Fentanyl depression of respiration: comparison with heroin and morphine

Short Title: Fentanyl depression of respiration

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Non standard abbreviations

AUC	Area under the curve
NorBNI	nor-Binaltorphimine

Key words

Fentanyl; morphine, heroin, naloxone; diprenorphine; respiratory depression

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2 tables

ABSTRACT

Background and Purpose:

Fentanyl overdose deaths have reached 'epidemic' levels in North America. Death in opioid overdose invariably results from respiratory depression. In the present work we have characterized how fentanyl depresses respiration and by comparing fentanyl with heroin and morphine, the active breakdown product of heroin, we have sought to determine the factors, in addition to high potency, that contribute to the lethality of fentanyl.

Experimental Approach:

Respiration (rate and tidal volume) was measured in awake, freely moving mice by whole body plethysmography

Key Results

Intravenously administered fentanyl produced more rapid depression of respiration than equipotent doses of heroin or morphine. Fentanyl depressed both respiratory rate and tidal volume. Fentanyl did not depress respiration in μ opioid receptor knock-out mice. Naloxone, the opioid antagonist widely used to treat opioid overdose, reversed the depression of respiration by morphine more readily than that by fentanyl whereas diprenorphine, a more lipophilic antagonist, was equipotent in reversing fentanyl and morphine depression of respiration. Prolonged treatment with morphine induced tolerance to respiratory depression but the degree of cross tolerance to fentanyl was less than the tolerance to morphine itself.

Conclusion and Implications:

We propose that several factors (potency, rate of onset, lowered sensitivity to naloxone and lowered cross tolerance to heroin) combine to make fentanyl more likely to cause opioid overdose deaths than other commonly abused opioids. Lipophilic antagonists such as diprenorphine may be better antidotes than naloxone to treat fentanyl overdose.

Bullet Point Summary

What is already known

- Fentanyl and related analogues are potent opioids responsible for many overdose deaths in intravenous opioid users in North America

What this study adds

- On intravenous administration fentanyl depresses respiration faster than heroin or morphine
- Fentanyl depresses both respiratory rate and tidal volume
- Naloxone is weaker at reversing fentanyl depression of respiration than that produced by morphine whereas diprenorphine reverses both equally.
- Fentanyl breaks through tolerance to respiratory depression induced by prolonged morphine treatment

What is the clinical significance

- Fentanyls are more likely to cause overdose due to their fast rate of onset, potency and relative insensitivity to reversal by naloxone.

INTRODUCTION

Since 2013 there has been a dramatic rise in acute opioid overdose deaths involving new synthetic opioids, primarily the fentanyls (fentanyl and structurally related medicinal and illicit drugs), in North America (NIH, 2019). Of the over 60,000 opioid overdose deaths in the USA in 2017 almost 30,000 involved fentanyls, exceeding those involving heroin or prescription opioids such as oxycodone and hydrocodone. Elsewhere, in Europe, fentanyl deaths have been recorded in Estonia (for some time fentanyls were the main street opioids available in that country), and there have been sporadic outbreaks of fentanyl-related deaths in the UK, Germany and Finland (EMCDDA, 2018). Ease of synthesis (cf. the need to grow swathes of opium poppies to produce heroin), high potency (smaller quantities need to be shipped by comparison with heroin) and ease of purchase on the dark web make the fentanyls attractive to suppliers of illicit opioids (Fairbairn et al., 2017). Fentanyls are frequently mixed with heroin to increase its potency (Griswold *et al.*, 2017; Marinetti *et al.*, 2014). A recent development is the addition of fentanyls to cocaine products and to illicit prescription opioid and benzodiazepine tablets (Green and Gilbert, 2016; Sutter *et al.*, 2017). Death in fentanyl overdose results primarily from depression of respiration (Mathers *et al.*, 2013; Pierce *et al.*, 2015). Fentanyls and other opioid agonists depress respiration by acting on μ opioid receptors at various sites to reduce the response to raised pCO_2 and lowered pO_2 and thus reduce the drive to breathe (Pattinson, 2008). This reduction in respiratory drive results in a decrease in the rate of breathing and in periods of apnea (cessation of breathing) which in extremis results in death.

