentanyl (HDF). Below each plot are voltage outputs from the plethysomography chambers used to generate the data points.

c. Poincaré plots of n vs $n+1$ for breath frequency for baseline, the 1st minute of hypoxia, and hyperoxia in a typical B6 mouse. n , plotted on the x-axis, refers to a certain breath; it is

plotted against $n+1$, the subsequent breath, on the y-axis. Baseline, resting breathing in room air; hypoxia minute 1, breathing during the first minute of hypoxic exposure with 8% O₂; hyperoxia, breathing during the first two minutes after addition of 100% O₂. *Left*, vehicle (saline). *Right*: 0.04 mg/kg fentanyl (LDF). Below each plot are voltage outputs from the plethysomgraphy chambers used to generate the data points.

d. Poincaré plots of n vs $n+1$ for breath frequency for baseline, the 1st minute of hypoxia, and hyperoxia in a typical B6 mouse. n , plotted on the x-axis, refers to a certain breath; it is plotted against $n+1$, the subsequent breath, on the y-axis. Baseline, resting breathing in room air; hypoxia minute 1, breathing during the first minute of hypoxic exposure with 8% O₂; hyperoxia, breathing during the first two minutes after addition of 100% O₂. *Left*, vehicle (saline). *Right*: 0.4 mg/kg fentanyl (HDF). Below each plot are voltage outputs from the plethysomgraphy chambers used to generate the data points.

Figure 3.

- a. Slowed breaths in the A/J (n=12). Values less than ½ the frequency of the mean of the time period of a given gas challenge are given and shown as mean $\pm$ SEM. Mice were treated with vehicle (saline), LDF, or HDF. Results were measured at baseline (before injection with fentanyl), room air (after injection with fentanyl), hypoxia minute 1 (1st minute after challenge with 8% O₂), hypoxia minute 5 (5th minute after challenge with 8% O₂), and hyperoxia (within 2 minutes after challenge with 100% O₂). Significant difference between a dose and vehicle: * p<0.05 **Significant difference exists between strains for all treatments and conditions (as compared to B6 data displayed in Fig. 3b): p<0.01.**
- b. Slowed breaths in the B6 (n=12). Format is the same as in Figure 3a. Significant difference between a dose and vehicle: * p<0.05, ** p<0.01. **Significant difference exists between strains for all treatments and conditions (as compared to A/J data displayed in Fig. 3a): p<0.01.**

Figure 4.

4a–d. Hey's Plots for A/J and B6 with saline, LDF and HDF (n=12 for each strain). Baseline frequency vs tidal volume marker for both treatments is black; remaining markers, in order, represent post-injection, hypoxia minute 1, hypoxia minute 5, and hyperoxia and match color scheme of the in-figure key.

Figure 5.

a. Protocol 2: Mean frequency (breaths per minute) for A/J mice (n=6) injected with naloxone and either vehicle (saline) or HDF. Results were measured at baseline (before injection with vehicle or HDF), room air (after injection with vehicle or HDF), Post-Naloxone Room Air (after injection with naloxone), hypoxia minute 1 (1st minute after challenge with 8% O₂), hypoxia minute 5 (5th minute after challenge with 8% O₂), and hyperoxia (within 2 minutes after challenge with 100% O₂). No significant difference found between a dose and vehicle. Significant difference between strains (as compared to B6 data displayed in Fig. 1d): ## p<0.01.

b. Protocol 2: Mean frequency (breaths per minute) for B6 mice (n=6) injected with naloxone and either vehicle (saline) or HDF. Results were measured at baseline (before injection with vehicle or HDF), room air (after injection with vehicle or HDF), Post-Naloxone Room Air (after injection with naloxone), hypoxia minute 1 (1st minute after challenge with 8% O₂), hypoxia minute 5 (5th minute after challenge with 8% O₂), and hyperoxia (within 2 minutes after challenge with 100% O₂). No significant difference between a dose and vehicle. Significant difference between strains (as compared to A/J data displayed in Fig. 1b): ## p<0.01.

