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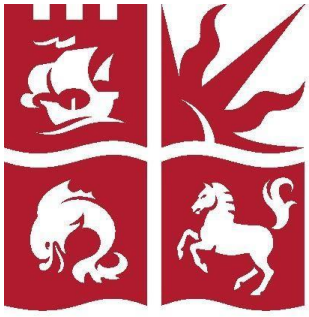

University of BRISTOL

Studies in opioid induced respiratory depression

Rakulan Santhakumar

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of MSc by Research in Physiology and Pharmacology in the Faculty of Life Sciences and the school of School of Physiology, Pharmacology and Neuroscience

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Abstract

The main cause of fatality from opioid overdose is opioid induced respiratory depression (OIRD), with much of the street heroin being cut with fentanyl. Heroin habits often follow drug abstinence-relapse cycles. Currently, overdose is reverse by antagonising the μ-opioid receptor with naloxone. This may lead to opioid withdrawal syndrome, which can induce aggression, a problem to first responders and clinicians. Clinically, balancing analgesia and respiratory depression can present itself to be difficult. AMPA receptor mediators show promise in reversing respiratory depression without affecting analgesia.

Heroin and fentanyl dose-dependently depressed respiration in male CD1 mice with heroin doing so through depressing respiratory rate and fentanyl through depressing respiratory rate and tidal volume. The idea that 6-MAM over morphine is the active metabolite of heroin is relatively new. In this study, 6-MAM induced OIRD. It has been previously shown that heroin fentanyl mixtures act synergistically in decreasing oxygen saturation within the brain. A heroin-fentanyl mixture failed to show additivity or synergy in causing respiratory depression as a ceiling effect in response likely occurred. Investigating alternative OIRD reversing agents and the role of the AMPA receptor during opioid induced respiratory depression. Ketamine has been shown to reverse opioid induced respiratory depression to some of the fentanyls but has yet been shown to reverse heroin induced respiratory depression (HIRD). Ketamine (10, 30mg/kg i.p.) and the metabolite, 2R,6R-hydroxynorketmaine (10, 30mg/kg i.p.) failed to reverse HIRD. Tianeptine 30mg/kg i.p. induced OIRD equipotent to heroin 10mg/kg i.p. and showed to induce analgesia (measured in tail flick latency). The decrease in potency could be attributed to AMPA receptor potentiation. Respiratory depression and analgesia showed reversal by naloxone (0.3 mg/kg i.p.), indicating μ-opioid receptor agonism. Many users claim that they perceive an enhanced rate of tolerance to euphoria following periods of abstinence. After heroin pre-treatment using osmotic mini-pumps an enhanced rate of tolerance development to respiratory depression after a 6-day abstinence period is indicated. However, robust tolerance was not seen with the current protocol. When mice were pre-treated with methadone, this enhanced rate was not present. Indicating a PKC mediated mechanism as heroin metabolites are said to induce desensitisation of the μ-opioid receptor.

This thesis reports a variety of emerging novel fields within the field of opioid induced respiratory depression and provides an insight into a possible future direction of opioid induced respiratory depression mediating agents and the process of understanding the real-world use of heroin and incorporating behaviours into experimental design.

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I would like to thank my supervisors Professor Graeme Henderson and Professor Eamonn Kelly for their help, advice and the opportunity to conduct this research. Thank you Dr Rob Hill for passing your experimental knowledge and practical abilities onto me and for succumbing to my minesweeper high score. I'd also like to thank Dr Joanna Kesten and those at the Bristol Drug's Project for your expertise and input.

I'd like to thank my friends who stayed on for a fourth year in Bristol, my school friends for keeping me sane, in addition to my Cambridge housemates at The Cottages. Finally, a thank you to my family, especially my late grandmother, for their copious amounts of support.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .... [redacted] ..... DATE: 18/09/2020

Table of Contents

Table of Figures

Table of Tables

Table of Abbreviations

%MPE	Percent maximum possible effect
6-MAM	6-Monoacetylmorphine
AMPAr	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AUC	Area under the curve
BDP	Bristol Drugs Project
CA3	cornu Ammonis 3
cAMP	cyclic adenosine monophosphate
CYP4A4	Cytochrome P450 3A4
CYP2B6	Cytochrome P450 2B6
DAMGO	[D-Ala2, N-MePhe4, Gly-ol]-enkephalin
DOPr	δ-opioid receptor
GDP	Guanosine-5'-diphosphate
GIRK	G-protein-coupled inwardly-rectifying potassium channels
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinases
GTP	Guanosine-5'-triphosphate
HEK	293 Human endothelial kidney 293 cells
i.p.	Intraperitoneal
i.v.	Intravenous
JNK	c-Jun N-terminal Kinase
KO	Knock out
KOPr	κ-opioid receptor
LC	Locus coeruleus

M3G	Morphine-3-gluconoride
M6G	Morphine-6-gluconoride
MOPr	μ-opioid receptor
MV	Minute volume
NMDA	N-methyl-D-aspartate receptor
OIRD	Opioid induced respiratory depression
ORL-1	Opioid-like-receptor
OST	Opioid substitution therapy
PKC	Protein Kinase C
PKA	Protein Kinase A
RR	Respiratory Rate
s.c.	Subcutaneous
TV	Tidal Volume
VSCC	Voltage sensitive calcium channels

1.0 Introduction

1.1 Background to Opioid Use

The issue of substance abuse is not limited to modern times but has been well described throughout human history. British importation of opium from British India into China in the 1760s caused widespread addiction in China. This caused widespread social and health consequences for the country and its residents (Lu et al., 2008). In 1874 diacetylmorphine (commonly known as heroin) was developed in Germany with the belief that it could be used as an analgesic and cough suppressant with non-addictive properties (Sneader, 1998).

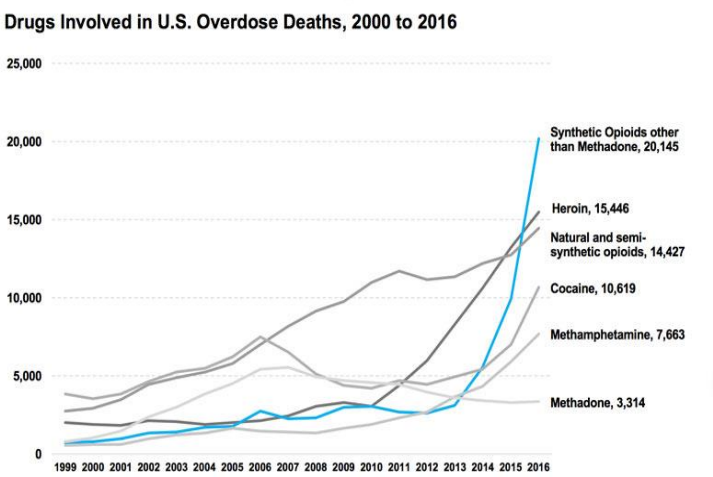

Figure 1.1 Yearly overdose deaths in the U.S. With opioids totalling over 50,000 deaths in 2016 (NIDA, 2017)

With the advancement of technologies that allow for easier production of opioids in combination with individual socio-economic pressures and the commonality of opioid prescriptions; the prevalence of drug abuse has only increased (Monnat, 2016). The level of opioid abuse has reached epidemic levels within the United States of America. This epidemic cannot solely be attributed to heroin abuse, but also to the raised levels of prolonged and/or high dose opioid prescriptions in patients with chronic illness – mainly chronic (non-cancer) pain (Guy et al., 2017). Figure 1.1 illustrates the increase in opioid-related deaths over the last two decades, with a huge increase in the number of deaths caused by synthetic opioids (mainly fentanyls) which has, since 2015, overtaken the number of deaths attributed to heroin overdoses. The euphoria and analgesia associated with opioids is also paired with side effects that include physical dependence, constipation, and respiratory depression. With respiratory depression being the leading cause of death from opioids (White and Irvine, 1999).

1.2 Heroin

The structure of heroin is very similar to that of morphine, the two extra acetyl groups at positions 3 and 6 gives it the name diacetylmorphine. It has very low affinity and efficacy at the μ-opioid receptor (MOPr) (Kelly, 2013), instead working as a highly lipid soluble pro-drug which allows for more rapid penetration of the brain - supposedly more so than its metabolites (Inturrisi et al., 1983; Corbett et al., 2006). Heroin is rapidly metabolised into 6-monoacetyl-morphine, also known as 6-MAM, which is then metabolised into morphine which exerts its main pharmacological effects. Both 6-MAM and morphine show similar affinity to the MOPr (Goldberger et al., 1994). Morphine is then further metabolised by a process called glucuronidation to produce the metabolites morphine-6-glucuronide (M6G) which is active at MOPr and morphine-3-glucuronie (M3G) (de Gregori et al., 2012).

1.3 Fentanyl

Fentanyl is a synthetic opioid agonist at the MOPr, 50-100 times more potent than heroin (Volpe et al., 2011). It is commonly used in the medical setting for its analgesic and sedative effect via its MOPr activity. Therefore, these qualities, and its fast rate of onset make it an ideal drug for breakthrough pain, commonly experienced by cancer patients (Mishra et al., 2009). However, it is for these same qualities that it is often found combined with street sold heroin to create the perception of a higher quality and more potent form of heroin. There are also recorded cases of people knowingly abusing fentanyl itself. Manufacturing techniques have improved the synthesis of fentanyl (Valdez, Leif and Mayer, 2014). Drug traffickers are able to take advantage of fentanyl's potency in order to discretely transport the substance in smaller packages as "a little goes a long way". Fentanyl carries with it a high risk of overdose when not used with caution; small increases in the weight of fentanyl administered can lead to larger, potentially life-threatening respiratory depression (Krinsky et al., 2011).

Currently, it is understood that fentanyl metabolism mainly occurs within the liver, via the hepatic enzyme cytochrome p450 3A4 (CYP3A4). Here, it is dealkylated into norfentanyl with other p450 isoforms (P450s 1A2, 2b6, 2C9, 2D6, 2E1 and 3A4) (Smith, 2009). More common prescription opioids, such as oxycodone, are also metabolized hepatically and it has been suggested they would compete for the CYP3A4 enzyme (Gallego et al., 2007). The danger here is that polydrug use of these substances would most likely enhance the physiological effect of fentanyl making overdose more likely. This would exacerbate the opioid crisis as prescription opioids are often a gateway for opioid addiction. Similarly, methadone, used in replacement therapy to treat heroin addiction, also competes for this

metabolic pathway (Smith, 2009). Thus, there is a risk in administering methadone to patients who may go on unknowingly to use heroin laced with fentanyl.

1.4 Opioid Receptors

The agonists described above, and their active metabolites bind primarily to the μopioid receptor (MOPr). The MOPr is one of four classes of opioid receptors, the others being the κ-opioid receptor (KOPr), δ-opioid receptor (DOPr) and opioid like receptor (ORL-1). Many opioid ligands have differing affinities to each of these receptors but for drug abuse it is their activity at MOPr which is important. MOPr knockout mice show a lack of responses to respiratory depression, antinociception and reward (a measure for euphoria) (Matthes et al., 1996; Romberg et al., 2003). Opioid receptors are G-protein coupled receptors (GPCRs) which largely signal through G_i/G_o proteins. G-proteins contain three subunits: α, β and γ. The receptor consists of seven transmembrane domains which span the cellular membrane. When an agonist binds to the extracellular domain, a conformational change in structure occurs as activation energy is lowered by the docking. This triggers G-protein dissociation from the receptor to interact with the respective substrates leading to a signalling cascade (Williams et al., 2013).

1.4.1 Activation of Opioid Receptors

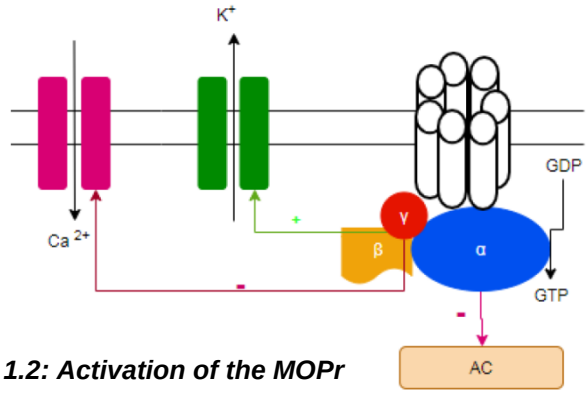

Figure 1.2: Activation of the MOPr

The binding of an agonist causes the inhibition of N-type voltage sensitive calcium channels and G-coupled inward-rectifying potassium channel via the dissociated β/γ subunit complex. Adenylyl cyclase is in turn inhibited by the dissociated α-subunit .

Morphine, the prototypic opioid agonist binds to the MOPr in the orthosteric binding pocket, leading to a conformational change in its structure. This activates the G-protein, causing guanosine-5'-diphosphate (GDP) to be exchanged with guanosine-5'-triphosphate (GTP) on the intracellular α-subunit (illustrated in Figure 1.2). This in turn causes it to dissociate from the β/γ- subunit complex. The α-subunit inhibits intracellular adenylyl cyclase, thus inhibiting cyclic adenosine monophosphate (cAMP) synthesis. The β/γ complex inhibits N-type voltage sensitive calcium channels (VSCC); preventing an influx of calcium ions (Soldo and Moises, 1998). This occurs simultaneously alongside the β/γ- subunit complex activating G-protein-coupled-inward-rectifying potassium (GIRK) channels that cause an efflux of potassium ions (North et al., 1985). Inhibition of calcium entry leads to a reduction in neurotransmitter

release whereas activation of GIRK channels leads to cell hyperpolarization, secondary to the decreased concentration of positive ions within the cell and therefore decreased neuronal excitability.

1.5 Desensitisation of the MOPr and Tolerance

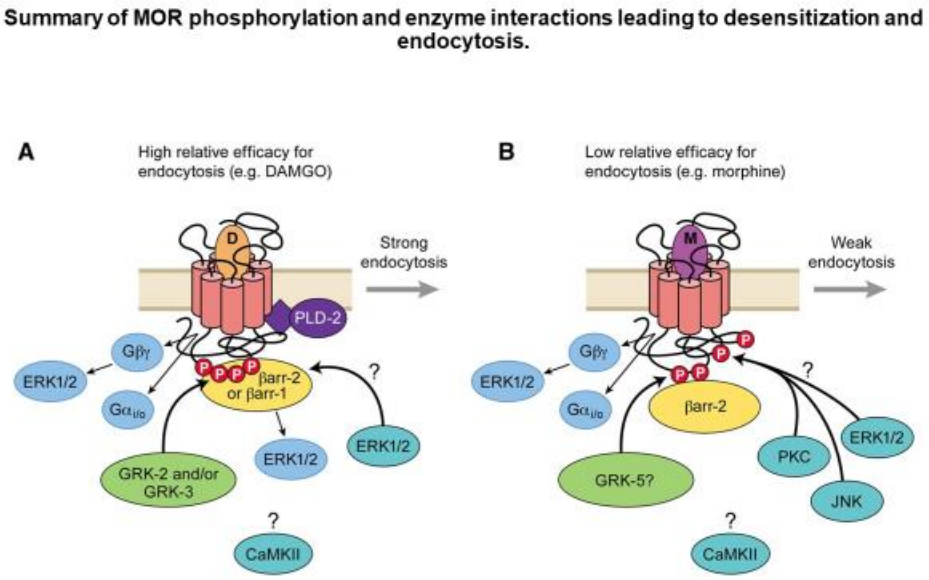

Figure 1.3 Summary of MOPr phosphorylation and other interactions via various proteins causing desensitisation. *Figure obtained from review by Williams et al (2013)*

Desensitisation, internalization and the recycling of receptors are regulated by various proteins. There is strong evidence that desensitisation of MOPr is largely due to receptor phosphorylation (Kelly et al., 2008). While several kinases have been implicated in MOPr desensitisation (see Figure 1.3) the two main kinases involved in this process appear to be G-protein receptor kinases (GRKs) and isoforms of protein kinase C (PKC). The recruitment of these proteins appears to be ligand dependent (Bailey et al., 2006). Evidence of this has been shown in the form of bias to [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) in recruiting GRK in locus coeruleus neurons (LC), whereas morphine was shown to recruit PKC. PKCα knockout mice were shown not to show morphine desensitisation, but still showed DAMGO desensitisation (Bailey et al., 2006). The desensitisation pathway has been of great interest within the literature, especially with regards to how desensitisation can be linked to tolerance; an observable adaptation where the effect of a drug is reduced following repeated exposure over a period of time (Johnson et al., 2006; Levitt and Williams, 2012).

There is evidence that MOPr desensitisation occurs before the onset of tolerance and that desensitisation is required for both development and maintenance of cellular tolerance

(Bailey et al., 2009). A past study injected a slow releasing bolus dose of morphine 200mg/kg in rats, over a 3-day period, inducing cellular tolerance caused by MOPr desensitisation in LC neurons. The LC neurons were then incubated in a 1uM morphine within artificial cerebral spinal fluid, which maintained tolerance. Challenge doses of morphine caused a significant decrease in morphine response in comparison to naïve controls. By washing the LC neurons to remove the presence of morphine, MOPr desensitisation reversed as did tolerance, suggesting that desensitisation is a pre-requisite in establishing morphine tolerance (Bailey et al., 2009). Continuous PKC activity is essential in the maintenance of morphine-induced MOPr desensitisation. Within the same study, PKC inhibitors administered to LC neurons before and after desensitisation have been showed to reverse and decrease MOPr desensitisation respectively. This further demonstrates that continuous desensitisation is required for the maintenance of cellular tolerance.

There has been contradictory evidence as to whether desensitisation and tolerance are interlinked. Some investigations suggest that desensitisation and tolerance to opioids occurs as separate events. Morphine is said to be inefficient at inducing acute desensitisation in neurons (Alvarez et al., 2002; Bailey et al., 2003; Dang et al., 2005) This, in combination with the degree of tolerance seen with morphine does not support the weight that desensitisation has on the phenotype of tolerance. In one study, osmotic mini-pumps were used to pre-treat rats with morphine over a period of 6 days (Levitt and Williams, 2012). After the prolonged pre-treatment, the LC response to morphine was measured through voltage clamp recordings of GIRK channels. The LC neurons from pre-treated rats showed a significantly decreased response to morphine in comparison to naïve controls. This tolerance was present even with the absence of morphine. This presented itself as “long-lasting cellular tolerance” (Levitt and Williams, 2012). The LC neurons were then maintained in plasma concentrations of morphine, where a further decrease in response was seen, which represented desensitisation. Desensitisation could therefore enhance existing long-lasting cellular tolerance; with both phenomena existing as mutually exclusive events. In this study, a PKC inhibitor reversed desensitisation but not long-lasting cellular tolerance to morphine. Indicating the mechanism for desensitisation is an independent event (Levitt and Williams, 2012).