A number of factors may contribute to why the fentanyls are so deadly. Their high potency means that only small amounts are required to produce profound effects and

thus even a small error in weighing out the drug can result in too much being taken. Rapid penetration into the brain can result in overdose levels being reached more quickly than with heroin. Deaths in heroin overdose may take more than 30 min to occur after injection (Darke *et al.*, 2016), providing a window of opportunity for intervention (administration of the opioid antagonist naloxone). In contrast, fentanyl overdose deaths can occur very quickly before there is an opportunity to administer naloxone (Burns *et al.*, 2016). Fentanyls induce muscle stiffness (Benthuysen *et al.*, 1986; Streisand *et al.*, 1993) including in intercostal and diaphragm muscles, often referred to as 'wooden chest', and this is likely to make it harder to breathe. There have been several reports suggesting that depression of respiration by fentanyls shows reduced sensitivity to reversal by naloxone (Fairbairn *et al.*, 2017; Lynn *et al.*, 2018; Peterson *et al.*, 2016; Schumann *et al.*, 2008). In one report, several cases were recorded in which multiple doses of naloxone were required before recovery of respiration following a fentanyl overdose (Somerville *et al.*, 2017). Furthermore, a low level of cross tolerance between heroin and fentanyls may allow the fentanyls to retain potency to depress respiration in individuals tolerant to heroin-induced respiratory depression. In the present paper we have characterized how fentanyl depresses respiration in mice and have sought to determine how the factors described above may contribute to fentanyl overdose.

METHODS AND MATERIALS

Animals: Male CD-1 mice weighing approximately 30 g were obtained from Charles River (UK). Breeding pairs of μ opioid receptor knock-out mice (*Oprm1*^{tm1Kff}, stock number 007559) were obtained from the Jackson Laboratory (USA) and offspring bred in house at the University of Bristol. Both male and female μ opioid receptor knock-out mice weighing between 25 and 30 g were used in this study. Male and female wild type C57BL/J mice weighing approximately 30 g were purchased from Charles River (UK). All mice were maintained at 22 °C on a reversed 12 h dark-light cycle with food and water available *ad libitum*. All experiments were performed in the dark (active) phase. Mice were randomly ascribed to treatment groups with the experimenter blinded to the drug treatment or knock-out/wild type phenotype as appropriate.

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive (2010/63/EU) and the University of Bristol ethical review document, as well as the ARRIVE and British Journal of Pharmacology guidelines.

Measurement of respiration: Respiration was measured in freely moving mice using plethysmography chambers (EMKA Technologies, France) supplied with a 5% CO_2 in air mixture (BOC Gas Supplies, UK) as described previously *(*Hill *et al., 2016;* Hill *et al*., 2018). The day prior to experimentation mice were habituated to plethysmograph chambers for 30 min whilst breathing air. On the day of experimentation a 20 min baseline respiration period was recorded prior to administration of an opioid agonist challenge. Rate and depth of respiration were recorded and averaged over 1 or 5 min periods (except immediately after drug injection when the time period was 0.5 or 3 min

respectively) and converted to minute volume (rate x tidal volume). Tidal volume was calculated from the raw inspiration and expiration data in concert with compensation accounting for internal plethysmograph chamber variations in temperature and humidity as well as reference atmospheric pressure according to the Drorbaugh and Fenn Formula (Drorbaugh and Fenn., 1955). Drugs were administered i.v. or i.p. as indicated. Changes in body temperature induced by opioid agonists was not monitored during the plethysmography experiments.

Measurement of locomotor activity: A beam break rig (Linton Instrumentation, UK) was used to assess the locomotor activity of mice. An automated data logging suite (AMON Lite, Linton Instrumentation, UK) was used to track the movement of mice throughout the experimental session. On the day prior to locomotor assessment each mouse was placed in a fresh cage and allowed to explore the cage for 30 min. On the experimental day the mouse was again allowed to explore the cage for 30 min before drug administration. Locomotor activity was then measured for 30 min following drug administration. Mice had access to water *ad libitum* but had no access to food in either session in order to dissuade rearing and climbing behaviour.

Drug administration: In most experiments we have used i.p. injection as this is a simple and relatively non-stressful means of drug administration to mice. However, in those experiments designed to determine rate of onset of effect opioid agonist drugs were administered i.v. with mice restrained in a clear plastic tube while opioid or vehicle was administered by tail vein injection in a 0.1 ml volume.

Reversal of opioid respiratory depression by opioid antagonists: Respiration was recorded for 20 min followed by an acute i.p. injection of opioid or vehicle. Respiration was then recorded for 20 min following opioid/vehicle administration, allowing maximal depression of respiration to occur. Naloxone or diprenorphine was then administered (20 min after opioid/vehicle) by i.p. injection on the opposite side of the peritoneal cavity to the opioid/vehicle injection.

Induction of morphine tolerance: Tolerance to morphine respiratory depression was induced by 3x 100 $mg.kg^{-1}$ morphine injections, administered 12 h apart followed by subcutaneous implantation of morphine-filled osmotic mini-pumps delivering 45 $mg.kg^{-1}.day^{-1}$ morphine for a total of 6 d as described previously (Hill *et al.*, 2016; Hill *et al.*, 2018). Control mice were injected with saline and implanted with minipumps that were filled with saline.