Figure 6.

a. Poincaré plots of n vs $n+1$ for breath frequency for baseline, the 1st minute of hypoxia, and hyperoxia in a typical A/J mouse. n , plotted on the x-axis, refers to a certain breath; it is plotted against $n+1$, the subsequent breath, on the y-axis. Baseline, resting breathing in room air; hypoxia minute 1, breathing during the first minute of hypoxic exposure with 8% O₂; hyperoxia, breathing during the first two minutes after addition of 100% O₂. *Left*, vehicle (saline). *Middle*: 0.4 mg/kg fentanyl (HDF). *Right*: 0.4 mg/kg fentanyl (HDF) and 1.0 mg/kg naloxone. Below each plot are voltage outputs from the plethysomography chambers used to generate the data points.

b. Poincaré plots of n vs $n+1$ for breath frequency for baseline, the 1st minute of hypoxia, and hyperoxia in a typical B6 mouse. n , plotted on the x-axis, refers to a certain breath; it is plotted against $n+1$, the subsequent breath, on the y-axis. Baseline, resting breathing in room air; hypoxia minute 1, breathing during the first minute of hypoxic exposure with 8% O₂; hyperoxia, breathing during the first two minutes after addition of 100% O₂. *Left*, vehicle (saline). *Middle*: 0.4 mg/kg fentanyl (HDF). *Right*: 0.4 mg/kg fentanyl (HDF) and 1.0 mg/kg

naloxone. Below each plot are voltage outputs from the plethysomgraphy chambers used to generate the data points.

Figure 7.

a. Slowed breaths in the A/J (n=6). Values less than ½ the frequency of the mean of the time period of a given gas challenge are given and shown as mean $\pm$ SEM. Mice were treated with intraperitoneal injections of vehicle (saline) + naloxone, or HDF + naloxone. Results were measured at baseline (before injection with fentanyl), room air (after injection with fentanyl), post-naloxone (after injection with naloxone, still with room air), hypoxia minute 1 (1st minute after challenge with 8% O₂), hypoxia minute 5 (5th minute after challenge with 8% O₂), and hyperoxia (within 2 minutes after challenge with 100% O₂). Significant difference between a dose and vehicle: * p<0.05 **Significant difference exists between strains for all treatments and conditions (as compared to B6 data displayed in Fig. 3b): p<0.01**

b. Slowed breaths B6 (n=6). Format of figure is the same as described in Figure 7a. No significant difference between a dose and vehicle. **Significant difference exists between strains for all treatments and conditions (as compared to A/J data displayed in Fig. 7a): p<0.01.**

Figure 8.

a–b. Hey Plots for A/J and B6 with saline and HDF, each with naloxone (n=6 for each strain). Baseline frequency vs tidal volume marker for both treatments is black; remaining markers in series match the in-figure key.

Table 1

Baseline ventilatory characteristics of Experiment 1

	N	Age (weeks); M $\pm$ SEM; *p<0.05	Mass (g); M $\pm$ SEM *p<0.05	Baseline Frequency (bpm); M $\pm$ SEM *p<0.05	Baseline Tidal Volume (a.u); M $\pm$ SEM *p<0.05	Baseline Pauses; M $\pm$ SEM *p<0.05
A/J	12	13 $\pm$ 0.4	21.7 $\pm$ 0.5	211.2 $\pm$ 2.6	11.9 $\pm$ 0.6	0.08 $\pm$ 0.03
B6	12	8 $\pm$ 0	27.4 $\pm$ 1.1	189.7 $\pm$ 2.7	8.1 $\pm$ 0.3	4.3 $\pm$ 0.5

Table 2

Baseline ventilatory characteristics of Experiment 2

	N	Age (weeks); M±SEM *p<0.05	Mass (g); M±SEM *p<0.05	Baseline Frequency (bpm); M±SEM *p<0.05	Baseline Tidal Volume (a.u.); M±SEM p>0.05	Baseline Pauses; M±SEM *p<0.05
A/J	6	13 ± 0.4	22.5 ± 1.4	226.2 ± 6.4	12.7 ± 1.4	0
B6	6	8 ± 0	26.4 ± 3.4	205.5 ± 6.4	9.1 ± 0.6	2.2 ± 0.7

Table 3

Pauses in B6 Mouse by Treatment

	Baseline; M±SEM	Room Air; M±SEM	Post-Naloxone (if given); M±SEM	Hypoxia Min 1; M±SEM	Hypoxia Min 5; M±SEM	Hyperoxia; M±SEM
Sal_LDF	4.6 ± 1.1	0.3 ± 0.2	-	0	3 ± 2.8	1.5 ± 0.9
LDF	4.0 ± 0.9	0.3 ± 0.3	-	0.5 ± 0.4	1.3 ± 0.5	1.5 ± 1.0
Sal_HDF	5.4 ± 1.5	0.5 ± 0.5	-	0	0.3 ± 0.1	0.8 ± 0.4
HDF	3.3 ± 0.7	0	-	0.3 ± 0.1	0	0.3 ± 0.3
Sal_HDF	2.5 ± 1.4	0.5 ± 0.3	0	0.3 ± 0.2	0.3 ± 0.3	0
HDF + Nal	1.8 ± 0.7	0.3 ± 0.2	0	0.2 ± 0.2	0.3 ± 0.2	0.5 ± 0.3