Many heroin users cycle through periods of heroin use, detox, abstinence, substitution therapy and relapse (Scott et al., 2011). Anecdotal accounts of intravenous heroin users that tolerance to heroin occurs more rapidly during relapse after a period of abstinence (Personal communication from Prof Graeme Henderson following discussions with heroin users at the Bristol Drugs Project). This may be attribute to both psychological and biological factors. A potential physiological component may be that users are more confident using after previous

experience and thus take higher doses more often after relapsing relative to their initial use. Or that they relapse in the company of ongoing users and thus initiate taking doses similar to those who had built up tolerance due to a lack of abstinence. A qualitative study by Kesten et al, 2020 at the Bristol Drugs project is attempting to unearth possible reasonings for the psychological component of this perceived enhanced rate of tolerance development.

The biological component of this perceived phenomenon has not previously been investigated within the literature. Potential mechanisms may involve an upregulation of PKC. Chronic (3 weeks) morphine administration has been shown to induce this upregulation and thus increase the level of desensitisation upon MOPr agonist administration (as discussed in Chapter 1.5). Methadone induced MOPr desensitisation has been suggested to be GRK and arrestin mediated (McPherson et al., 2010; Lowe et al., 2015). With methadone tolerance not being able to be reversed by the PKC inhibitor calphostin c (Withey et al., 2017). Thus, methadone may not contribute to an upregulation of PKC. This is further supported with evidence that ethanol reverses tolerance to respiratory depression for morphine but not methadone or buprenorphine (Hill et al., 2016). Possibly due to ethanol inhibiting PKC activity and thus acting as a “desensitisation inhibitor” (Slater et al., 1997; Rex et al., 2008; Hill et al., 2016). This highlights how methadone and buprenorphine trigger independent desensitisation mechanisms (Kellyet al., 2008) . Methadone and buprenorphine could provide a useful tool in investigating whether PKC upregulation contributes to an enhanced rate of development of tolerance.

1.6 Opioid Respiratory control

A 1923 study in decerebrate cats showed that the brainstem is vital for respiratory control (Montandon et al., 2011). The brainstem consists of the midbrain, the pons and the medulla oblongata (Lalley et al., 2014). Within the brainstem there are various nuclei involved in respiratory rhythm including the Kolliker-fuse, the parabrachial complex and the pre-Bötzinger complex. The pre-Bötzinger complex is found within the medulla oblongata and has been suggested to be vital in the generation of respiratory rhythm. This has been demonstrated using microdialysis of fentanyl dorsal to these neurons which abolished respiration (Lonergan et al., 2003). However, it should be noted that fentanyl was added dorsally to the pre-Bötzinger complex and not directly within the structure. Furthermore, the lipophilic nature of fentanyl would mean that its administration would not be localized to that area alone. More recently, it has been suggested that the pre-Bötzinger complex is not essential for respiratory rhythm generation (Qi et al., 2017). Another group had shown that endomorphin-1 microinjected directly into the pre-Bötzinger complex did not decrease

phrenic nerve activity (O'Regan and Majcherczyk, 1982); the nerve which stimulates respiratory motor components. Following contradictions of the lack of importance of the pre-Bötzinger complex as a major site of opioid action, in 2017 a study showed that the MOPr agonist, endomorphin-2 administration within this region decreased breathing frequency and amplitude in rats, with the MOPr antagonist, β-funaltrexamine being able to prevent the effects of endomorphin-2 (Pattinson, 2008), highlighting again the importance of this region within the respiratory network that it is an opioid sensitive region .

Respiration is precisely monitored by homeostatic mechanisms. Central and peripheral chemoreceptors are able to detect a decrease in blood pH caused by an increase in blood levels of carbon dioxide (CO_2) (Zhang et al., 2007), either due to an increase in metabolic rate or decrease in respiration. Central chemoreceptors can be found within the pre-Botzinger complex, the nucleus tractussolitarus, raphe nucleus and the pons (Lahiri et al., 2006). MOPr agonists have been shown to affect chemoreception within the raphe, by decreasing response to CO_2 by up to 28% (Pokorski and Lahiri, 1981). Peripheral chemoreceptors are located within the carotid bodies and aorta and are the main mechanism in detecting hypoxia (Purves et al., 2001). These receptors also can be modulated by opioids. Enkephalin, a MOPr agonist, has been shown (in cats), to decrease carotid body chemoreceptor activity which can be reversed through naloxone antagonism (Clark and von Euler, 1972). Action potentials from the carotid body propagate to the nucleus tractussolitarus (Takakura et al., 2006) along a mechanical stretch of tissue (Lalley et al., 2014). Peripheral mechanoreceptors detect the degree of inflation of the lung. Once their stretch threshold has been reached, action potentials are sent via the vagus nerve to the medulla and the apneustic center in the pons. This inhibits inspiration, allowing expiration to occur, this is called the Herring Breuer Reflex (Wamsley, 1983; Lahiri et al., 2006).

There is, however, a clear lack of definitive understanding over the weight each nucleus has over respiratory rhythm. However, as with most structures within the brain, the role of nuclei are likely to be interlinked to allow for synchronous operating (Schmid et al., 2017; Hill et al., 2020). MOPr can be found abundantly throughout the nuclei involved in respiratory rhythm and can also be located in chemoreceptive tissues such as glomus cells (Wheeler et al., 2015). Application of MOPr agonists decrease the ability of cells to meet the thresholds required to generate action potentials due to downstream signalling effects (refer to section 1.4.1). The decrease in neuronal output decreases the activity of respiratory nuclei within the brainstem thus inducing respiratory depression. Furthermore, the decrease in CO_2 sensitivity of chemoreceptors caused by opioids prevents the rectifying increase in respiratory neuronal activity to increase respiration.

Opioid induced respiratory depression is a result from MOPr activation, instead of DOPr, KOPr and ORL-1. Transgenic mice without MOPr do not show respiratory depression when MOPr agonists are applied (morphine and fentanyl) (Hill et al., 2018). MOPr are abundant throughout the respiratory system within the brain. The cellular mechanisms behind opioid induced respiratory depression has been well debated within the literature. Initially, it was thought that β-arrestins and G-protein coupled receptor kinases were limited to receptor desensitisation, internalization and recycling (Whistler and von Zastrow, 1998). It was later hypothesised that b-arrestin2 downstream signalling could mediate opioid induced respiratory depression, independent of G proteins (Reiter et al., 2012). However, this hypothesis has mostly been disproved. Transgenic mice with the inability to recruit β-arrestin2 via GRK phosphorylation still showed respiratory depression to morphine and fentanyl (Kliewer et al., 2019). Furthermore, it was also shown that b-arrestin2 knockout mice still showed morphine and fentanyl induced respiratory depression (Kliewer et al., 2020). Both studies contradict the validity of the b-arrestin2 hypothesis and indicate that opioid induced respiratory depression is b-arrestin2 independent. Significant evidence has been collated to support the hypothesis that respiratory depression occurs via the downstream effect of G_i protein activation of GIRK activation (see Chapter 1.4.1), to induce hyperpolarization. Mice that lacked GIRK2, a subunit of neuronal GIRK channels, were hypothesized to lack GIRK activity. These mice did not show DAMGO induced respiratory depression, indicating that GIRK conductance plays a vital role in opioid induced respiratory depression (Montandon et al., 2016).

1.7 AMPA Receptors in Respiratory Control

Naloxone is currently used by emergency personal and first responders as the reversal agent of choice in opioid induced respiratory depression and overdose (Wheeler et al., 2015). It is a competitive MOPr antagonist, causing rapid reversal of heroin induced respiratory depression when administered intravenously or intramuscularly (Skolnick, 2018). Naloxone can cause sudden-onset acute withdrawal syndrome; negatively impacting users who may display aggression after its administration and may also prevent them from seeking further medical aid (Schnur, 1991). Furthermore, this withdrawal syndrome can potentiate the urge for users to abuse opioids again in order to inhibit the unpleasant side effects of withdrawal syndromes, which in turn makes a future overdose more likely (Nowak et al., 1984; Sun, 1998). In cases where overdoses are caused by a combination of heroin and fentanyl, naloxone has been shown to not be as effective in reversing fentanyl induced respiratory depression in comparison to respiratory depression caused by heroin (Fairbairn,

Coffin and Walley, 2017; Hill et al., 2020). This shows the importance and the necessity for new and more effective overdose treatments. One potential approach is utilizing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) targeting ligands.

The excitatory synaptic transmission mechanism within the brain is largely due to glutamate neurotransmission. The three recognized glutamate-gated ion channels include the N-methyl-D-aspartate receptor (NMDA), kainite and AMPAr. AMPAr are tetrameric cation influx channels (Blanke and VanDongen, 2008). Activation of the AMPAr allows for some postsynaptic depolarization; removing the magnesium block of NMDA receptors to allow for NMDA activation and further postsynaptic depolarization will induce neuronal firing (Nowak et al., 1984).

AMPArs have been shown to influence the respiratory network. Local application in the pre-Bötzinger complex of an AMPAr antagonist blocked inspiratory drive. AMPA, an AMPAr agonist, induces a depolarizing current in neurons within the pre-Bötzinger complex (Ge and Feldman, 1998). AMPAkines bind allosterically to AMPAr to increase the open probability of this ionotropic receptor and thus increase its activity. In vitro, both brainstem-spinal cord and medullary slices from perinatal rat pups were bathed in the MOPr agonist Tyr-D-Ala-Gly-Me-Phe-Gly-ol-enkephalin (DAGO) to suppress neuronal output. The administration of the AMPAkine, CX546 into the bath significantly increased neuronal output of both the brainstem-spinal cord and medullary slices, suggesting an increase in rhythmic generation frequency (Ren et al., 2006). Within the same study, CX546 was shown to reverse fentanyl induced respiratory depression in vivo. A supporting study showed that alfentanil-induced respiratory depression was also reversed with application of another AMPAkine CX717 in humans. CX717 did not affect the analgesic effects of alfentanil (Oertel et al., 2010). The reversal of analgesia by naloxone is common within clinical settings (Gan et al., 1997). Postoperative titrating doses of opioids can lead to severe respiratory depression and although a MOPr antagonist (such as naloxone) would reverse this, it would also reverse the analgesic effects (Sofia and Harakal, 1975). A non MOPr targeting AMPAkine could prove useful in reversing respiratory depression whilst maintaining the opioid's analgesic properties.

1.8 Ketamine

Ketamine is a racemic mixture consisting of (S)- and (R)- enantiomers with mainly NMDA antagonist activity (Clements et al., 1982). It is most commonly known for its dissociative anaesthetic properties (Dundee et al., 1970). Aside from this, as early as the 1970s,

ketamine was suggested to have antidepressant effects (Sofia and Harakal, 1975), and more recently in 2018, a nasal spray of (S)-ketamine, was approved for the treatment of depression by the Food and Drug Administration (FDA) (Canuso et al., 2018). In contrast to many opioids, ketamine has not been shown to cause clinically significant respiratory depressive effects (Zanos et al., 2016).

It has been well documented that ketamine undergoes hepatic metabolism through N-demethylation to (R,S)-norketamine via cytochrome P450 enzymes CYP2B6 and CYP3A4 (Kharasch and Labroo, 1992; Yanagihara et al., 2001; Desta et al., 2012). CYP2B6 demethylates both enantiomers in a near equal efficacious manner and CYP3A4 has a (S)-ketamine bias (Portmann et al., 2010).(R,S)-norketamine is rapidly metabolised into (2R,6R;2S:6S)-hydroxynorketamine and dehydronorketamine. With the hydroxynorketamine metabolites being active agonists at the AMPAR. Reportedly (2R,6R)-hydroxynorketamine does not produce dissociative effects (Zanos et al., 2016). Furthermore, (2R,6R)-hydroxynorketamine but not (2S,6S)-hydroxynorketamine, has been shown to have large amplitude mediated bursts within hippocampal CA3, so is thought to have more therapeutic potential (Chen et al., 2020).

1.9 Tianeptine and potential respiratory control

Tianeptine is an atypical antidepressant with evidence to suggest that it acts both as a full MOPr agonist and as a positive allosteric modulator at the AMPAr (Labrid et al., 1988). As previously described, AMPAkines have potential for stimulating opioid induced respiratory depression (Ren et al., 2006). Tianeptine, although not strictly an AMPAkine, has the same effect of potentiating AMPA activity (Szegedi et al., 2011). As such, it could be clinically useful as an analgesic agent without the risk of significant respiratory depression as seen in traditional opioid treatment (Paul et al., 1990).

2.0 Materials and Methods

2.1 Animals

Male CD-1 mice (Charles River Laboratories, UK), with an average weight of ~30 g were used. Upon arrival, they were housed for a minimum of 5 days in social groups of 4 prior to any experimental procedures being carried out. The exception was where fighting occurred between the animals when the decision was made to singly house them. Cage temperature was kept between 19-23°C at a relative humidity of 45-65%. Mice were provided with sawdust, bedding, and a cardboard tube for enrichment. A reverse light cycle was used due to the nocturnal nature of the animals, with the dark period running from 8:15am to 8:15pm. Light periods had a light intensity between 350-400 lux (at bench level). All procedures were carried out during the dark cycle under red light to ensure that the mice were in their active phase during the experiments.

All procedures were performed in accordance with the UK Animals Scientific Procedures Act 1986, the European Communities Council Directive (86/609/EEC) and the University of Bristol ethical review document.

2.2 Drugs

Heroin (diacetyl morphine hydrochloride; NIDA), 6-MAM in acetonitrile solution (6-monoacetylmorphine; NIDA) buprenorphine hydrochloride (Tocris, UK), methadone hydrochloride (Sigma Aldrich, UK), fentanyl citrate (Sigma Aldrich, UK), ketamine hydrochloride (Sigma Aldrich, UK),(2R,6R)-hydronorketamine (Tocris,UK) and tianeptine (a gift from Professor Emma Robinson, University of Bristol) were all dissolved in sterile saline for challenge i.p. doses and tail vein injection experiments. The heroin that was used in osmotic mini pumps for prolonged administration was dissolved in distilled water and sonicated to ensure that it had fully dissolved.

2.3 Drug injections

2.3.1 Intraperitoneal injections

Mice were scruffed at the base of the neck and a 27-gauge needle was used to inject 0.1ml of drug solution in the ventral-caudal quadrant of the body. Where multiple intraperitoneal

injections (i.p. injections) were required they were carried out on alternating sides of the intraperitoneal space. This prevented bruising, stress and haemorrhaging.

2.3.2 Tail vein Injections

The tail (caudal) vein was used for intravenous injections (i.v.). Mice were restrained in a clear restraining tube where xylene was swabbed onto the tail to promote vasodilation. A tail vein illuminator (Braintree Scientific, Massachusetts) was placed beneath the tail to highlight the vein. A 29-gauge needle was then used to inject into the caudal vein. All i.v. injections were carried out with 0.1ml drug solution, apart from 6-MAM and its respective control which used 0.2ml due to low available stock solution concentrations of 6-MAM.

2.4 Measuring Respiration in Unrestrained Mice

Unrestrained whole-body plethysmography was used to measure the respiration of mice. Four plethysmography chambers (EMKA Technologie, France) with differential pressure transducers, temperature and humidity controllers were used. A mass flow controller was set to deliver 0.5L/min of a dry 95% air plus 5% CO_2 gas mixture (BOC Industrial Gases, UK). This gas mixture prevented the mice from falling asleep. The level of CO_2 does not cause stress to the mice (Hill et al., 2016). Corticosterone is secreted in mice in under stressful conditions, this may have an impact on respiration (Barlow et al., 1975). Figure 2.1 obtained from Rob Hill shows that a 5% CO_2 in air mixture does not cause significant levels of elevated plasma corticosterone. Furthermore, it has been suggested that concentrations of CO_2, less than 8% does not induce a stress response and thus minimises the effect on respiration (Johnson et al., 2013). The chambers were calibrated according to EMKA guidelines for mice. The pressure transducers were set to detect mouse inspiration and expiration as negative and positive pressure changes, respectively. This was achieved by injecting 1ml of air with a syringe into each chamber.

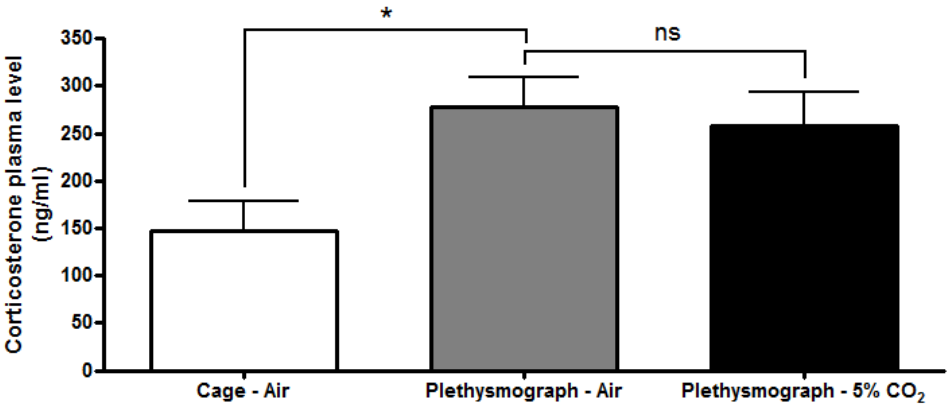

Figure 2.1 The effects of air, CO_2 or home cage treatment on plasma corticosterone levels in mice. Blood samples collected from truncated mice (n=8) were collected and analysed to measure corticosterone levels. Mice breathing air and mice breathing an air to 5% CO_2 in air mixture showed now significant difference in corticosterone levels. Both plethysmography chamber animals had significantly higher levels of corticosterone plasma levels when compared to mice kept in their home cage breathing air. Groups compared using an unpaired two tailed Student's T-test. *p<0.05. Figure obtained from Rob Hill.

In order to prevent stress from the mice being in a novel confined space, mice were habituated to the chambers 24 hours before the day of an experiment. During habituation, mice breathed room air at a pressure of 0.5 bar and a four-way air pump delivered room air to these chambers at identical rates. Each mouse was placed in the same chamber during habituation and on the day of experiments for consistency in measurements. Whilst in the chambers, mice had unrestricted access to drinking water and were in the chambers for a maximum of 1 hour to prevent their airways from drying out. Between recordings each chamber was cleaned and dried to remove faeces and urine in order to prevent any undue stress to the next mouse going into the chamber.

In the Results section of this thesis respiration is represented as minute volume; a composite measurement calculated by the multiplication of tidal volume and respiratory rate with humidity and temperature being considered in real-time. This was calculated in iox2 (EMKA Technologie, France). Tidal volume is the volume of air inspired during a single breath and respiratory rate is the number of complete inspiration-expiration cycles per minute. The minute volume following i.p. and i.v. injections were measured by averaging data from each inspiration expiration cycle into 5-minute and 1-minute intervals respectively. On the day of experiments, mice were placed into their respective chambers for 20 minutes to record baseline minute volume. Challenge doses of drugs were injected i.p. or i.v. within a 5-minute

window and then the mice were placed back into their chambers and respiration measured for a further 30 minutes.