Experimental design and data analysis: Data from previous experiments where respiratory depression and locomotor activity were measured either following acute opioid administration in naïve mice or following pump implantation were subjected to post hoc power analyses using G*Power (version 3.1.9). Our calculations indicated that for depression of respiration n=6 (acute experiments) or n=7 (pump experiments) and for locomotor activity n=8 for each individual group would produce a significant result if an actual effect occurred.

For each mouse the change in each behavioural parameter (respiratory rate, tidal volume, minute volume and locomotor activity) following acute drug administration has been calculated as the percentage of the pre-drug baseline as described previously (

Hill *et al.*, 2016; Hill *et al.*, 2018; Withey *et al.*, 2017). Area under the response versus time curve (AUC) was determined using a 100% baseline as described previously (Hill *et al.*, 2016). Overall changes from a single factor were analysed using a One-way ANOVA with Bonferroni's post-test. Statistical significance is assumed when $P<0.05$. Only where $P<0.05$ in One- or Two-way ANOVA analyses were post-hoc comparisons made between parametric variables. Data were excluded only where values were different by greater than two standard deviations from the mean (as outlined in the appropriate figure legends). GraphPad Prism 5 was used for all statistical analyses. All data are displayed as mean ± standard error of the mean (s.e.m.). The data and statistical analyses (on group sizes of $n>5$ where each n is an independent value) comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018).

Drugs and chemicals: Heroin hydrochloride (diacetyl morphine hydrochloride), morphine hydrochloride (both from Macfarlane Smith, UK), fentanyl citrate, naloxone hydrochloride, naltrindole hydrochloride and norbinaltorphimine dihydrochloride (all from Sigma Aldrich, UK) were dissolved in sterile saline. Diprenorphine (Tocris, UK) was dissolved in water by adding an equivalent amount of hydrochloric acid and subsequently diluted in sterile saline.

Nomenclature of targets and ligands: Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.,* 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al*., 2017).

RESULTS

Depression of respiration by fentanyl, heroin and morphine.

To study the rate of onset of respiratory depression fentanyl, heroin and morphine, were administered i.v. to mice. Morphine (7.5 $mg.kg^{-1}$), heroin (7.5 $mg.kg^{-1}$) and fentanyl (112 $\mu g.kg^{-1}$) each depressed minute volume significantly, reaching approximately the same degree of respiratory depression 10 - 15 min after injection (Fig. 1A). Saline injection did not significantly alter respiration. The half-time to reach maximum depression of minute volume was determined for each agonist by fitting a single exponential curve (Fig. 1A). Fentanyl had the fastest rate of onset and morphine the slowest (Table 1). The rate of onset of respiratory depression correlated with the lipophilicity of each drug (Table 1).

Both morphine and heroin depressed respiration by decreasing the rate of respiration, having no effect on tidal volume (Fig. 1B & C). As previously reported (Hill *et al.*, 2016; Hill *et al.*, 2018) , tidal volume was maintained due to the prolongation of inspiration by apneustic compensation. However, fentanyl depression of respiration resulted from both a decrease in the rate of respiration (Fig. 1B) and a decrease in tidal volume (Fig. 1C). As the rate of onset of the decrease in rate of respiration was the same for both heroin and fentanyl (Fig. 1B) then the faster depression of minute volume by fentanyl (see Fig. 1A) seems likely to be due to the rapid decrease in tidal volume (Fig. 1C).

We have previously demonstrated the dose-dependency of depression of respiration by morphine and oxycodone in mice breathing 5% CO_2 in air (Hill *et al.*, 2016; Hill *et al.*, 2018). Heroin (1 - 90 $mg.kg^{-1}$ i.p.) produced a dose-dependent depression of minute volume and respiratory rate (Fig. 2A, E & G) but only slightly decreased tidal

volume at the highest dose of 90 mg.kg^{-1} (Fig. 2C, F & G). The potency of heroin to depress minute volume was similar to that previously reported for morphine (Hill *et al.*, 2016). Fentanyl (0.05 - 1.35 mg.kg^{-1} i.p.) produced a dose-dependent depression of tidal volume, respiratory rate and minute volume (Fig. 2B, D, E, F & G). To depress minute volume fentanyl was approximately 70-fold more potent than heroin.