2.5 Induction of Opioid Tolerance and the Redevelopment of Tolerance Following a Period of Abstinence/Detox

2.5.1 Surgical implantation of osmotic mini-pumps for prolonged opioid administration

For prolonged opioid treatment osmotic mini-pumps 2001D (ALZET®) were used which have a reservoir of 200µL and a flow rate of 1µL/hr; enough to deliver drugs for 7 days. Mice were anaesthetised using 3% isoflurane in oxygen within an anaesthetic box. After they had lost consciousness and their righting reflex, they were then placed headfirst into a nose cone to provide further anaesthetic. A scavenging unit collected excess anaesthetic gas. The tail and feet of mice were pinched to determine whether they had lost their nociceptive reflexes. Once nociceptive reflexes had been lost, the fur on the back at the base of the neck was shaved to expose the skin. A 1 cm incision in the skin was made at the base of the neck and straight forceps were used to form a subcutaneous pocket on the dorsal flank. The pump was then inserted with the site of drug delivery facing caudally. Incisions were closed using Clay Adams 9mm wound clips (VetTech Solutions LTD) and were treated with veterinary wound powder (Battles). Anaesthetic administration was terminated, and mice were monitored in a recovery cage alone until natural exploratory behaviour and righting reflexes had returned. They were subsequently returned to their home cages and were reviewed 24 hours post-surgery; if deemed necessary (inflammation, showing signs of scratching) opioid free wound powder was re-applied at this stage to prevent infection.

2.5.2 Initial induction of tolerance in naïve mice.

A previous study using similar osmotic mini-pumps containing methadone showed that pre-injections before pump implantation would enhance the development of tolerance (Quillinan et al., 2011) In the present experiments, for the prolonged pre-treatment of heroin, mice were injected i.p. 3 times (12 hr intervals) with 100mg/kg heroin to progress the induction of tolerance. After the third injection, a mini-osmotic pump was implanted. Heroin was dissolved in distilled water at a concentration of 56.35mg/ml to deliver 45mg/kg/day (see Figure 2.2 for previous work done within the lab). Saline solution pre-treated mice were used as the control group. Groups receiving methadone prolonged treatment received 3 injections

12 hours apart (5mg/kg, 7.5mg/kg and 7.5mg/kg) prior to pump implantation. The pump was filled with 75mg/ml methadone in saline solution to deliver 60mg/kg/day. Buprenorphine prolonged treatment groups received 3 saline injections 12 hours apart prior to the implantation of pumps containing a buprenorphine in saline solution concentration of 6.25mg/ml to deliver 5mg/kg/day. Mice with buprenorphine pumps were treated with saline as it has been previously been reported that priming the mice with buprenorphine injections had no significant impact on the induction of tolerance (Quillinan et al., 2011).

All pumps were left in place after surgical implantation to administer drug for 6 days. After 6 days a challenge dose of 10mg/kg heroin i.p. was injected and respiration was measured as previously described, to assess the presence of an initial significant degree of tolerance. This part of the experiment is portrayed in yellow in Figure 2.3.

2.5.3 Abstinence period and the redevelopment of tolerance in prolonged opioid-treated mice.

After the challenge dose to assess that the initial onset of tolerance was administered, the pumps were removed under general anaesthesia (3% isoflurane inhalation). Another incision was created more caudally than the initial incision and the pump was then pushed through

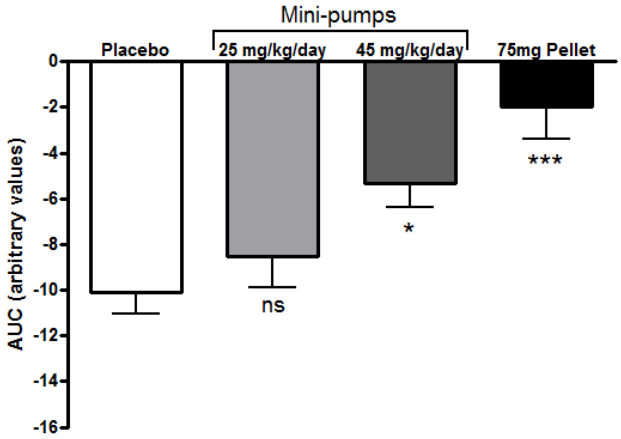

Figure 2.2: Levels of tolerance to respiratory depression during a 6-day prolonged morphine pre-treatment. *Mice were implanted with either a placebo pellet, a 75mg morphine pellet, or osmotic mini-pumps (delivering 25 or 45 mg/kg/day of morphine). AUC analysis showed that only a 45mg/kg/day mini-pump and 75mg pellet showed a significant level of tolerance. Groups were compared using a one-way ANOVA with Bonferroni's multiple comparisons *P<0.05 vs control ***P<0.001 vs control n=6-8 Figure from Rob Hill (University of Bristol).*

this incision. The wound was then closed in the same way as described following implantation. Pumps were then visually inspected to see whether the appropriate level of drug had been administered, by measuring the remaining volume left in the pumps. The mice were checked 24 hours after pump removal and were left in their home cage for 5 days for an abstinence period, shown in orange in Figure 2.3.

After 5 days of detox, a twice daily injection protocol was used to examine the redevelopment of tolerance lost during the abstinence period by administering 10mg/kg

heroin i.p., represented in Figure 2.3 in green. On the first day of the protocol, respiration was measured after the first dose was administered in the morning. This was followed by an evening injection which was given after 9 hours. During the next 4 days, morning and evening doses were given but respiration was measured only following the evening dose for 5 days to study the re-development of tolerance to respiratory depression.

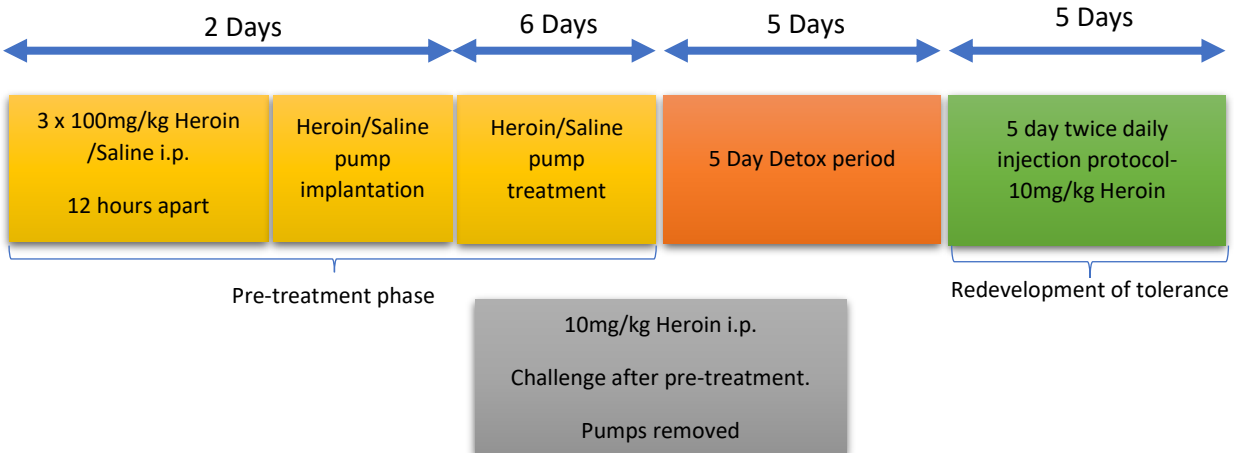

Figure 2.3: Timeline of the induction and redevelopment of heroin tolerance after a period of abstinence. *Each colour represents different experimental phases of this study.*

2.6 Measuring Acute Antinociception

A water bath set to 52±0.5°C was used to induce a thermal nociceptive response whilst minimising supraspinal influence. Mice were scruffed and the tip of their tail placed 1.5cm under the surface of the water. As the tail was placed in the water a stopwatch was started, once the tail flick response occurred, the stopwatch was stopped. A 15 second maximum cut-off was used to prevent thermal damage to the tails of the mice. Nociception data are presented both as tail flick latency (seconds) and as percentage of the maximum possible effect (%MPE). %MPE was calculated using the following equation:

$$\%MPE = \frac{(Post\ Drug\ latency - Baseline\ latency)}{(Cut - off\ latency)} X\ 100$$

Mice had their baseline tail flick latency measured and then were placed into the plethysmography chambers. 10 minutes later they were injected with either naloxone 0.3mg/kg or saline i.p. and again returned to their respective chambers. After a further 10 minutes, they were injected with tianeptine within a 5-minute window before being placed back into their chambers. Respiration was then measured for a further 20 minutes. At 20 minutes, tail flick latency was re-measured. This timeline is illustrated in Figure 2.4.

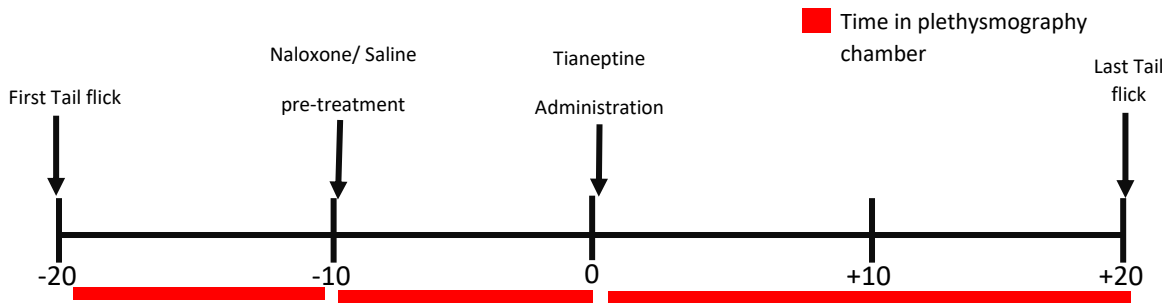

Figure 2.4: Timeline of Tail Flick experiment with drug pre-treatment whilst measuring respiration. *Measurement of the initial tail flick before naloxone pre-treatment allows for analysis of tail flick in response to tianeptine after pre-treatment of naloxone and saline.*

2.7 Data Analysis

All experiments were performed and analysed blind to prevent experimenter bias from influencing the data. Acute opioid effects, attempting to reverse opioid induced respiratory depression and nociception experiments had an n=6. The redevelopment of tolerance experiments had an n=9 and tail vein experiments had an n=12. All experimental techniques used in this thesis had been extensively used previously within our laboratory. This generated a large sample of data for power analysis in order to calculate an appropriate n number for initial experiments. Power analysis was carried out using G*Power version 3.1.9. After preliminary experiments, the data that was collected by myself was then used to recalculate the power analysis to accurately power the experiments based on the technical ability of the individual over the technical ability of previous experimenters within the lab. Raw data were normalised to show response as a percentage of baseline, with baseline being set to 100%. This was deemed appropriate due to the variation between animals within a group and between groups of animals across the experimental period.

There is a possibility that mice that exhibit a lower or higher baseline respiration would respectively exhibit decreased or increased response to opioid induced respiratory depression, explained by a potential “ceiling” effect on the mechanism. This would produce skewed data as mice with a higher baseline minute volume would appear to show a greater

reduction and therefore more profound respiratory depression and vice versa. Mice would have to be screened prior to their use in experiments to select mice with similar baselines. This would encourage the use and termination of more mice.

In order to investigate possible skew due to a possible baseline artefact and to reduce the number of animals used in screening, previous work undertaken by Rob Hill whilst at the University of Bristol was reviewed (Figure 2.5). This work shows that when mice were injected with 10mg/kg morphine, both grouped mice (a) and the individual mice within each of these groups (b) had shown no correlation between baseline MV and maximum depression of respiration. Raw data is still presented in Chapter 3, to support a fuller

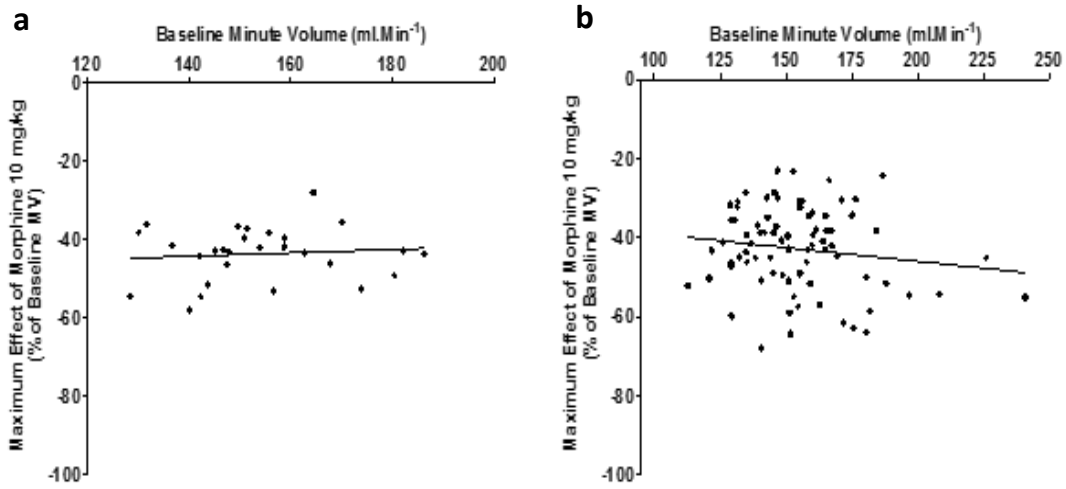

Figure 2.5: The initial baseline of MV does not correlate with the maximum depression of respiration by morphine. *MV baseline was plotted against maximum effect.* ***(a)*** *shows this data from grouped mice and* ***(b)*** *shows this for each individual mouse. A linear regression was calculated and a correlation co-efficient of* R^2*=0.009 and* R^2*=0.023 was found for* ***(a)*** *and* ***(b)*** *respectively. No correlation between baseline MV and maximum depression of respiration was seen in this data. Figure obtained from Rob Hill.*

understanding of the data collected.

The raw baseline response did not include the first data point (at 5 minutes) during the initial time interval, as mice were usually getting accustomed to being handled and moved from their home cage to the plethysmography chamber in this initial period. The data from the next 3-time intervals were then averaged to give a raw baseline value. Each raw data point after drug administration was then divided by this baseline value and then multiplied by 100 to produce a set of normalised data, represented as a percentage of baseline.

To analyse the overall effect of a drug on MV/TV/RR, the area under the curve (AUC) was calculated in GraphPad Prism 8 using the normalised percentage of baseline over time. This allowed for the quantification of the effect of respiration over the duration of the experiment.

This was calculated by setting the baseline across the response to 100% of baseline MV/TV/RR. The following equation was used:

AUC= Time (measured as minutes post drug) X Percentage Change in Baseline respiration

The AUC was calculated for each individual mouse to then be used to calculate a mean and standard error. This would remove a level of variance within a group of mice.

A single exponential was chosen in order to give an estimate $t_{1/2}$ (half life) representing the rate of onset were calculated from the one phase decay function for each individual mouse and then averaged to give a mean t1/2 and standard error of the mean. A biexponential did not improve the fit of the curve for heroin, 6-MAM and fentanyl.

To calculate the predictive summative effect of heroin fentanyl mixtures, individual heroin and fentanyl responses at each one-minute time interval were summated together and plotted.

A one-way ANOVA was used to compare multiple groups to different doses of drugs as drug dose was the only variable in the experiment such as when analysing area under the curve. Bonferroni's multiple comparisons was used to correct for normally distributed data, where data were not normally distributed, Dunnett's analysis was used instead. A two-way ANOVA was used to compare two or more groups of mice with two independent variables with Bonferroni multiple comparisons used (used in ketamine and tianeptine experiments in Chapter 4 and comparisons of baseline measurements in Chapter 5). The presence and absence of a drug acts as two levels of variance. GraphPad Prism 8 (GraphPad software, San Diego, USA) was used for all statistical analyses.

3.0 Acute Effects of Heroin and Fentanyl on Respiration

3.1 Introduction

Heroin and fentanyl both act on the MOPr, which is involved in the regulation of breathing, pain and euphoria These drugs are often abused for their euphoria inducing effects. However, both heroin and fentanyl can provoke respiratory depression which is the most common cause of death in opioid overdoses. It is widely accepted that fentanyl is almost 50 times more potent than heroin (Poklis, 1995; Scholz et al., 1996). Drug users often unknowingly buy heroin laced with fentanyl as the addition of fentanyl creates the illusion that the heroin is of a higher quality. As such, 45.9% of all opioid related deaths involve synthetic opioids (mainly fentanyl), with a large majority of users choosing to inject intravenously. Opioids may act on the same receptors, however, they do not all produce identical observable effects. They differ in their potency and rate of onset.

3.1.1 Chapter aims

The aims of this chapter are:

i. To characterise the potency of heroin and fentanyl in depressing respiration.
ii. Compare and characterise the differences between intraperitoneal (i.p.) injections of heroin and fentanyl and intravenous (i.v.) injections.
iii. To investigate whether heroin and fentanyl mixtures have an additive response composed of heroin and fentanyl's individual responses or whether a synergistic potentiation of respiratory depression occurs.

3.2 Results

3.2.1 The effect of intraperitoneally administered Heroin on respiration

Bolus single doses of diamorphine hydrochloride (heroin) were administered i.p. to groups of CD1 mice breathing a mixture of 5% CO_2 and 95% air. Respiratory function (measured by minute volume (MV, min/ml)) was calculated prior to and after heroin administration**.** The results showed that increases in the dose of heroin administered (in increments between 1-100mg/kg) correlated with increasing levels of respiratory depression; illustrated by decreasing MV (Figure 3.1a).

Natural variability in baseline respiratory function between each group and between individual mice within groups makes direct comparisons between groups difficult. In order to minimize this effect, individual mice had their post-injection respiratory parameters expressed as a percentage of their pre-injection baselines. This is demonstrated in Figure 3.1b which further reiterates that higher doses of intraperitoneal heroin induce more profound respiratory depression. Saline had no significant effect on respiration. In groups injected with 1-10mg/kg heroin, respiratory depression begins to recover from 20 minutes post-injection. However, in the group injected with 100mg/kg, in the time measured the respiratory function remained almost static at around 40% of baseline.