Effects of fentanyl and heroin on locomotor activity

In mice, at high doses, morphine and other μ opioid receptor agonists increase locomotor activity (Hill *et al.*, 2016; Lessov *et al.*, 2003; Valjent *et al.*, 2010). In the present study we examined the effect on locomotor activity of doses of heroin and fentanyl that produced similar levels of depression of respiratory rate (Fig 2E) and observed that heroin (90 mg.kg^{-1} i.p.) increased locomotor activity whereas, fentanyl (1.35 mg.kg^{-1} i.p.) decreased locomotor activity (Fig. 3).

Antagonism of fentanyl depression of respiration

It has been suggested that in overdose in humans the depression of respiration by fentanyl is less effectively reversed by naloxone compared to that by heroin (Peterson *et al.*, 2016; Schumann *et al.*, 2008; Somerville *et al.*, 2017). To investigate antagonist reversal of opioid respiratory depression, we administered equipotent doses of morphine, the main active breakdown product of heroin, and fentanyl (10 mg.kg^{-1} and 0.15 mg.kg^{-1} i.p. respectively) to mice, allowed maximal depression of respiration to develop over 20 min and then administered naloxone or diprenorphine. Naloxone (0.3 mg.kg^{-1} i.p.) rapidly antagonised the depression of respiration induced by morphine, with full reversal being apparent 5 min after naloxone administration (Fig. 4A). In contrast, the same dose of naloxone did not fully reverse the respiratory depression induced by fentanyl (Fig. 4A). Naloxone (1 mg.kg^{-1} i.p.) also did not fully reverse the

respiratory depression induced by fentanyl (Fig. 4B) but did fully reverse respiratory depression induced by morphine. Only when naloxone (3 mg.kg^{-1} i.p.) was administered did full reversal of the respiratory depression induced by fentanyl occur (Fig. 4C), i.e. a 10-fold greater dose of naloxone was required to reverse fentanyl respiratory depression compared to that by an equi-potent dose of morphine. On the other hand, when naloxone (0.3 mg.kg^{-1} i.p.) was administered 20 min prior to fentanyl or morphine the response to either opioid was attenuated although the fentanyl response was less affected than that of morphine (Fig. 4F).

In contrast to naloxone, diprenorphine (0.03 mg.kg^{-1} i.p.) reversed equipotent doses of morphine and fentanyl to the same degree (Fig. 4D). However, administration of a higher dose of diprenorphine (0.09 mg.kg^{-1} i.p.) more rapidly reversed morphine and fentanyl depression of respiration (Fig. 4E).

Fentanyl has been reported to be a relatively selective agonist at μ opioid receptors showing 100-fold and 400-fold higher affinity for the μ opioid receptor over κ and δ opioid receptors respectively (Toll *et al.*, 1998). To examine the possibility that there may be a δ opioid receptor component to fentanyl's respiratory depressant activity we have assessed the ability of the δ opioid antagonist naltrindole to prevent the effect of fentanyl on minute volume, respiratory rate and tidal volume in CD-1 mice. Pre-treatment with naltrindole (10 mg.kg^{-1}) failed to prevent the depression of minute volume, respiratory rate and tidal volume produced by fentanyl (1.35 mg.kg^{-1})(Fig. 5); indeed, naltrindole slightly potentiated the effect of fentanyl on tidal volume (Fig. 5C) but not on respiratory rate (Fig. 5B). Furthermore, pre-treatment of CD-1 mice with the κ opioid receptor antagonist NorBNI (10 mg.kg^{-1} i.p.) 24 h prior to fentanyl

administration did not affect the respiratory depressant effects of fentanyl (1.35 mg.kg^{-1}) (Fig. 5D, E & F). We have previously reported that this treatment with NorBNI prevented the antinociceptive response to the κ opioid receptor agonist U69593 in the tail flick latency assay and that U69593 alone did not depress respiration (Hill *et al.*, 2018).

Finally, we examined the ability of fentanyl to depress respiration in μ opioid receptor knock-out mice (C57BL/J background strain). Administration of fentanyl (1.35 mg.kg^{-1} i.p.) to the wild type background strain of mice depressed minute volume by ~80%, respiratory rate by ~60% and depressed tidal volume by ~30% (Fig. 5G, H & I). Administration of the same dose of fentanyl to μ opioid receptor knock-out mice produced no depression of either minute or tidal volume (Fig. 5E and F).