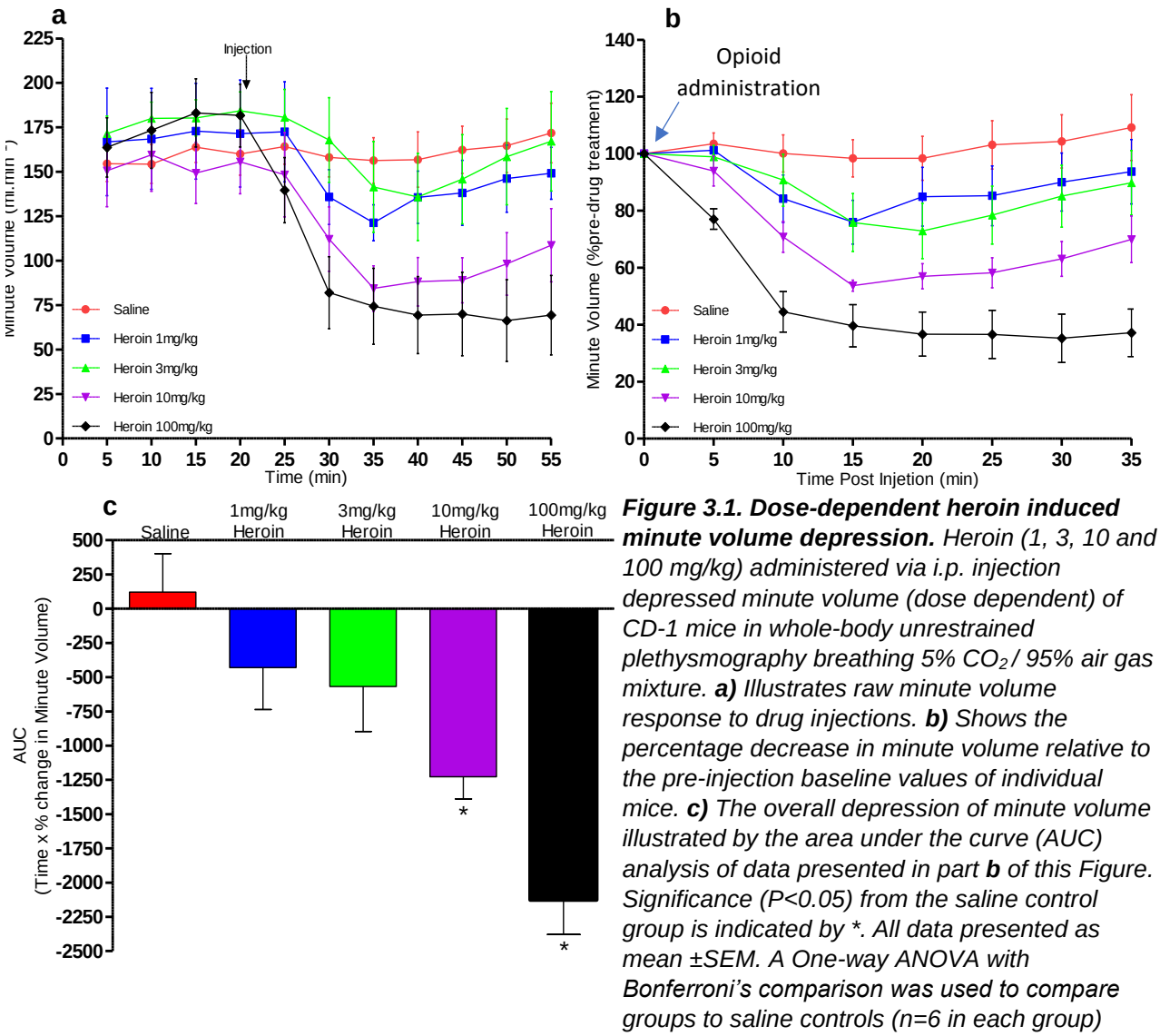

Figure 3.1. Dose-dependent heroin induced minute volume depression. *Heroin (1, 3, 10 and 100 mg/kg) administered via i.p. injection depressed minute volume (dose dependent) of CD-1 mice in whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture.* ***a)*** *Illustrates raw minute volume response to drug injections.* ***b)*** *Shows the percentage decrease in minute volume relative to the pre-injection baseline values of individual mice.* ***c)*** *The overall depression of minute volume illustrated by the area under the curve (AUC) analysis of data presented in part* ***b*** *of this Figure. Significance (P<0.05) from the saline control group is indicated by *. All data presented as mean ±SEM. A One-way ANOVA with Bonferroni's comparison was used to compare groups to saline controls (n=6 in each group)*

The AUC calculated from the data in Figure 3.1b is presented in Figure 3.1c. Here we see that in groups administered 10-100mg/kg heroin there is a significant decrease in MV relative to the saline control. Doses of 1-3 mg/kg heroin did not produce an AUC significantly

different from the saline control. Thus, a dose of 10mg/kg heroin was chosen to be used in further experiments where a response from i.p. heroin is required.

MV is a composite measure calculated from TV and RR. The effect of heroin on each of these factors separately is illustrated in Figures 3.2 -3.3. The raw data suggests that 1-3mg/kg heroin causes a slight increase in TV whereas 10mg/kg heroin appears to cause a slight decrease in TV (Figure 3.2a). Administration of 100mg/kg heroin clearly demonstrates a decrease in TV. Figure 3.2b uses normalised data where variable baselines have been removed to compare the percentage change in TV pre- and post-injection. The AUC in all of the heroin-injected groups were not significantly different compared to saline controlled mice

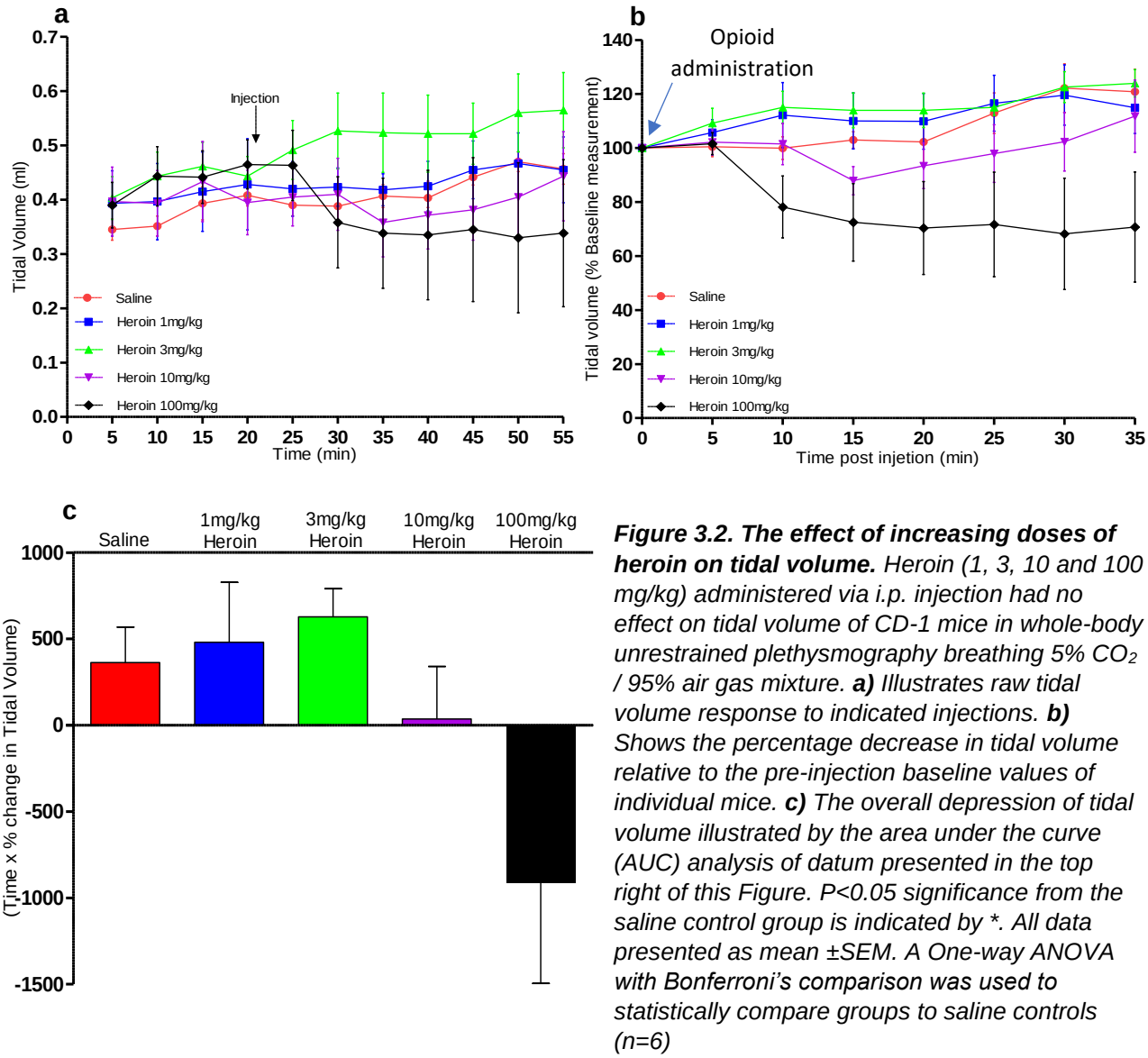

Figure 3.2. The effect of increasing doses of heroin on tidal volume. *Heroin (1, 3, 10 and 100 mg/kg) administered via i.p. injection had no effect on tidal volume of CD-1 mice in whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture.* ***a)*** *Illustrates raw tidal volume response to indicated injections.* ***b)*** *Shows the percentage decrease in tidal volume relative to the pre-injection baseline values of individual mice.* ***c)*** *The overall depression of tidal volume illustrated by the area under the curve (AUC) analysis of datum presented in the top right of this Figure. P<0.05 significance from the saline control group is indicated by *. All data presented as mean ±SEM. A One-way ANOVA with Bonferroni's comparison was used to statistically compare groups to saline controls (n=6)*

(Figure 3.2c). Heroin had no statistically significant effect on TV of mice at the doses used. Upon closer inspection on the distribution of individual mouse data, two mice had very low TV (9% of baseline) thus explaining the large SEM in heroin 100mg/kg. The raw data

presented in Figure 3.3a suggests that there is a decrease in RR in response to i.p. heroin administration. The decrease in RR occurs in a dose-dependent manner, as shown in Figure 3.3b, with higher doses of heroin causing a greater percentage decrease in baseline RR. Figure 3.3c reiterates that heroin induced respiratory depression is dose dependent, shown by a decrease in the AUC (increases in the negative direction). The AUC of mice injected with 3-100mg/kg is significant compared to the saline control. In measuring the effects of heroin on the composite factors of MV (RR and TV), we are able to deduce that heroin induced respiratory depression is mostly due to an effect of heroin on by RR and not TV.

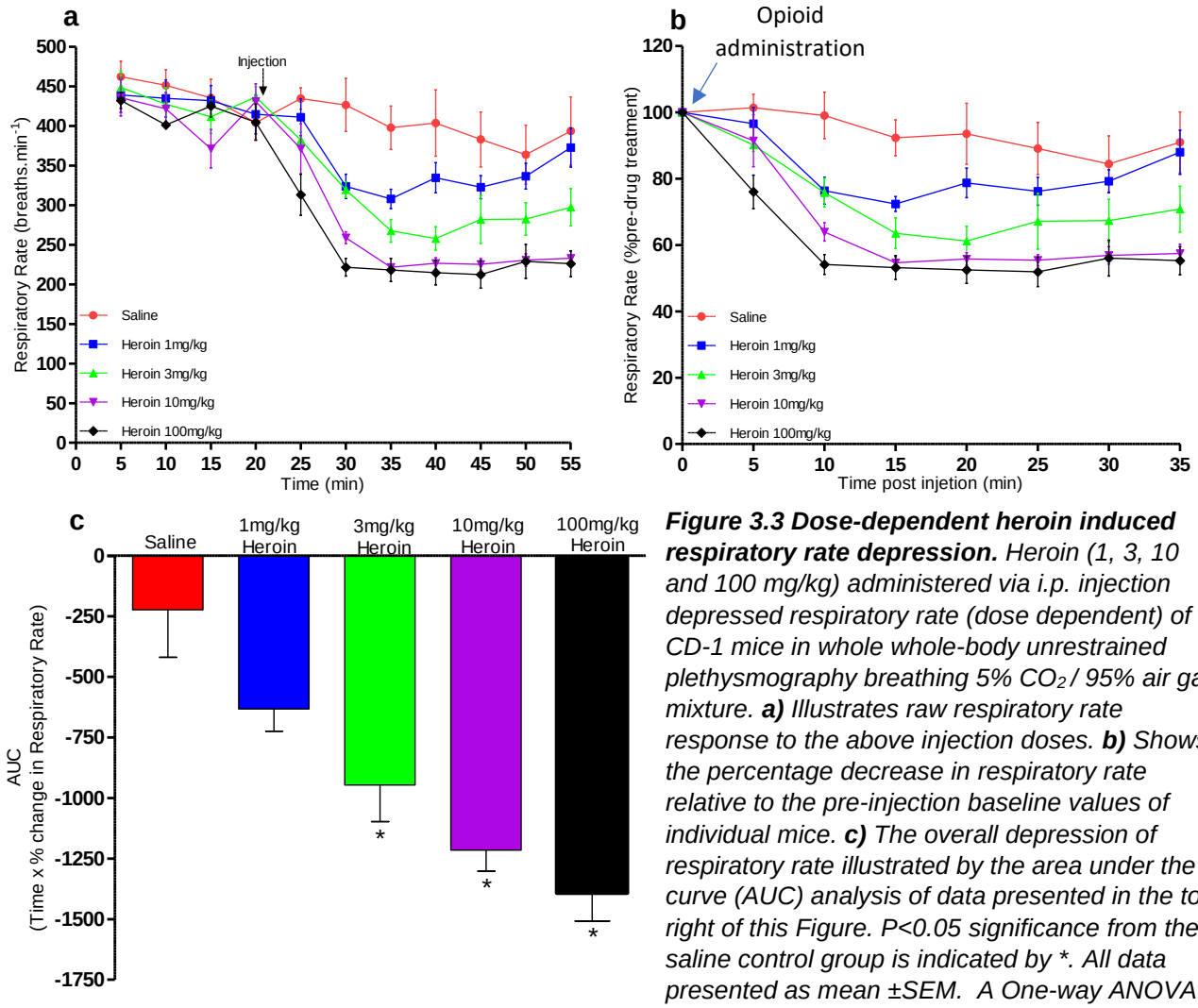

Figure 3.3 Dose-dependent heroin induced respiratory rate depression. *Heroin (1, 3, 10 and 100 mg/kg) administered via i.p. injection depressed respiratory rate (dose dependent) of CD-1 mice in whole whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture.* ***a)*** *Illustrates raw respiratory rate response to the above injection doses.* ***b)*** *Shows the percentage decrease in respiratory rate relative to the pre-injection baseline values of individual mice.* ***c)*** *The overall depression of respiratory rate illustrated by the area under the curve (AUC) analysis of data presented in the top right of this Figure. P<0.05 significance from the saline control group is indicated by *. All data presented as mean ±SEM. A One-way ANOVA with Bonferroni's comparison was used to statistically compare groups to saline controls (n=6)*

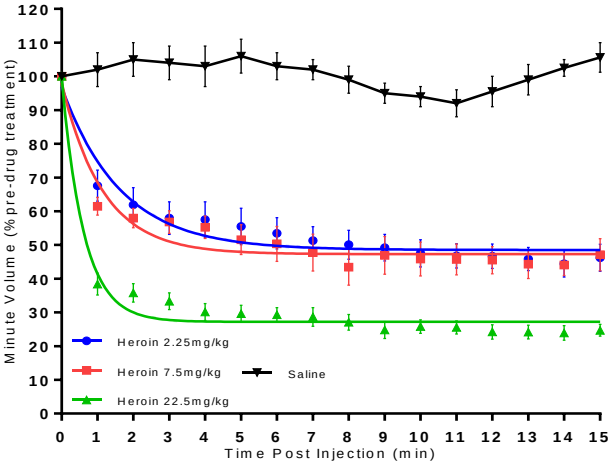

Figure 3.4. Dose dependent i.v. heroin induced minute volume depression. *Heroin (2.25, 7.5, 22.5 mg/kg) administered via i.v. injection depressed minute volume of CD-1 mice in whole whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture. (n=9) Saline data are reproduced from previous work in the lab by Dr Rob Hill. Responses have been fitted to a single exponential, R^2= 0.631, 0.629 and 0.898 respectively All data presented as mean ±SEM. All data presented as mean ±SEM (n=9) Saline data from previous work by Dr Rob Hill (n=12)*

3.2.2 The effect of intravenously (i.v.) administered Heroin on respiration

With i.p. administration the drug is administered into the peritoneal cavity from where it is absorbed into surrounding tissues via diffusion. Next the drug is distributed away from the peritoneal cavity, such as the liver where some first pass metabolism occurs before entering the systemic circulation (Turner et al., 2011). This step is absent in i.v. injections as the drug enters the systemic circulation immediately, bypassing metabolism that occurs in the liver, in theory allowing for a larger concentration of the injected drug to reach target tissues. This allows for the rate of onset of a response to be studied without being limited by rate determining steps such as absorption and metabolism. Experiments were therefore conducted with i.v. opioid administration via the lateral tail vein of CD1 mice and results compared to their effects when administered i.p. In measuring the response to intravenously injected opioids we may be able to draw conclusions which are useful to the study of opioid abuse in humans; most of which are often abused intravenously (Perekopskiy et al, 2019).

CD1 mice were restrained using a plastic restraining tube to access their tail vein as a point of administration. All three doses of heroin 2.25mg/kg, 7.5mg/kg and 22.5mg/kg significantly depressed respiratory function (Figure 3.4). The dose of 22.5mg/kg heroin depressed MV the most and doses of 7.5mg/kg and 2.25mg/kg heroin depressed MV to a similar extent with no significant difference between the two. When comparing Figure 3.3a and Figure 3.4, it is evident that the rate of onset of i.v. heroin administration is faster than that of i.p. heroin administration. The peak response in the i.v. experiments occurred between 4-6 minutes post administration versus after 15-20 minutes for i.p administration as seen in Figure 3.3b. These experiments also show that heroin is more potent when administered i.v. than i.p. An i.p. dose 4.4-fold higher than the i.v dose produced a smaller response; 100mg/kg i.p. heroin

reduced MV to 36% of pre-drug levels, whereas 22.5mg/kg i.v. heroin reduced MV to 29% of pre-drug levels. A single exponential function was used to fit a curve for i.v. administration of heroin in order to estimate the rate of onset of these drugs. A biexponential did not improve the curve fitting for any of the data points. The rapid onset of action gives $t_{1/2}$ values that are shorter than the sampling interval of 1 minute and so are only estimates. By shortening sampling intervals, the resolution of the data would increase and thus a more accurate estimate could be produced.

The effect of the 6-MAM on respiratory depression is shown in Figure 3.5. A 6-MAM 6.66mg/kg i.v. dose in acetonitrile produced a response similar to that of 22.5mg/kg heroin. Acetonitrile, the vehicle for 6-MAM, produced a small decrease in MV relative to that seen with 6-MAM administration. Therefore, the potency of 6-MAM can not be accurately compared to that of heroin. There was no significant difference in the rate of onset between heroin and 6-MAM portrayed by the $t_{1/2}$ calculations in Table 3.1.