Interactions between heroin, morphine and fentanyl

Opioid users are not thought to use fentanyl as their primary drug of choice, rather they predominantly use heroin to which fentanyl has been added to enhance the "quality" of a given batch of heroin (Ciccarone, 2009; Dasgupta *et al.*, 2013). They are therefore likely to have already developed some degree of tolerance to heroin. We therefore investigated the degree of cross tolerance to fentanyl produced by prolonged pre-treatment with morphine, the main active breakdown product of heroin. In control mice that were implanted with a pump containing saline an acute challenge with morphine (10 or 90 mg.kg^{-1} i.p.) produced respiratory depression of 40% and 60% respectively, whereas in mice that had received prolonged pre-treatment with morphine the response to the 10 mg.kg^{-1} challenge dose of morphine was completely abolished and that to 90 mg.kg^{-1} markedly attenuated (Fig. 6A & B). These data

demonstrate that the morphine pre-treatment had produced significant tolerance. In contrast morphine pre-treated mice showed significantly less cross tolerance when challenged with fentanyl (Fig. 6C - F). At the lower challenge dose of fentanyl (0.15 $mg.kg^{-1}$) the depression of respiration was partially reduced but to a lesser extent than the equipotent challenge dose of morphine (Fig. 6C & E). At the higher challenge dose of fentanyl (1.35 $mg.kg^{-1}$) respiratory depression was the same as in non-morphine treated animals (Fig. 6D & F).

DISCUSSION

In the present study we observed that in the mouse fentanyl more rapidly depressed respiration than heroin and that with fentanyl the depression of respiration involved both a decrease in the frequency of breathing and a decrease in tidal volume whereas with heroin only at the highest dose was a small effect on tidal volume observed. Fentanyl was approximately 70x more potent that heroin or morphine in depressing respiratory rate; we observed a similar relative potency of fentanyl and morphine to produce antinociception in the same mouse strain (Hill, 2019). We also observed that the depression of respiration by fentanyl required higher doses of the opioid antagonist naloxone to be reversed than did the depression induced by morphine, the active breakdown product of heroin. In contrast diprenorphine reversed fentanyl and morphine depression of respiration to the same extent. The depression of respiration by fentanyl is mediated by the μ opioid receptor because it was not observed in μ opioid receptor knock-out mice (see also Schmid et al., 2017). Finally, prolonged pre-treatment of mice with morphine produced less cross tolerance to fentanyl than the tolerance to morphine itself.

The higher *in vivo* potency of fentanyls compared to other opioids is well documented as a factor in their lethality. However, we observed in this study that fentanyl was not only approximately 70x more potent than morphine and heroin when depressing mouse respiration, but it also had a significantly faster rate of onset to depress respiration. A fast rate of onset has been reported in fentanyl-using human populations with fentanyl suggested to produce lethal respiratory depression as quickly as 2 min following injection (Burns *et al.*, 2016; Green and Gilbert, 2016). This would make effective intervention with naloxone in fentanyl overdose more difficult to achieve.

Schmid *et al.*, (2017) have proposed that fentanyl's ability to depress respiration results from it being an 'arrestin biased' μ opioid receptor agonist (i.e. it is better at recruiting and signalling through arrestin than activating G protein signalling) and that opioid depression of respiration is mediated by arrestin signalling as proposed by Raehal *et al.* (2005). We have previously reported that fentanyl does not exhibit arrestin bias in arrestin translocation and GTPγS binding assays (McPherson *et al.*, 2010; Rivero *et al.,* 2012). Furthermore, in transgenic mice in which all the phosphorylation sites on the C tail of the μ opioid receptor had been mutated to alanine and which are therefore not phosphorylated by G protein receptor kinases (GRKs) and do not recruit and bind arrestins, fentanyl still depressed respiration (Kliewer *et al.*, 2019). This observation brings into question the concept that opioid depression of respiration is a function of arrestin signalling (Montandon and Slutsky, 2019).

We have previously reported that in the mouse the depression of respiration by several μ opioid receptor agonists, morphine, oxycodone and methadone, results from a reduction in respiratory rate and at the doses tested did not involve a decrease in tidal

volume (depth of breathing) (Hill *et al.*, 2016; Hill *et al.*, 2018; Withey *et al.*, 2017). In the present study however, we observed that fentanyl depressed both respiratory rate and tidal volume whilst heroin was only observed to reduce tidal volume slightly at the highest dose tested. The decrease in tidal volume produced by fentanyl may result from an increase in respiratory muscle stiffness and/or changes in phrenic motor activity during inspiration expiration (Campbell et al., 1995). Both fentanyl and alfentanil have been reported to cause profound muscle stiffness in rodents by an action on μ opioid receptors in the CNS (Weinger et al., 1991; Lui et al., 1993). Conversely, the hyper locomotor activity induced by heroin in mice may stimulate respiration and obscure any decrease in tidal volume. This would not occur with the high dose of fentanyl we tested as it reduced locomotor activity but may occur at lower doses which do enhance locomotor activity (Kleiwer et al., 2019; Varshneya et al., 2019).