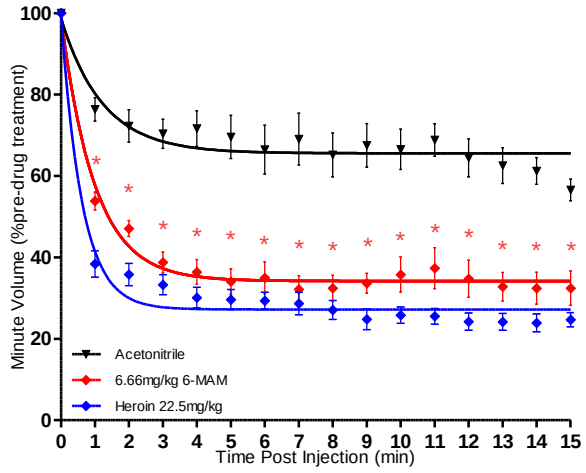

Figure 3.5. A comparison between i.v. 6-MAM and heroin induced minute volume depression. *6-MAM (6.66 mg/kg) administered via i.v. injection depressed minute volume, to almost the same extent as heroin (22.5mg/kg). A significant difference between acetonitrile control and 6-MAM indicated with *. All data presented as mean ±SEM. A two-way ANOVA with bonferonni's multiple comparisons was used to compare significance between groups. *P<0.05 (n=9)*

Drug	$t_{1/2}$ *(min)*
Heroin 22.5mg/kg	0.45±0.01
6-MAM 6.66mg/kg	0.75±0.13

Table 3.1: $t_{1/2}$ values calculated from one phase decay of heroin and 6-MAM induced respiratory depression from Figure 3.5. *There was no significant difference between half-lives. Half-lives were calculated from a single exponential fit. An unpaired two tailed t-test was used to compare groups. All data presented as mean ±SEM. *P<0.05 (n=9)*

3.2.3 The effect of intraperitoneally administered fentanyl on respiration

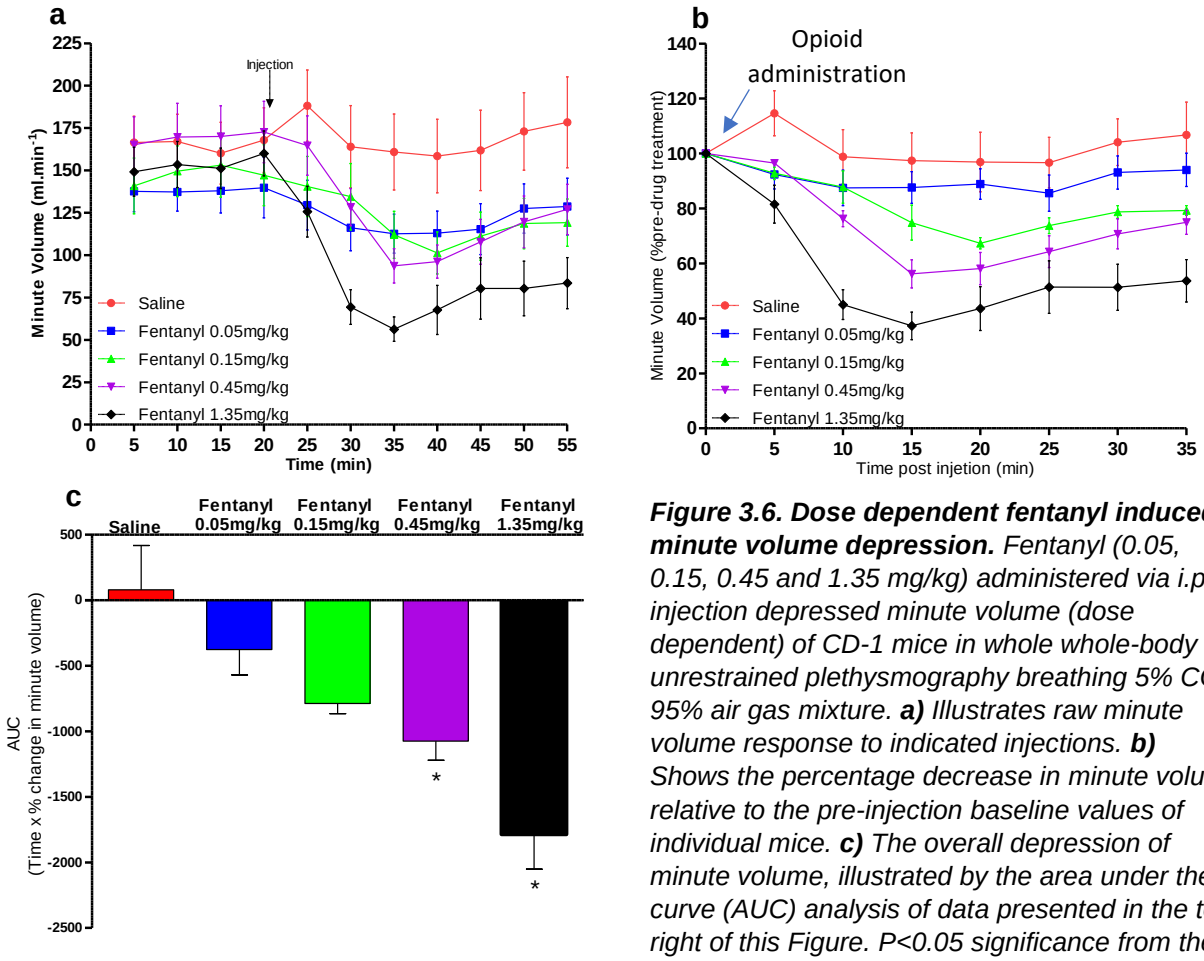

Figure 3.6. Dose dependent fentanyl induced minute volume depression. *Fentanyl (0.05, 0.15, 0.45 and 1.35 mg/kg) administered via i.p. injection depressed minute volume (dose dependent) of CD-1 mice in whole whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture.* ***a)*** *Illustrates raw minute volume response to indicated injections.* ***b)*** *Shows the percentage decrease in minute volume relative to the pre-injection baseline values of individual mice.* ***c)*** *The overall depression of minute volume, illustrated by the area under the curve (AUC) analysis of data presented in the top right of this Figure. P<0.05 significance from the saline control group is indicated by *. All data presented as mean ±SEM. A One-way ANOVA with Bonferroni's comparison was used to statistically compare groups (n=6)*

Bolus single i.p. doses of fentanyl were administered to mice. With increasing doses of fentanyl (0.05-1.35 mg/kg) leading to increasing levels of respiratory depression. Figure 3.6a demonstrates that MV decreases to a greater extent as fentanyl doses increase. This is supported by Figure 3.6c, which uses the AUC from the normalised values in Figure 3.6b of the percentage change in MV relative to the pre-injection baseline to illustrate the dose-response relationship between MV depression and fentanyl dose.

Simultaneously, TV was measured in the same mice. Figure 3.7a shows no depressive effect of fentanyl (0.05- 0.15 mg/kg) on TV, however 0.45mg/kg appears to decrease TV and 1.35mg/kg dose shows a larger depression of TV. When the data were normalized (Figure 3.7b), -1.35mg/kg shows clear depression of TV, peaking at 15 minutes post-injection. Using normalised results, Figure 3.7c shows that only the higher dose of 1.35mg/kg fentanyl significantly depressed TV in comparison to saline. The other component of MV, RR, was

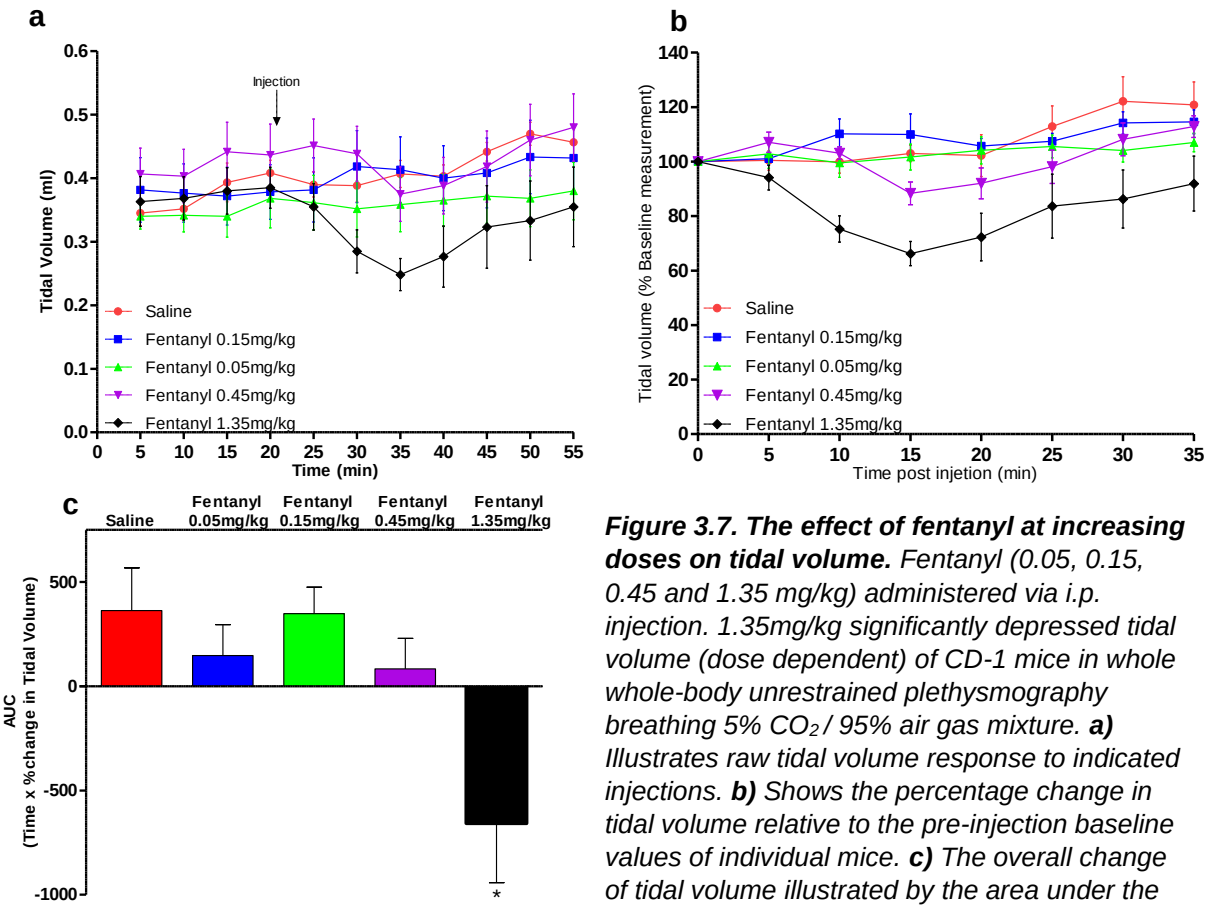

Figure 3.7. The effect of fentanyl at increasing doses on tidal volume. *Fentanyl (0.05, 0.15, 0.45 and 1.35 mg/kg) administered via i.p. injection. 1.35mg/kg significantly depressed tidal volume (dose dependent) of CD-1 mice in whole whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture.* ***a)*** *Illustrates raw tidal volume response to indicated injections.* ***b)*** *Shows the percentage change in tidal volume relative to the pre-injection baseline values of individual mice.* ***c)*** *The overall change of tidal volume illustrated by the area under the curve (AUC) analysis of data presented in (**b**). P<0.05 significance from the saline control group is indicated by *. All data presented as mean ±SEM. A One-way ANOVA with Bonferroni's comparison was used to statistically compare groups (n=6)*

also measured concurrently in the same mice. The raw data (Figure 3.8a) demonstrates that there is an increase in the depression of RR as fentanyl dose increases. When the data are measured as a percentage of their baseline measurement, the dose-dependent depressive effects on RR can be seen even more clearly. In Figure 3.8b, the effects of 0.05mg/kg fentanyl were similar to control and doses 0.15-1.35mg/kg caused RR depression. Figure 3.8c shows that as the fentanyl dose increases, AUC further decreases, implying further depression of RR. Doses of 0.15-1.35mg/kg of fentanyl were shown to have AUCs significantly different from controls.

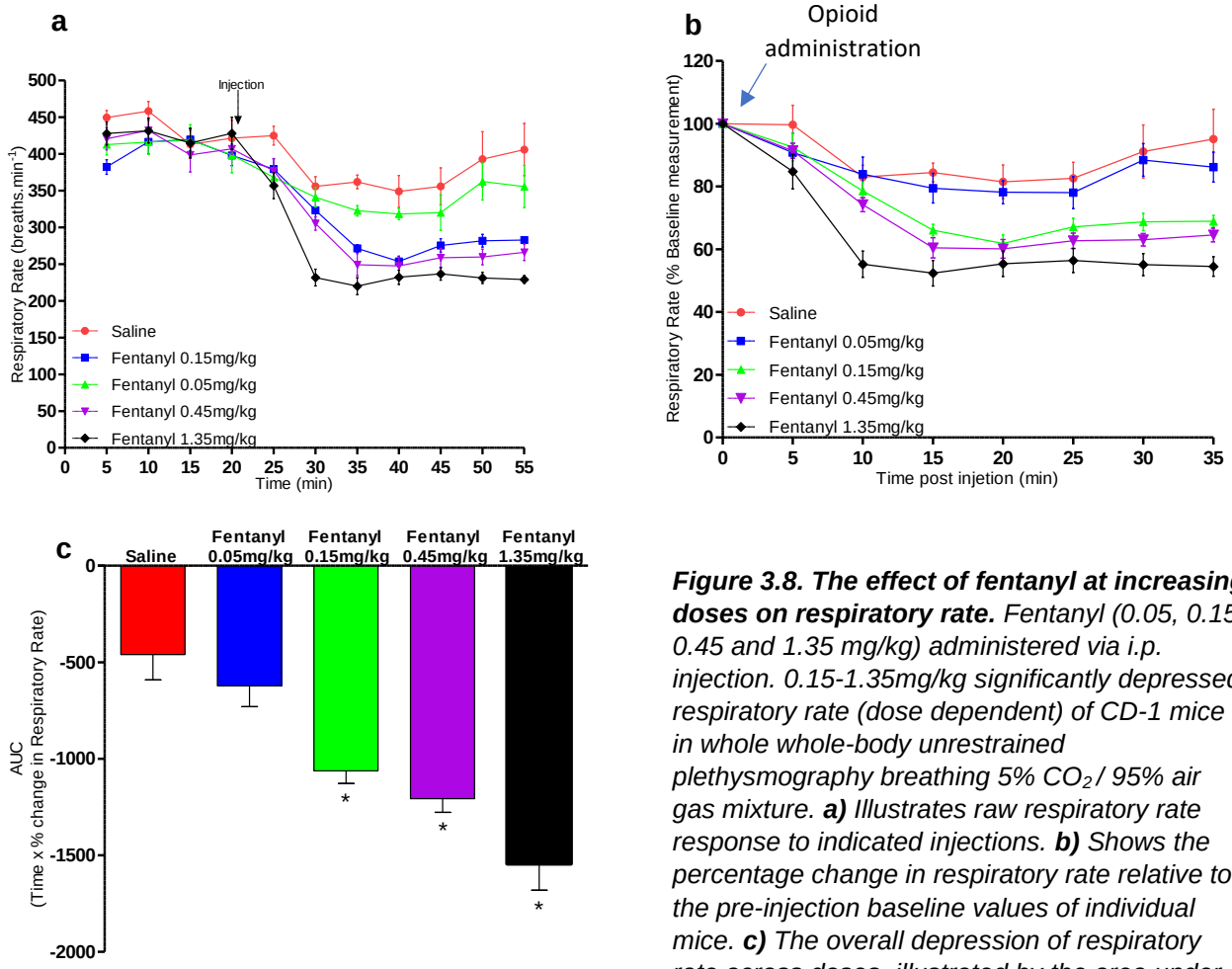

Figure 3.8. The effect of fentanyl at increasing doses on respiratory rate. *Fentanyl (0.05, 0.15, 0.45 and 1.35 mg/kg) administered via i.p. injection. 0.15-1.35mg/kg significantly depressed respiratory rate (dose dependent) of CD-1 mice in whole whole-body unrestrained plethysmography breathing 5% CO_2 / 95% air gas mixture.* ***a)*** *Illustrates raw respiratory rate response to indicated injections.* ***b)*** *Shows the percentage change in respiratory rate relative to the pre-injection baseline values of individual mice.* ***c)*** *The overall depression of respiratory rate across doses, illustrated by the area under the curve (AUC) analysis of datum presented in the top right of this Figure. P<0.05 significance from the saline control group is indicated by *. All data presented as mean ±SEM. A One-way ANOVA with Bonferroni's comparison was used to statistically compare groups (n=6)*

3.2.4 The effect of intravenously administered fentanyl on respiration

The fentanyl dose-dependent depression of MV can also be seen with i.v. fentanyl. This can be seen in Figure 3.9 where 0.3375mg/kg reduced MV to 32% of baseline, 0.1125mg/kg reduced MV to 53% of baseline and 0.0375mg/kg reduced MV to 59%. The peak effect of i.v. doses occurred more rapidly post injection relative to the equivalent injection of the i.p. dose. For example, 1.35mg/kg i.p. fentanyl produced its peak effect of 40% of baseline (Figure 3.6b), 10-15 minutes post injection. Whereas the equivalent i.v. dose of 0.3375mg/kg

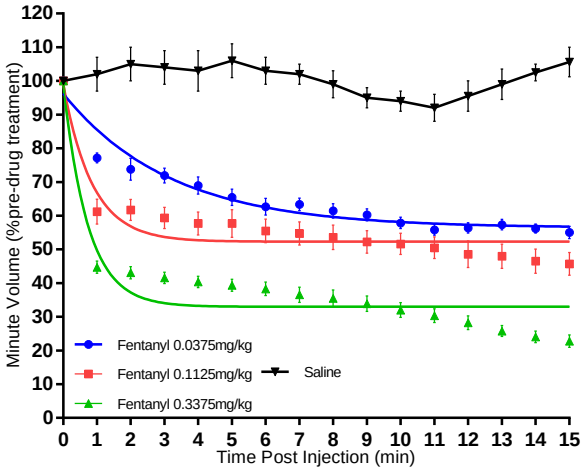

Figure 3.9. Dose dependent i.v. fentanyl induced minute rate depression. *Fentanyl (0.0375, 0.1125, 0.3375 mg/kg) administered via i.v. injection depressed minute volume (dose dependent) of CD-1 mice in whole whole-body unrestrained plethysmography breathing 5% CO_2/ 95% air gas mixture. Responses have been fitted to a single exponential. All data presented as mean ±SEM (n=9) Saline data from previous work by Dr Rob Hill (n=12)*

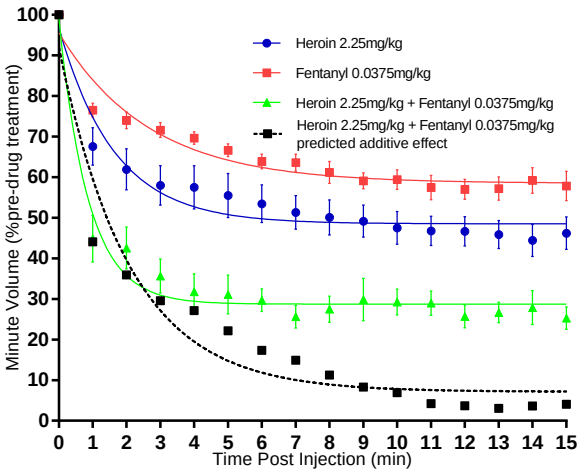

Figure 3.10. The effect of fentanyl and heroin mixtures on normalised minute volume. *Fentanyl 0.0375mg/kg + heroin 2.25mg/kg mixture administered via i.v. injection depressed minute volume to a pre-calculated sub additive effect in CD-1 mice during whole whole-body unrestrained plethysmography breathing 5% CO_2/ 95% air gas mixture. Responses have been fitted to a single exponential. All data presented as mean ±SEM (n=9)*

produced its peak effect of 35% of baseline, 5 minutes after administration. A dose of 1.35mg/kg i.p. fentanyl reduced MV to 40% of baseline, whereas a 4x lower i.v. dose of 0.3375mg/kg reduced MV to 36%. Similar to the results obtained from i.v. and i.p. heroin administration, from these experiments we find that fentanyl is at least 4-fold more potent and a 3-fold more rapid in onset when administered i.v versus i.p.

3.2.5 The effect of intravenous heroin and fentanyl mixtures on respiration

Figure 3.10 shows the data for individual drugs, heroin (2.25mg/kg) and fentanyl (0.0375mg/kg), taken from Figure 3.4 and Figure 3.6 respectively. Both cause a decrease in MV post injection. These low doses were chosen to form the mixture as a preventative overdose measure. To test for additivity, a predicted additive effect was calculated (see Chapter 2.7) which gave a predicted peak depression of MV of 8% of baseline (black fitted line in Figure 3.10. In the experimentally obtained drug mixture data the mixture only decreased MV to 30% of baseline, data points during the first 4 minutes post injection were not significantly different to the predicted additive effect, suggesting additivity during the onset of response. However, the data between 5-15 minutes post injection were significantly less than that predicted by summation, showing a lack of additivity. No true conclusions can be made due to the possibility of a ceiling effect at 30% of baseline.

3.3 Discussion

3.3.1 Heroin's effect on respiratory depression

The analysis of the present data showed that heroin induced a dose-dependent reduction of minute volume in mice. A dose response curve obtained from our data identified that 10mg/kg i.p. heroin was a sub-maximal dose with significant MV depression. Subsequently, this dose was chosen for further experiments as a suitable challenge dose to induce a significant level of respiratory depression in future experiments (See Chapters 4 & 5). The administration of heroin lead to a marked decrease in RR, which was the main driver of MV reduction, versus insignificant changes in TV. Previous experiments in the laboratory have shown that activation of the MOPr by morphine and oxycodone to induce respiratory depression is caused by a decrease in RR (Hill et al., 2016, 2018). Unilateral microinjection of the endogenous tetrapeptide endomorphin-2 (EM2) into the pre-Bötzinger complex decreased breathing frequency, but also amplitude (Qi et al., 2017). As mentioned in previous studies within the lab, tidal volume was maintained through the prolongation of inspiration via apneustic compensation. This is thought to occur due to an increase in the duration of inspiratory phase of breathing in order to compensate for a reduction of RR (Hill et al., 2016, 2018).

When we compare the data obtained in the experiments using i.p. and i.v heroin, it can be seen that the rate of onset of i.v. heroin depression of respiration is at least 3.5 fold more rapid than with i.p. administration (for the doses used). This is thought to be due to fact that i.p. injections first require absorption of the drug into the mesenteric vessels and through the hepatic portal vein into the liver, where it then undergoes first pass metabolism before entering systemic circulation, (Turner et al., 2011) finally reaching the respiratory centre in the brain. With i.v. administration, heroin bypasses absorption into the systemic system (Turner et al., 2011). When comparing the responses to each dose of heroin, at least a 4-fold lower dose of i.v. heroin is required to achieve the same level of respiratory depression as i.p. heroin. Due to the more rapid rate of onset via i.v. administration, a higher concentration of drug reaches the brain faster. The slower onset following i.p. administration prevents as high of a concentration being present in the brain tissue, due to metabolic activity and elimination methods such as fat sequestration (Ummenhofer et al., 2000). When the heroin metabolite, 6-MAM, was intravenously administered to mice, respiratory depression (MV) approached the values achieved with 22.5mg/kg heroin i.v. ,it should be noted, that acetonitrile induced respiratory depression, therefore the comparison of 6-MAM in acetonitrile and heroin in saline solution cannot be carried out. A wider range of doses of

6-MAM would have been used. However, only a maximum dose of 6.66mg/kg i.v. 6-MAM was available and due to time restrictions, the response to other doses were not explored (Andersen et al., 2009a). The literature proposes that the high lipophilicity of fentanyl is one of the main contributing factors to its relative potency (Scott et al., 1991). LogP values, used as a measure of drug lipophilicity, for both heroin and 6-MAM are near identical with logP values of 1.58 and 1.55 respectively (Avdeef et al., 1996). This implies that differences in lipophilicity does not explain the extent of the difference in potency of these two drugs. There is also evidence to show that heroin is rapidly enzymatically cleaved into 6-MAM in the blood, before crossing the blood brain barrier (Boix et al., 2013; Gottås et al., 2013). Furthermore, peak heroin concentrations found in the blood and in the brain after i.v. administration were orders of magnitude lower than 6-MAM (Gottås et al., 2013). Boix et al (2013) used a pharmacokinetic model of mice (subcutaneous administration) to show that the rate of heroin passing the blood brain barrier was 150-fold lower than the conversion rate of heroin into 6-MAM in the blood (Boix et al., 2013). Heroin has negligible MOPr activity (Inturrisi et al., 1983). So all the effect of heroin is after the conversion of heroin into 6-MAM and subsequently morphine. After i.v. administration of 1.3mg heroin, they observed the peak brain ECF (extracellular fluid) 6-MAM concentrations were 7.3 fold higher than peak morphine concentrations and reached peak concentration 5 folder quicker. This indicates that during intravenous heroin administration, 6-MAM is the main opiate passing through the blood brain barrier (Andersen et al., 2009). This is not supported in this thesis as we found no significant difference between the rate of onset of heroin and 6-MAM. If 6-MAM passes through the BBB (blood brain barrier) instead of heroin it is likely to have a faster rate of onset as the heroin metabolism step would have been skipped. The data in this thesis suggests that heroin also can also pass into the brain as rapidly, however a likely reasoning for the lack of this observation may be that the resolution of the data collected could not distinguish between these rates.

3.3.2 The effect of fentanyl on respiration

Similar to the results seen with i.p. heroin, i.p. fentanyl induced respiratory depression (a reduction in MV) occurring in a dose-dependent manner. Furthermore, we also observed a dose-dependent reduction in RR. Fentanyl's mechanism of action through activation of MOPr is likely to follow a similar signalling pathway as heroin's active metabolites in causing respiratory depression. Interestingly, unlike any dose of heroin, a significant decrease in TV was observed with the highest dose of fentanyl administered (1.35mg/kg), supporting a previous paper from the laboratory (Hill et al., 2020). It has been documented in humans that

fentanyls can cause muscle stiffness; this includes respiratory muscles such as the intercostal muscles and diaphragm (Streisand et al., 1993). Stiffness in these muscles would mechanically limit TV due to a reduction in maximum lung volume. This is also compounded by fentanyl's ability to reduce phrenic nerve innervation of respiratory muscles, as seen in rats (Campbell et al., 1995) which would also contribute to the decrease in TV (see Figure 3.7).

The single exponential fit for fentanyl i.v. appears to be a poor fit. However, this produced a better fit than a bi exponential fit. The documented rapid rate of onset (Peng, 1999) and the estimated rapid onset rate seen in this thesis, is likely to have caused this poor fit as the sampling intervals were not of a high enough resolution. Thus, it would provide useful to repeat fentanyl i.v. characterisation with a smaller sampling interval.

3.3.3 Heroin and fentanyl mixtures

In this thesis, a combination of i.v. heroin and fentanyl was used to model the mixtures of these two opioids often found in street use. In doing so, it was examined whether there was any synergistic potentiation or additivity between these drugs following i.v. administration. Responses to i.v. fentanyl were first characterised to calculate the potential additive effect of the mixture to compare with the actual response given by the mixture of both. This led to the decision to use the lowest doses of heroin and fentanyl in assessing these effects, whilst keeping the risk of overdose in the mice low.

The mixture of heroin and fentanyl produced a sub-additive response. Possibly due to a ceiling effect, like seen with nalbuphine (Romagnoli et al., 1980). Hill et al (2016) showed that buprenorphine pre-treatment had an antagonistic effect on morphine induced respiratory depression (Hill et al., 2016). This effect was due to the high affinity, low efficacy partial agonist action of buprenorphine, inhibiting the response to morphine (Lewis, 1985a). Heroin's active metabolites – 6-MAM and morphine- having lower agonist efficacy than fentanyl, could also act as partial agonists at MOPr to reduce the fentanyl component of the response in the mixture. The importance of this finding is that it would predict that street sold heroin and fentanyl might be less dangerous than fentanyl alone. However, this depends on the relative amounts of each drug which is unknown on the street.

3.4 Conclusion

These experiments concur with previous literature which show that heroin and fentanyl produce dose-dependent respiratory depression. Furthermore, this study demonstrated that i.p. administration of heroin and fentanyl was not as potent in comparison to when they are

administered intravenously. The heroin metabolite, 6-MAM, also depressed respiration and appeared to be more potent than heroin itself. The proposed synergistic effect of a heroin and fentanyl mixtures (Solis et al., 2017) was not seen, in fact a reduced effect from that predicted by additivity was instead observed. Repeating this last experiment with a wider range of doses of the heroin-fentanyl mixtures would be useful to further investigate this phenomenon.

4.0 Ketamine and tianeptine

4.1 Introduction

Currently naloxone is used to reverse opioid induced respiratory depression (Skolnick, 2018), in both clinical settings (Boyer, 2012a) and in public settings by first responders. Naloxone induces opioid withdrawal syndrome (OWS) which causes a proportion of addicts to become agitated and in some cases aggressive. This withdrawal syndrome is often unpleasant and often has the unwanted effect of causing users to seek a further “hit” to escape these side effects (Boyer, 2012b). Thus, there is a cause to find a new reversal agent for opioid induced respiratory depression that does not lead to OWS which has the secondary effect of protecting the safety of the user, first responder and medical staff.

The (S)-ketamine enantiomer has been suggested to reverse opioid induced respiratory depression (Mildh et al., 1998a), either through NMDA blockade or through another action of its active metabolite 2R,6R-hydroxynorketamine. In the present study, a racemic mixture of ketamine was chosen to investigate its effect on reversing opioid induced respiratory depression. In doing so, the effect of both enantiomers and both enantiomer metabolites would be given the potential to reverse respiratory depression.

Tianeptine is thought to have MOPr agonistic activity (Gassaway et al., 2014) with the ability of acting potentiating the AMPAr through phosphorylating the receptor (Svenningsson et al., 2007). AMPAr activation and modulation by phosphorylation has been suggested to be crucial for rhythm generation within the pre-Bötzinger complex. A combination of efficacy for these receptors could prove clinically useful in inducing analgesia in patients for pain management, with reduced respiratory depressive side effects.

4.1.1 Chapter aims

I. Investigate whether racemic ketamine can reverse heroin induced respiratory depression.
II. Investigate whether the known human ketamine metabolite, 2R,6R-hydroxynorketamine, can reverse heroin induced respiratory depression.
III. To characterise the atypical antidepressant, tianeptine, with suggested MOPr and AMPAractivity in relation to respiratory depression.

4.2 Results

4.2.1 The effect of ketamine in reversing heroin induced respiratory depression

As previously mentioned, the aim of the investigation was whether ketamine reverses heroin induced respiratory depression. As this thesis shows that the doses of ketamine used does not reverse heroin induced respiratory depression, saline pre-treatment with saline and saline pre-treatment with ketamine was not investigated. This would show the effect of ketamine alone on respiratory depression.

Respiratory depression was induced with an i.p. injection of 10mg/kg heroin and then followed 20 minutes later by an i.p. injection of 1mg/kg ketamine. Figures 4.1a & d show that a 10mg/kg injection of heroin produced a significant decrease in MV versus the control group in which saline was administered. The injection of ketamine (1 or 10 mg/kg) did not reverse heroin induced depression of MV to any observable degree.

RR is shown in Figure 4.1c to significantly decrease with the pre-treatment of 10mg/kg heroin. With the administration of 1mg/kg ketamine, there was no effect on RR. Figure 4.1f shows similarities to Figure 4.1c, ketamine 10mg/kg did not reverse 10mg/kg heroin induced RR depression, nor did it influence saline controls.

We also found that 10mg/kg heroin did not cause a significant decrease in TV (Figure 4.1b). With the administration of 1mg/kg ketamine, there was little to no effect on TV in heroin pre-treated mice. The data at 30 and 35 minutes were significantly different to the saline control. However, this is most likely due to an increase in TV in saline control animals, suggesting that ketamine could perhaps increase TV in naïve mice. Figure 4.1e shows that the heroin challenge appeared to induce a decreased TV, opposing data collected in Chapter 3 which suggested that TV was not influenced by heroin administration. Ketamine 10mg/kg seemed to cause a slight increase in TV of saline pre-treated mice and a lack of reversal for respiratory depression was seen in heroin pre-treated mice.

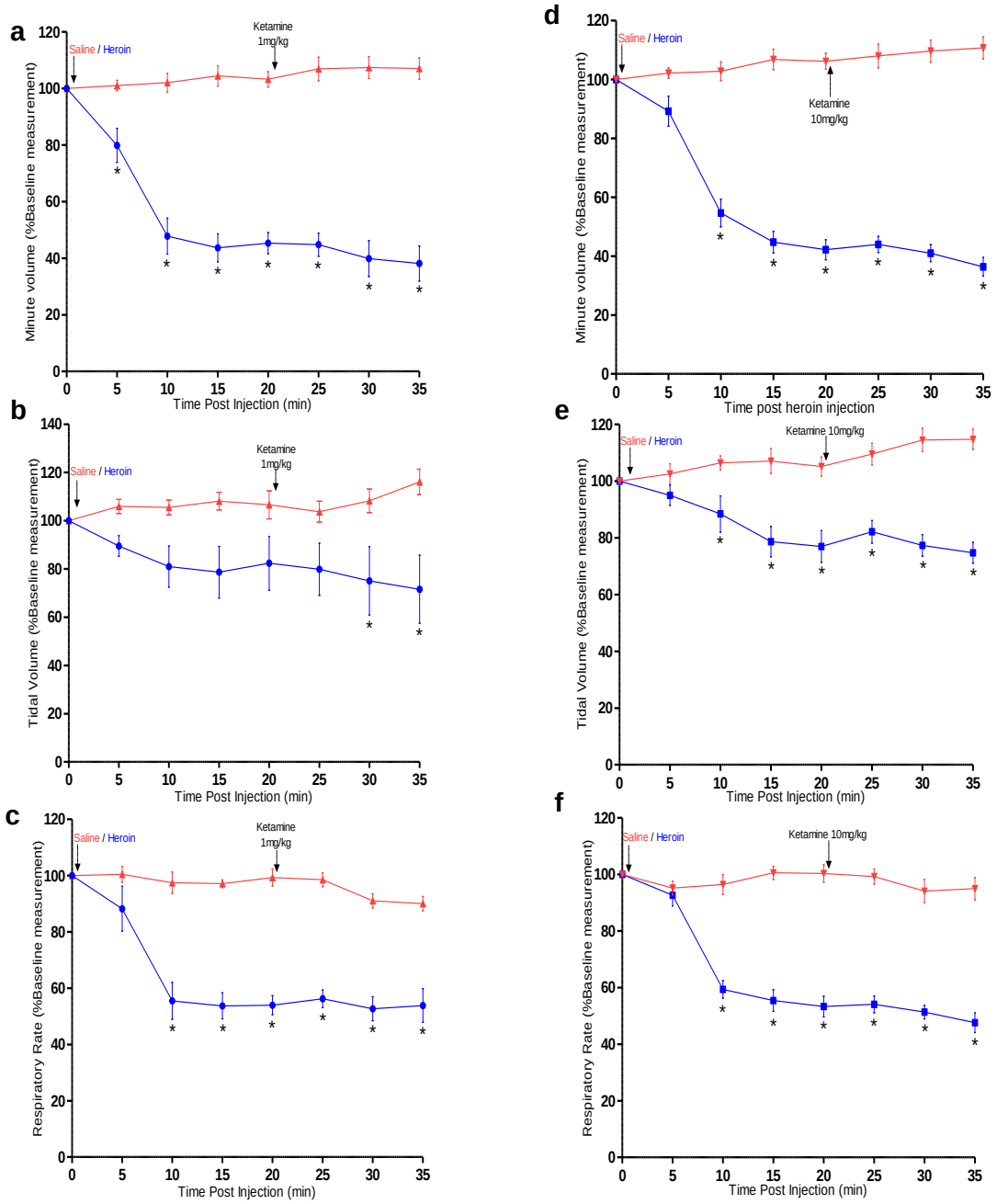

Figure 4.1: The effect of ketamine on heroin induced respiratory depression* (a-f).** *Submaximal doses of heroin (10mg/kg i.p.) or saline were administered to mice 20 minutes prior to ketamine administration. Data were normalised to maximum respiratory response prior to the heroin injection* ***(a) *Ketamine 1mg/kg i.p. had no significant effect on minute volume or saline.* ***(b)*** *Ketamine 1mg/kg i.p. had no significant effect on tidal volume in heroin or saline pre-treated mice.* ***(c)*** *Ketamine 1mg/kg i.p. had no significant effect on respiratory rate in heroin or saline pre-treated mice.* ***(d)*** *Ketamine 10mg/kg i.p. had no significant effect on minute volume in heroin or saline pre-treated mice.* ***(e)*** *Ketamine 10mg/kg i.p. had no significant effect on tidal volume in heroin or saline pre-treated mice.* ***(f)*** *Ketamine 10mg/kg i.p. had no effect on respiratory rate in both heroin and saline pre-treated mice. All data presented as mean ±SEM. A two-way ANOVA with Bonferroni's corrections was used for statistical comparison between heroin and saline before and after ketamine administration. *indicates p<0.05 compared to saline. n=6 for each group.*

4.2.2.1 Experimental troubleshooting of the effect of ketamine in reversing heroin-induced respiratory depression

The literature suggests that (S)-ketamine can cause a significant reduction in heroin induced brain hypoxia (Jonkman et al., 2018), however, the results obtained in the present study indicate that there was no effect of (R,S)-ketamine on respiration. To exclude whether the lack of effect of ketamine was due to the use of a non-efficacious dose, a human error in making the ketamine solutions or a faulty batch of ketamine, it was decided to re-conduct the experiments with 10mg/kg ketamine and a higher dose of 30mg/kg ketamine and with a different batch of ketamine known to be efficacious in other studies.