The δ opioid antagonist naltrindole potentiated the fentanyl depression of tidal volume and thus also minute volume, without affecting the depression of respiratory rate. We have previously reported that naltrindole does not alter the depression of minute volume by morphine or oxycodone (Withey et al., 2017). This would imply that the effect of naltrindole on fentanyl depression of tidal volume is not an off target effect as any such off target effect would be expected to be observed against all the opioid agonists. Rather it implies that the fentanyl depression of tidal volume involves the release of endogenous opioid(s) on to δ receptors. Given the possible role of muscle stiffness in the depression of tidal volume it is interesting to note that that δ opioid agonists have been shown to inhibit alfentanil-induced muscle stiffness (Vankova *et al.,* 1996)

In man, intravenous administration of high doses of fentanyl and alfentanil produces skeletal muscle rigidity resulting in stiffness of the chest wall (Benthuysen *et al.*, 1986; Streisand *et al.*, 1993; Waller *et al.*, 1981). Brain micro injection studies in rats have implicated several brain regions - locus coeruleus, basal ganglia, nucleus raphe pontis and periaqueductal grey - as sites of action of fentanyls to induce muscle rigidity (Blasco *et al.*, 1986; Lui *et al.*, 1989; Lui *et al.*, 1990; Slater *et al.*, 1987; Weinger *et al.*, 1991; Widdowson *et al.*, 1986) and have shown that it is mediated by activation of μ, and not δ or κ opioid receptors (Vankova *et al.*, 1996). It is likely therefore that in humans intravenous injection of fentanyl results in both a decreased drive to breathe and a mechanical resistance to breathing both of which would contribute to overdose death (Burns *et al.*, 2016). Signs of muscle rigidity have been observed in opioid injectors who have presumably injected illicit fentanyls at a supervised drug injection facility in Vancouver (Kinshella *et al.*, 2018).

Our finding that naloxone less readily reversed respiratory depression by fentanyl compared with morphine confirms reports from studies in humans that more naloxone may be required to reverse a fentanyl overdose compared to a heroin overdose (Fairbairn *et al.*, 2017; Lynn *et al.*, 2018; Peterson *et al.*, 2016; Schumann *et al.*, 2008; Somerville *et al.*, 2017). In our study we allowed the depression of respiration by each agonist to reach maximum before naloxone was administered to mimic how the drugs would be administered in an overdose situation. Lower sensitivity of fentanyl to antagonism by naloxone cannot be explained simply by fentanyl having high affinity for the μ opioid receptor given that under competitive conditions the degree of antagonism does not depend on the affinity of the agonist but only upon the affinity

and concentration of the antagonist (Rang *et al.*, 2016). Furthermore, the lipophilic opioid antagonist, diprenorphine, a non-selective opioid antagonist (Corbett *et al.,* 1993) used in veterinary medicine to reverse the tranquilizing effects of etorphine and carfentanil in large animals, reversed both fentanyl and morphine depression of respiration equally. Why diprenorphine is better at antagonising fentanyl than naloxone is unknown at present. We can speculate that, due to its higher lipophilicity, diprenorphine may exhibit a differential drug concentration effect in the vicinity of the plasma membrane as described previously for dopamine antagonists (Gherbi *et al.,* 2018). Alternatively, fentanyl and diprenorphine may bind to the μ opioid receptor in a manner distinct from morphine and naloxone, as reported for certain ligands at M2, M3 and M4 muscarinic receptors (Dror *et al.,* 2013; Chan *et al.*, 2018).

We have previously demonstrated that tolerance develops to the respiratory depressant effects of μ opioid receptor agonists such as morphine, oxycodone and methadone (Hill *et al.*, 2016; Hill *et al.*, 2018). In the present study we observed that when morphine pre-treated mice were challenged with either fentanyl or morphine the degree of cross tolerance to fentanyl was less than the tolerance to morphine itself. A similar reduced level of cross tolerance between fentanyl and morphine has been reported in studies of rodent locomotor activity (Brase 1986) and antinociception (Paronis and Holzman, 1992; Bobeck *et al.,* 2019), although one study has reported equal cross tolerance between morphine and fentanyl on antinociception (Romero *et al.,* 2010). Cross tolerance develops between drugs acting at the same receptor, but the degree of cross tolerance will depend upon agonist intrinsic efficacy. Fentanyl has higher intrinsic efficacy (McPherson *et al.*, 2010) and so needs to occupy a smaller proportion of the available receptors to produce its response. It will therefore be less

affected by the loss of μ opioid receptor function - from either receptor desensitization, internalization or degradation – that underlies tolerance. The implication being that with opioid drug users fentanyl will be able to 'break through' tolerance induced by heroin, oxycodone and methadone and produce respiratory depression.

CONCLUSIONS

Our studies in mice indicate that in overdose by humans a number of factors may contribute to the high lethality of fentanyl. These include its high potency, rapidity of onset of action, depression of rate and depth of respiration, lower sensitivity to reversal by naloxone and reduced cross tolerance to other abused opioids.

Author contributions

R.H., W.L.D., E.K. and G.H. participated in research design.

R.H. and R. S. performed the experiments.

R.H., R.S. and G.H. analysed the data.

R.H., W.L.D., E.K. and G.H. participated in writing the manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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Opioid	Rate of onset ($t_{1/2}$, min)	ClogP
Fentanyl	0.54 ± 0.09*¶	3.62
Heroin	1.70 ± 0.68*	1.48
Morphine	4.64 ± 1.62	0.57

Table 1. Rate of onset of opioid depression of respiration following i.v. administration and calculated lipid solubility values (ClogP) for opioid agonists. The data for the onset of respiratory depression for opioid agonists were fitted to a single exponential (see Fig. 1A) to obtain the $t_{1/2}$ value for each opioid drug. Data are presented as the mean ± s.e.m; n = 12 for each drug. Statistical comparison was made by 1-way ANOVA with Bonferroni's comparison. * indicates statistical difference ($p<0.05$) from morphine and ¶ indicates statistical difference ($p<0.05$) from heroin [$F = 5.093$ (dfn = 2, dfd = 36)]. The ClogP value for each drug was calculated using Chem3D (PerkinElmer).

Figure Legends

Figure 1. Rate of onset of opioid respiratory depression. Respiratory parameters were monitored in mice receiving i.v. injection of fentanyl (112 μg.kg^{-1}), heroin (7.5 mg.kg^{-1}) morphine (7.5 mg.kg^{-1}) or saline. **A.** Fentanyl, heroin and morphine rapidly depressed minute volume (MV), the effect of the drugs reaching a similar steady state 10 - 15 min post-administration. Data for each drug are fitted to a single exponential. **B.** Fentanyl, heroin and morphine depressed respiratory rate. **C.** Heroin and morphine had no effect on tidal volume (TV), whereas fentanyl significantly depressed tidal volume [$F = 65.05$ (dfn = 3, dfd = 704)]. **A-C.** Saline injection did not alter any of the respiratory parameters. All data presented as mean ± s.e.m. Statistical comparison in **C** was made by 2-way ANOVA with Bonferroni's comparison. * indicates $p<0.05$ compared to saline. n=12 for each group.

Figure 2. Effect of heroin or fentanyl on mouse respiration. **A.** Heroin (1 - 90 mg.kg^{-1}, i.p.) dose-dependently depressed minute volume (MV). **B.** Fentanyl (0.05 - 1.35 mg.kg^{-1}, i.p.) dose-dependently depressed minute volume. **C.** Heroin only slightly depressed tidal volume (TV) at the highest dose tested (Data from one mouse have been excluded from all heroin 90 mg.kg^{-1} results due to the TV values being two standard deviations higher than the group mean). **D.** Fentanyl dose-dependently depressed tidal volume. In **C** [$F = 13.07$ (dfn = 4, dfd = 192)] & **D** [$F = 58.33$ (dfn = 3, dfd = 160)] statistical comparison was made by 2-way ANOVA with Bonferroni's comparison. * indicates $p<0.05$ compared to saline pre-treated mice. n=6 for each group. In **A** & **B** statistical significance was observed for most time points for each dose of heroin and fentanyl but *s have been omitted for clarity as the size of effect of each dose is clear from the dose-response graphs in **E** & **F**. **E**. Dose-response curves

for fentanyl and heroin depression of respiratory rate (data presented as peak depression of respiratory rate as calculated from experiments shown in **A** & **B**). **F**. Dose-response curves for fentanyl and heroin inhibition of tidal volume (data presented as peak depression of tidal volume calculated from experiments shown in **C** & **D**). **G.** Left hand trace - control respiratory trace in the absence of opioid. Middle trace – at maximum respiratory depression by heroin 90 $mg.kg^{-1}$. Right hand trace - at maximum respiratory depression by fentanyl 1.35 $mg.kg^{-1}$.

Figure 3. Change in mouse locomotor activity following heroin or fentanyl administration. **A.** Saline (i.p.), heroin (90 $mg.kg^{-1}$ i.p.) or fentanyl (1.35 $mg.kg^{-1}$ i.p.) were administered to mice and locomotor activity measured. Heroin caused a sustained increase in locomotor activity compared to saline, whereas fentanyl caused a decrease in locomotor activity compared to saline. **B.** Area under the curve (AUC) analysis of data in **A.** Statistical comparison in **B** made using 1-way ANOVA with Bonferroni's comparison. * indicates $p<0.05$ compared to saline. n=8 for each group.