In a new series of experiments, it was found that ketamine 10 or 30 mg/kg i.p. did not reverse heroin-induced respiratory depression, measured as MV, TV or RR (Figure 4.2). Figure 4.2b highlights the peak effect of heroin by averaging the data from individual mice at 15min and 20min post heroin administration. These timepoints were chosen as this was when the peak effect was observed in the experiments described in Chapter 3, Figure 3.3b.

The peak effect of ketamine was chosen to be 10- and 15-minutes post ketamine administration. These times were chosen based on a study by Levin-Arama et al, (2016) who showed that the rate of onset for response to i.p. ketamine averaged to 13 minutes across three different strains of male mice with a long duration of action (Levin-Arama et al., 2016). Furthermore, the decision to use peak onset times at 10 and 15 minutes was supported by findings that ketamine metabolites could be detected in the brain at peak concentrations 10 minutes post administration of ketamine (Zanos et al., 2018) **.** Heroin 10mg/kg i.p. induced a significant decrease in MV in both groups, there was no significant difference between heroin pre-treated mice before and after the administration of ketamine 10mg/kg. However if anything, ketamine 30mg/kg appeared to cause a further decrease in MV and TV in heroin treated animals. Both doses of ketamine had no significant impact on MV in saline pre-treated mice. Figure 4.2c shows that ketamine did not reverse the effect of heroin on TV. Looking at Figure 4.2d, it is evident that heroin 10mg/kg i.p. seemed to cause a significant decrease in TV compared to saline pre-treated mice, contradicting data discussed in Chapter 3. There was no significant difference of peak effect before and after ketamine 10mg/kg administration but a further decrease in TV after the administration of ketamine 30mg/kg was observed. Once more, ketamine did not reverse the heroin induced decrease in RR (Figure 4.2e). The data presented in Figure 4.2d shows that ketamine 10mg/kg did not have any significant effect of RR in heroin pre-treated mice. However, after the administration of ketamine 10 and 30mg/kg in saline pre-treated a significant decrease of

RR could be seen. Furthermore, ketamine 30mg/kg caused a significant decrease in RR in heroin pre-treated mice.

4.2.3 Effect of the ketamine metabolite, 2R,6R-hydroxynorketamine on heroin-induced respiratory depression

Next, the primary human metabolite of ketamine, 2R,6R-hydroxynorketamine, was investigated. Figure 4.3a illustrates that 2R,6R-hydroxynorketamine did not reverse heroin induced respiratory depression, i.e., MV remained reduced. Both 10 and 30mg/kg 2R,6R-hydroxynorketamine did not cause a significant change in MV after its administration (Figure 4.3b). Heroin induced TV depression was not reversed by either dose of 2R,6R-hydroxynorketamine (Figure 4.3c). However, in Figure 4.3d it appears that 2R,6R-hydroxynorketamine 10mg/kg seems to cause a significant increase in TV in saline pre-treated mice. This result was not reproduced when a higher dose of 30mg/kg was used. Neither dose caused a significant percentage change in TV in heroin pre-treated mice.

Both doses of 2R,6R-hydroxynorketamine did not reverse heroin induced RR depression (Figure 4.3e). RR significantly decreased in saline pre-treated mice following 2R,6R-hydroxynorketamine 10mg/kg administration, this decrease could not be seen when 30mg/kg was administered. Yet, there was no significant effect on RR after 2R,6R-hydroxynorketamine administration in heroin pre-treated mice (Figure 4.3f).

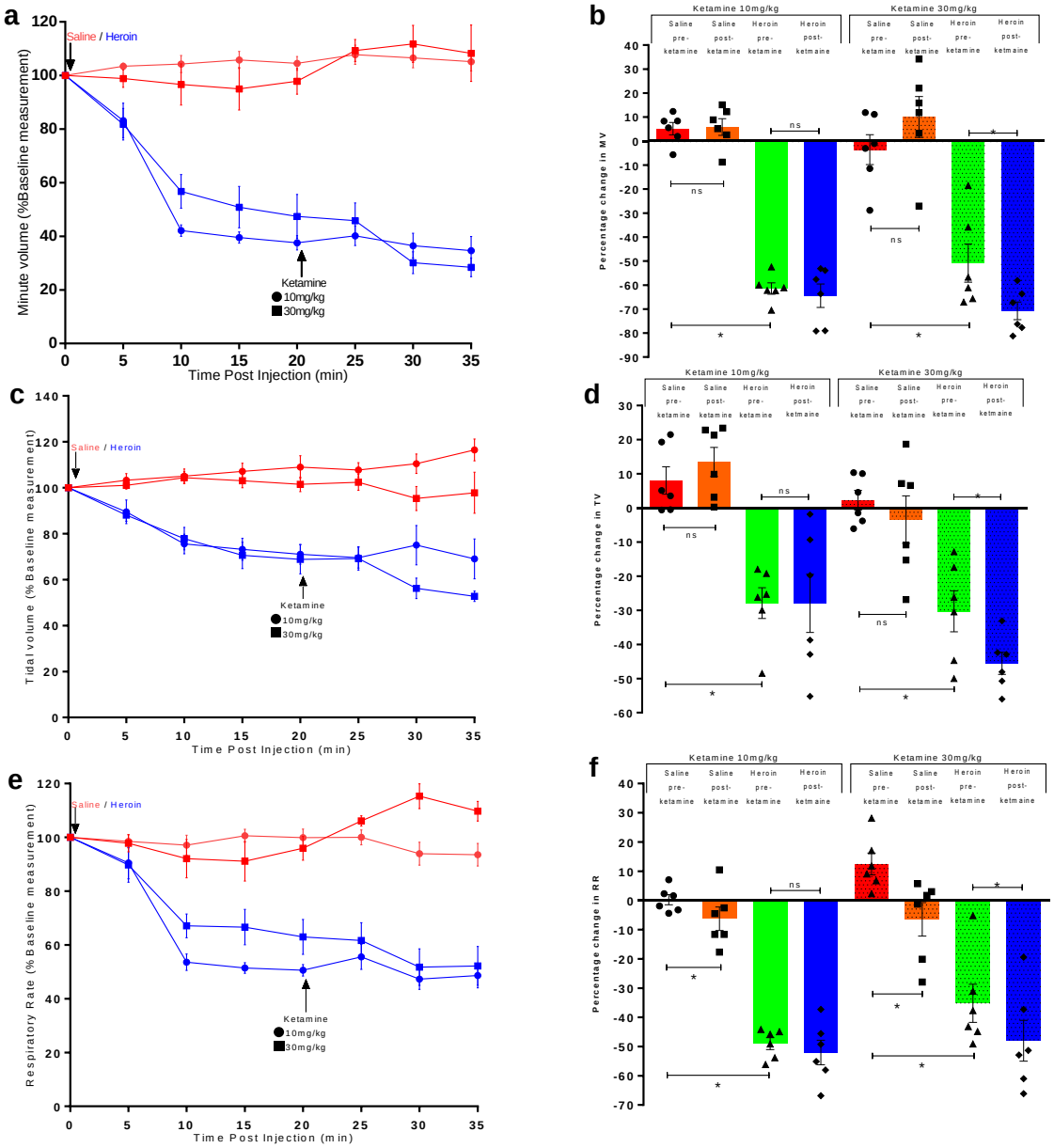

Figure 4.2: The effect of ketamine on heroin induced respiratory depression (a-f). *A submaximal dose of heroin (10mg/kg i.p.) or of saline were administered to mice 20 minutes prior to ketamine administration. Data were normalised to vales obtained prior to the administration of heroin or saline* ***(a)*** *Minute volume in heroin and saline pre-treated mice before and after ketamine 10 and 30 mg/kg i.p. administration. Ketamine did not reverse heroin induced minute volume depression.* ***(b)*** *The percentage change of minute volume in heroin induced respiratory depression. Ketamine 10mg/kg i.p. had no significant difference before or after administration. Ketamine 30mg/kg i.p. further caused a significant decrease in minute volume.* ***(c)*** *Tidal volume in heroin and saline pre-treated mice before and after ketamine 10 and 30mg/kg i.p. administration.* ***(d)*** *The percentage change of tidal volume of heroin induced respiratory depression. Ketamine 10 i.p. had no significant effect on tidal volume in heroin induced respiratory depression. Ketamine 30mg/kg i.p. caused a significant decrease in tidal volume in heroin pre-treated mice.* ***(e)*** *Respiratory rate in heroin and saline pre-treated mice before and after ketamine 10 and 30 mg/kg i.p. administration. Ketamine did not reverse heroin induced respiratory rate depression.* ***(f)*** *The percentage change of respiratory rate in heroin induced respiratory depression. Ketamine 10mg/kg i.p. had no significant difference before or after administration. Ketamine 30mg/kg i.p. further caused a significant decrease in respiratory rate. All data presented as mean ±SEM. A two-way ANOVA with Bonferroni's corrections was used for statistical comparison between heroin and saline after ketamine administration. *indicates p<0.05 compared to saline. n=6 for each group.*

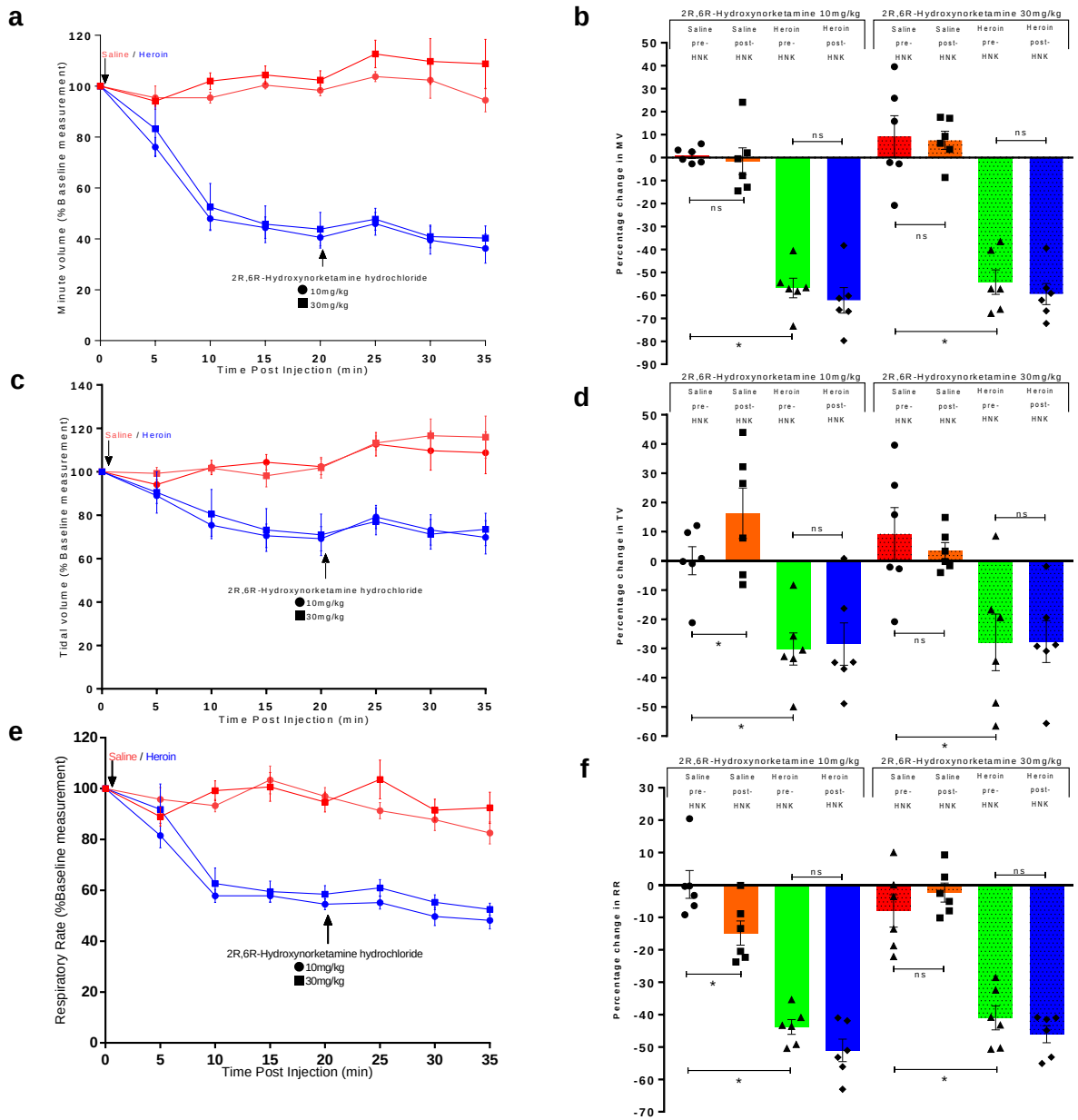

Figure 4.3: The effect of 2R,6R-hydroxynorketamine hydrochloride on heroin induced respiratory depression (a-f). *Submaximal doses of heroin (10mg/kg i.p.) or saline were administered to mice 20 minutes prior to 2R,6R-hydroxynorketamine administration. Data was normalised to maximum respiratory response prior to the induction of respiratory depression.* ***(a)*** *Minute volume in heroin and saline pre-treated mice before and after 2R,6R-hydroxynorketamine 10 and 30 mg/kg i.p. administration. 2R,6R-hydroxynorketamine did not reverse heroin induced minute volume depression.* ***(b)*** *The percentage change of minute volume in heroin induced respiratory depression. 2R,6R-hydroxynorketamine 10 and 30 mg/kg i.p. had no significant difference before or after administration.* ***(c)*** *Tidal volume in heroin and saline pre-treated mice before and after 2R,6R-hydroxynorketamine 10 and 30mg/kg i.p. administration.* ***(d)*** *The percentage change in tidal volume of heroin induced respiratory depression. 2R,6R-hydroxynorketamine 10 and 30mg/kg i.p. had no significant effect on tidal volume in heroin induced respiratory depression.* ***(e)*** *Respiratory rate in heroin and saline pre-treated mice before and after 2R,6R-hydroxynorketamine 10 and 30 mg/kg i.p. administration. 2R,6R-hydroxynorketamine did not reverse heroin induced respiratory rate depression.* ***(f)*** *The percentage change of respiratory rate in heroin induced respiratory depression 2R,6R-hydroxynorketamine 10 and 30mg/kg i.p. had no significant difference before or after administration. All data presented as mean ±SEM. A two-way ANOVA with Bonferroni's corrections were made for statistical comparison between heroin and saline after. 2R,6R-hydroxynorketamine administration. *indicates p<0.05 compared to saline. n=6 for each group.*

4.3.1 The effect of intraperitoneally administered tianeptine on respiration

Bolus doses of tianeptine were administered i.p. to CD1 mice breathing a mixture of 5% CO_2 and 95% air mixture. Figure 4.4a shows that tianeptine 10mg/kg had no effect on minute volume when compared to saline controls. Whereas tianeptine 30mg/kg decreased MV to a similar extent as heroin 10mg/kg. Normalised data in Figure 4.4a was used to calculate AUC in Figure 4.4b which confirmed no significant difference between tianeptine 10mg/kg and saline controls but a significant effect of tianeptine 30 mg/kg and heroin 10 mg/kg compared to saline controls. Tianeptine was approximately 3 times less potent than heroin at depressing MV at the doses tested.

Figure 4.4c shows that there was very little effect of tianeptine on TV. Tianeptine 10mg/kg appears to cause some increase in TV, however, Tianeptine 30mg/kg showed an apparent decrease in MV, again to no significant effect. Figure 4.4d reiterates how tianeptine, like heroin, has no significant influence on TV, discussed in Chapter 3.

Figure 4.4e clearly shows that tianeptine 10mg/kg had little effect on RR. Increasing the dose to 30mg/kg, showed similar effects to heroin 10mg/kg. When looking at the AUC data in Figure 4.4f, tianeptine 30mg/kg caused significant RR depression and was significantly better at depressing RR than tianeptine 10mg/kg, hinting at a dose-response relationship. Tianeptine 30mg/kg was equipotent to heroin 10mg/kg at depressing RR, so has a potency at least 3 times lower than the potency of heroin.

Given that tianeptine 30 mg/kg induced respiratory depression it was important to determine if this was mediated by MOPr. The effect of tianeptine with and without pre-treatment with naloxone was therefore examined. Figure 4.5a portrays that naloxone 0.3mg/kg i.p. pre-treatment 10 minutes before tianeptine 30mg/kg administration reduced the depression of MV compared to mice who had received saline pre-treatment. In naloxone pre-treated mice TV was still unchanged by tianeptine 30 mg/kg (figure 4.5b). Naloxone pre-treated mice showed significantly less depression of RR in response to tianeptine 30 mg/kg compared to saline pre-treated control mice (Figure 4.5c).

Given that tianeptine 30 mg/kg induced a naloxone-reversible depression of respiration we next examined whether at that dose the drug also induced antinociception and whether any antinociception observed was prevented by pre-treatment with naloxone. In saline pre-treated animals, there was a significant increase in tail flick latency by at least 4.5-fold.