Figure 4. Reversal of morphine and fentanyl respiratory depression by naloxone and diprenorphine. **A-C** Equipotent respiratory depressant doses of morphine (10 $mg.kg^{-1}$ i.p.) and fentanyl (0.15 $mg.kg^{-1}$ i.p.) were administered to mice before naloxone administration 20 min after saline or opioid agonist. **A.** Naloxone 0.3 $mg.kg^{-1}$ i.p. fully reversed morphine respiratory depression, but fentanyl respiratory depression was unaffected (-43.8 ± 4.8% pre-naloxone vs -28.1 ± 7.8% post-naloxone $p>0.05$) [$F = 55.3$ (dfn = 2, dfd = 15)]. **B.** Naloxone 1 $mg.kg^{-1}$ i.p. fully reversed morphine respiratory depression, whereas fentanyl was not reversed (-42.7 ± 5.4% pre-naloxone vs -28.9 ± 6.2% post-naloxone $p>0.05$) [$F = 14.98$ (dfn = 2, dfd = 15)].

C. Naloxone 3 mg.kg^{-1} i.p. fully reversed both morphine and fentanyl respiratory depression. **D.** Diprenorphine 0.03 mg.kg^{-1} i.p. reversed morphine and fentanyl respiratory depression to the same degree **E.** Diprenorphine 0.09 mg.kg^{-1} i.p. rapidly reversed both morphine and fentanyl respiratory depression back to baseline levels. All data presented as mean ± s.e.m. Statistical comparison of minute volume following naloxone administration made by 2-way ANOVA with Bonferroni's comparison. * indicates $p<0.05$ compared to saline. n=6 for each group.

Figure 5. Fentanyl depression of respiration results from activation of μ opioid receptors. A, B & C. Pre-treatment with naltrindole (10 mg.kg^{-1} i.p.) 20 min prior to fentanyl (1.35 mg.kg^{-1} i.p.) injection did not prevent fentanyl depression of minute volume (**A**), respiratory rate (**B**) or tidal volume (**C**). Instead naltrindole pre-treatment significantly enhanced fentanyl depression of both minute volume and tidal volume. **D, E & F.** Pre-treatment with NorBNI (20 mg.kg^{-1} i.p.) 24 h prior to administration of fentanyl did not alter fentanyl-induced depression of minute volume (**D**), respiratory rate (**E**) or tidal volume (**F**). **G.** Administration of fentanyl (1.35 mg.kg^{-1} i.p.) significantly depressed minute volume in wild-type background strain mice (MOP wild Type), but there was no effect in μ opioid receptor knock-out mice (MOP Knock Out) [$F = 933$ (dfn = 1, dfd = 80)]. **H.** Fentanyl (1.35 mg.kg^{-1} i.p.) also depressed respiratory rate in wild type mice but there was no effect in μ opioid receptor knock-out mice [$F = 414.1$ (dfn = 1, dfd = 80)]. **I**. Fentanyl (1.35 mg.kg^{-1} i.p.) depressed tidal volume in wild type mice but there was no effect in μ opioid receptor knock-out mice [$F = 170.9$ (dfn = 1, dfd = 80)]. All data presented as mean ± s.e.m. Statistical comparison made by 2-way ANOVA with Bonferroni's comparison. * indicates $p<0.05$ compared to saline pre-treated mice. n=6 for each group.

Figure 6. Cross tolerance to fentanyl following prolonged morphine administration. **A-B.** Acute morphine challenge (10 or 90 mg.kg^{-1} i.p.) induced less respiratory depression in morphine-treated mice compared to saline-treated controls. **C.** Acute fentanyl challenge (0.15 mg.kg^{-1} i.p.) induced less respiratory depression in morphine-treated mice compared to saline-treated controls. **D.** Acute fentanyl challenge (1.35 mg.kg^{-1} i.p.) induced the same level of respiratory depression in morphine-treated mice as was observed in saline-treated controls. **E-F.** The depression of respiration by fentanyl challenge (0.15 and 1.35 mg.kg^{-1}) was not reduced to the same extent as that by morphine challenge (10 or 90 mg.kg^{-1}) in morphine-treated animals. All data presented as mean ± s.e.m. Statistical comparison made by 2-way ANOVA with Bonferroni's comparison. **E** [F = 3.86 (dfn = 1, dfd = 24)] **F** [F = 25.7 (dfn = 1, dfd = 24)] * indicates $p<0.05$ compared to morphine. n=7 for each group.