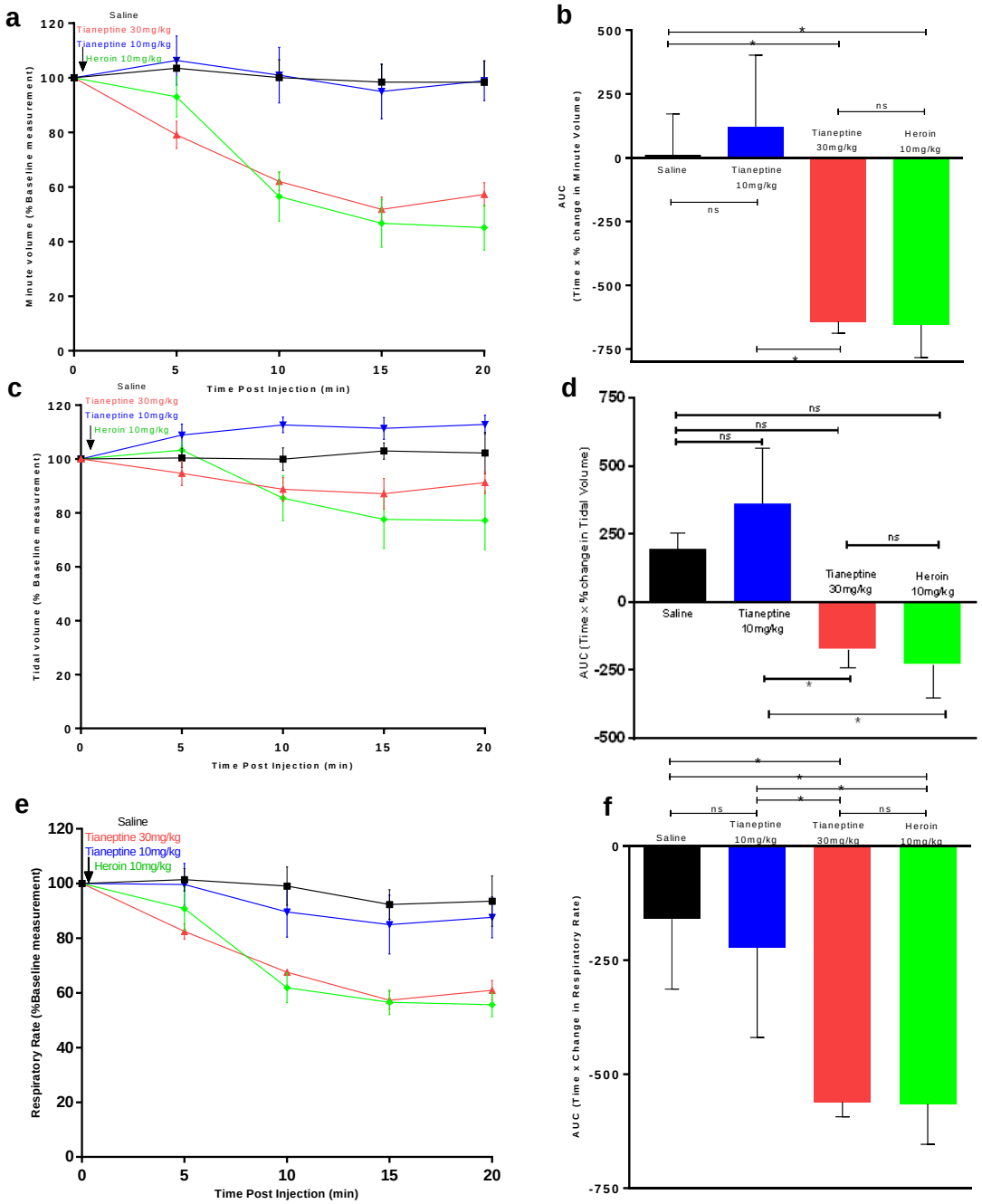

Figure 4.4: The effect of tianeptine on respiration. *Tianeptine (10 and 30 mg/kg) and heroin (10mg/kg) administered via i.p. injection on respiration of CD1 mice in whole-body unrestrained plethysmography breathing 5% CO_2/95% air-gas mixture.* ***a)*** *Shows the percentage decrease in minute volume relative to pre-injection baseline values of individual mice.* ***b)*** *The overall depression of minute volume illustrated by the area under the curve (AUC) analysis of datum presented in (a).* ***c)*** *Shows the percentage decrease in tidal volume relative to pre-injection baseline values of individual mice.* ***d)*** *The overall depression of tidal volume illustrated by the area under the curve (AUC) analysis of datum presented in (c).* ***e)*** *Shows the percentage decrease in respiratory rate relative to pre-injection baseline values of individual mice. P<0.05 significance indicated by *. A two-way ANOVA with Bonferroni's comparison was used to statistically compare groups (n=6)*

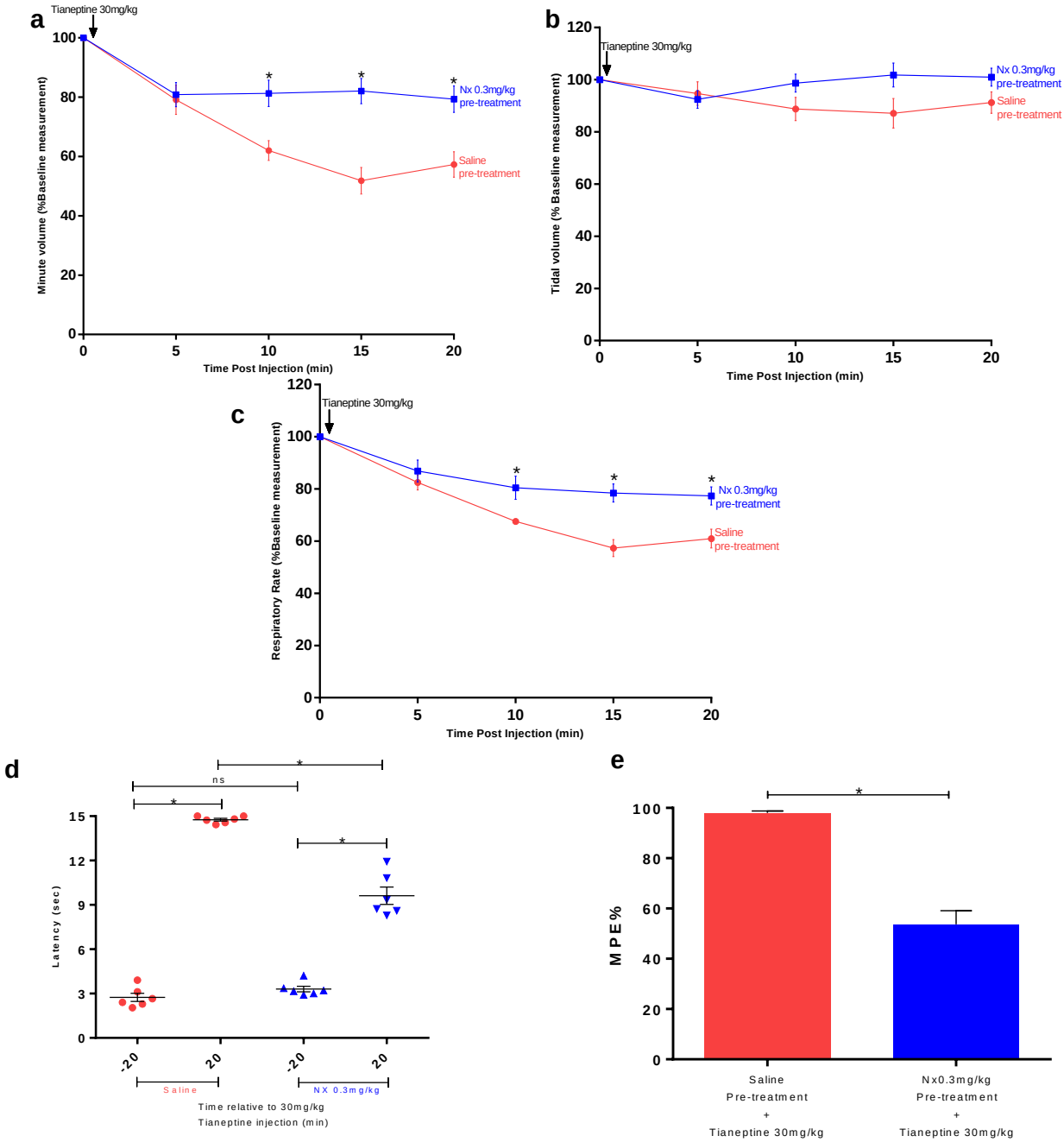

Figure 4.5: The effect of tianeptine on the MOPr activity phenotypes, respiratory depression and antinociception. *Tianeptine (30 mg/kg) and via i.p. injection on respiration and analgesia of saline and naloxone (0.3mg/kg) CD-1 mice in whole-body unrestrained plethysmography breathing 5% CO_2/95% air-gas mixture.* ***a)*** *Shows the percentage decrease in minute volume relative to pre-injection baseline values of individual mice. Tianeptine being significantly less effective at reducing minute volume in naloxone pre-treated mice.* ***b)*** *Shows the percentage decrease in tidal volume relative to pre-injection baseline values of individual mice. Whether mice were saline or naloxone pre-treatment caused no significant difference in tidal volume after tianeptine administration* ***c)*** *Shows the percentage decrease in respiratory rate relative to pre-injection baseline values of individual mice. Tianeptine being significantly less effective at reducing respiratory rate in naloxone pre-treated mice.* ***d)*** *The tail flick latency of saline and naloxone pre-treated mice before any administration of saline or naloxone and after tianeptine administration. Naloxone pre-treated mice had a significantly less tail flick latency than saline pre-treated mice post tianeptine administration* ***e)*** *The percent maximum possible effect of saline pre-treated mice were significantly greater than naloxone treated mice. %MPE was calculated from datum in* ***(d)****. %MPE was significantly lower in naloxone pre-treated mice. P<0.05 significance indicated by *. A two-way ANOVA with (a-c) Bonferroni's comparison was used to statistically compare groups and Dunnet's used for non-normally distributed data (d-e) (n=6)*

The 15 second cut-off point prevented the true maximum latency being reached by 2 mice within this group. In the group that received pre-treatment with naloxone 0.3 mg/kg the effect of tianeptine was reduced (Figure 4.5d & e). There was still a significant increase in tail flick latency following tianeptine (by about 3-fold), but no mice reached the cut-off point in this group. Both saline and naloxone pre-treated mice at time –20min that did not receive tianeptine had no significant difference in tail flick latency. %MPE was calculated from the data in Figure 4.5d and presented in Figure 4.5e, where the naloxone 0.3mg/kg pre-treatment group had a %MPE significantly lower than the saline pre-treated group following tianeptine 30mg/kg i.p. administration.

4.4 Discussion

In the present study, it was demonstrated that ketamine did not reverse heroin induced respiratory depression. A 30mg/kg dose of ketamine, however, was found to further induce respiratory depression in reducing both RR and TV. This could be due to possible MOPr activation by ketamine. S-ketamine has been shown to induce significantly more respiratory depression in wild type mice over MOPr-/- mice. These effects of S-ketamine have been recorded to start at 30mg/kg i.p. (Sarton et al., 2001), thus higher doses of ketamine were not investigated. The data does not endorse a previous study which reported that the racemic mixture of ketamine partially prevented a fentanyl induced depression of MV in humans (Mildh et al., 1998). Furthermore, in humans' low doses of ketamine have also been shown to stimulate breathing and RR (Eikermann et al., 2012). A more recent study has also shown that S-ketamine stimulates breathing through increasing CO_2 sensitivity which in turn leads to increased RR in healthy volunteers (Dahan et al., 2009). Which has been translated into a study where (S)-ketamine countered opioid induced respiratory depression (Jonkman et al., 2018). Notably, however, the studies mentioned used human volunteers, therefore physiological differences between species may explain why in this mouse study no reversal was observed.

S-ketamine has been shown to decrease noradrenaline reuptake into neurons (Aston-Jones et al., 2004). Almost half of all noradrenergic projections within the central nervous system may originate in the locus coeruleus (Magalhães et al., 2018). While noradrenergic neurons (A6 neurons) within the LC are not implicated in the control of respiration in conscious rats breathing air; respiratory depression is elicited in studies where A6 neurons were inhibited (Ge et al, 1998). The study further suggests that A6 neurons contribute to a CO_2-induced rise in inspiratory motor output. With NMDA inhibition, an increase in noradrenaline within synapses could increase neuronal output within the LC to stimulate breathing activity. Whilst

opioid induced respiratory depression would cause an increased concentration of CO_2 (H^+) within the blood, ketamine could be used to indirectly increase A6 neuronal activity to stimulate breathing.

The lack of reversal of heroin induced respiratory depression could be a heroin specific phenomenon, as both AMPAkines and ketamine reverse respiratory depression of fentanyl, remifentanil, and alfentanil (Ren et al., 2006; Oertel et al., 2010; Jonkman et al., 2018). As discussed in Chapter 3, studies have shown that fentanyl and alfentinil can induce muscle stiffness (Benthuysen et al., 1986; Streisand et al., 1993b; Weinger et al., 1991). Ketamine has been shown in rats to stabilize breathing by increasing genioglossus activity, thus increasing airway dilation and end-expiratory lung volume (Jordan et al., 2010). Potentially ketamine induced increased genioglossal activity could counteract fentanyl and alfentanil induced muscle stiffness (Benthuysen et al., 1986; Weinger et al., 1991; Streisand et al., 1993), resulting in the reversal of fentanyl and alfentanil respiratory depression.

AMPAkines, positive allosteric modulators of AMPA receptors, have been shown to stimulate breathing. The ampakine, CX546, has been shown to rescue fentanyl-induced respiratory depression in vivo, and it has been demonstrated to increase the frequency and amplitude of phrenic nerve activation (Ren et al., 2006b). 2R,6R-hydroxynorketamine also is an agonist at the AMPAr (Zanos et al., 2016; Aleksandrova et al., 2017), thus it would be expected that it would be able to reverse respiratory depression like other ampakines (Zanos et al., 2016b).There is evidence that 2R,6R-hydroxynorketamine can pass through the blood brain barrier as it has been detected in brain tissue after being administered intravenously (Leung and Baillie, 1986). However, like ketamine, 2R,6R-hydroxynorketamine was not able to reverse heroin induced respiratory depression. As described with ketamine, 2R,6R-hydroxynorketamine could have potential in reversing fentanyl-induced respiratory depression.

Results obtained in this study using tianeptine demonstrate that it causes respiratory depression, primarily through decreasing RR. Furthermore, through pre-treatment of the competitive opioid antagonist, naloxone, respiratory depression and antinociception was significantly decreased. This is supported by evidence within the literature which suggests that tianeptine is a MOPr agonist (Gassaway et al., 2014). Fentanyl has shown not to depress in respiration in MOPr knockout mice, indicating that opioid induced respiratory depression in the mouse is MOPr mediated instead of DOPr or KOPr (Hill et al., 2020). Therefore, pre-treatment of naloxone would be thought to influence tianeptine induced respiratory depression via the MOPr.

The antinociceptive properties of tianeptine have also been demonstrated in our study have also been demonstrated elsewhere (Kim et al., 2012; Gassaway et al., 2014). Furthermore, we found that tianeptine is at least 3-fold less potent than heroin, in inducing respiratory depression. However, at the 30mg/kg dose used tianeptine has been shown to induce significant hyperlocomotion (Samuels et al., 2017). Thus, it is possible that tianeptine is more potent than it appears in the present study due to a counteracting increased rate of respiration in response to the metabolic demands of hyperlocomotion.

In HEK293 cells expressing MOPr, tianeptine has been shown in to activate G-proteins with an EC_{50} of 194 +- 70nM (Gassaway et al., 2014) compared the EC_{50} of morphine which has been shown to be 660 +- 90nM (Hill et al., 2018). 10mg/kg heroin and morphine administered via intraperitoneal injection have been shown to be equipotent in inducing respiratory depression (Hill et al., 2020b). These published EC_{50} values of tianeptine and morphine opposes the degree of respiratory depression by tianeptine seen in this thesis as tianeptine would be expected to be more potent. When the EC_{50} of tianeptine was investigated in unpublished data within the laboratory, it was reported to be 10µM, making it less potent than morphine which supports the data seen in this thesis. Tianeptine's effect on AMPAr could be negligible, however, future experiments should focus on tianeptine's AMPAr activity on respiration should be done to confirm this.

4.5 Conclusion

Ketamine and its metabolite (2R,6R)- hydroxynorketamine did not reverse heroin induced respiratory depression in CD-1 mice. The literature suggests that (R,S)-ketamine and (S)-ketamine can reverse opioid induced respiratory depression, when fentanyl is the opioid under study. However, as heroin was used in this experiment it could suggest that the reversing effect of ketamine and 2R,6R-hydrixynorketamine could be a fentanyl specific phenomenon related to its ability to induce muscle stiffness, though further studies would be needed to confirm this. Our experiments also showed that tianeptine induces respiratory depression mainly through a reduction in RR, which like antinociception, could be reversed by naloxone pre-treatment. Tianeptine's lower relative potency (compared to heroin) cannot be attributed to its AMPA activity, and as such further experiments on this atypical antidepressant are required.

5.0 Investigating the redevelopment of heroin tolerance after a period of abstinence.

5.1 Introduction

There have been anecdotal accounts by intravenous heroin users that tolerance to heroin occurs more rapidly during relapse following a period of detox/abstinence compared to the tolerance that developed when they first used the drug (Personal Communication from Prof Graeme Henderson following discussions with heroin users at the Bristol Drugs Project). This could be due to psychological or biological factors, or a combination of both. In isolated neurons, accelerated MOPr desensitisation has been found in response to prolonged morphine treatment (Williams et al., 2013).

In a preliminary study, Rob Hill in our laboratory performed experiments to investigate whether this phenomenon could be observed in mice at the level of morphine-induced respiratory depression. He treated mice for 6 days by subcutaneous implantation of a morphine pellet. The pellet was then removed and mice 'detoxed' for 6 days. To simulate relapse the mice were then administered twice daily doses of morphine and respiration monitored. The dose of morphine chosen did not produce tolerance in naïve mice. However, in mice that had undergone morphine treatment and detox rapid development of tolerance was observed (Figure 5.1). These data suggest that morphine tolerance does develop more rapidly after a period of abstinence.

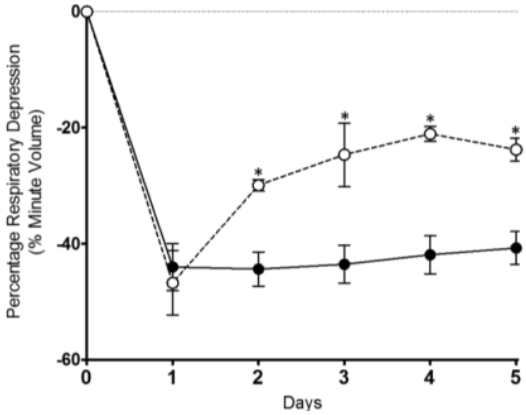

Figure 5.1 Morphine induced enhanced development of tolerance to respiratory depression following withdrawal and re-exposure to morphine*. Naïve mice (filled symbols) and mice previously pre-treated with morphine for 6 days and withdrawn for 4 days (filled symbols), received challenge twice daily injections of morphine (30mg/kg i.p.) and respiration was monitored for 30 min post 2nd challenge dose. Figure obtained from unpublished data from Rob Hill.*

Methadone and buprenorphine are used as opioid substitution treatments (OST) (Hayhurst and Durieux, 2016). OST with methadone and buprenorphine reduces the risk of death from overdose. However following detox from OST, the risk of death by overdose significantly increases (Cousins et al., 2016). It is unknown whether tolerance develops faster on relapse after OST treatment and detox or only from heroin/morphine.

By mimicking the behaviour of heroin users abstaining from heroin through entering rehabilitation, jail/prison or self-abstinence in a controlled experiment, the biological component of enhanced rate of tolerance redevelopment (if present) can be investigated. Morphine is said to cause desensitisation/tolerance via a PKC mediated pathway (Bailey et al., 2009), however methadone and buprenorphine induced desensitisation is thought to be PKC-independent (Lowe et al., 2015). The difference in desensitisation mechanism could prove useful in identifying whether PKC upregulation is responsible for the more rapid redevelopment of tolerance following a period of abstinence from heroin.

5.1.1 Chapter aims

I. The first aim of this study was to investigate whether the redevelopment of tolerance to heroin after a period of abstinence occurs at a faster rate than the initial development to heroin.
II. To characterise whether chronic methadone and buprenorphine pre-treatment also influences the subsequent development of heroin tolerance.

5.2 Results

5.2.1 The comparison of baseline measurements and the initiation of heroin tolerance.

In order to establish that the prolonged heroin pre-treatment process did not cause a shift in baseline respiration measurements of mice, the minute volume at baseline was measured immediately before and following heroin pre-treatment (mice administered heroin doses and osmotic mini-pump implantation as described in Methods. Figures 5.2a-b show that there

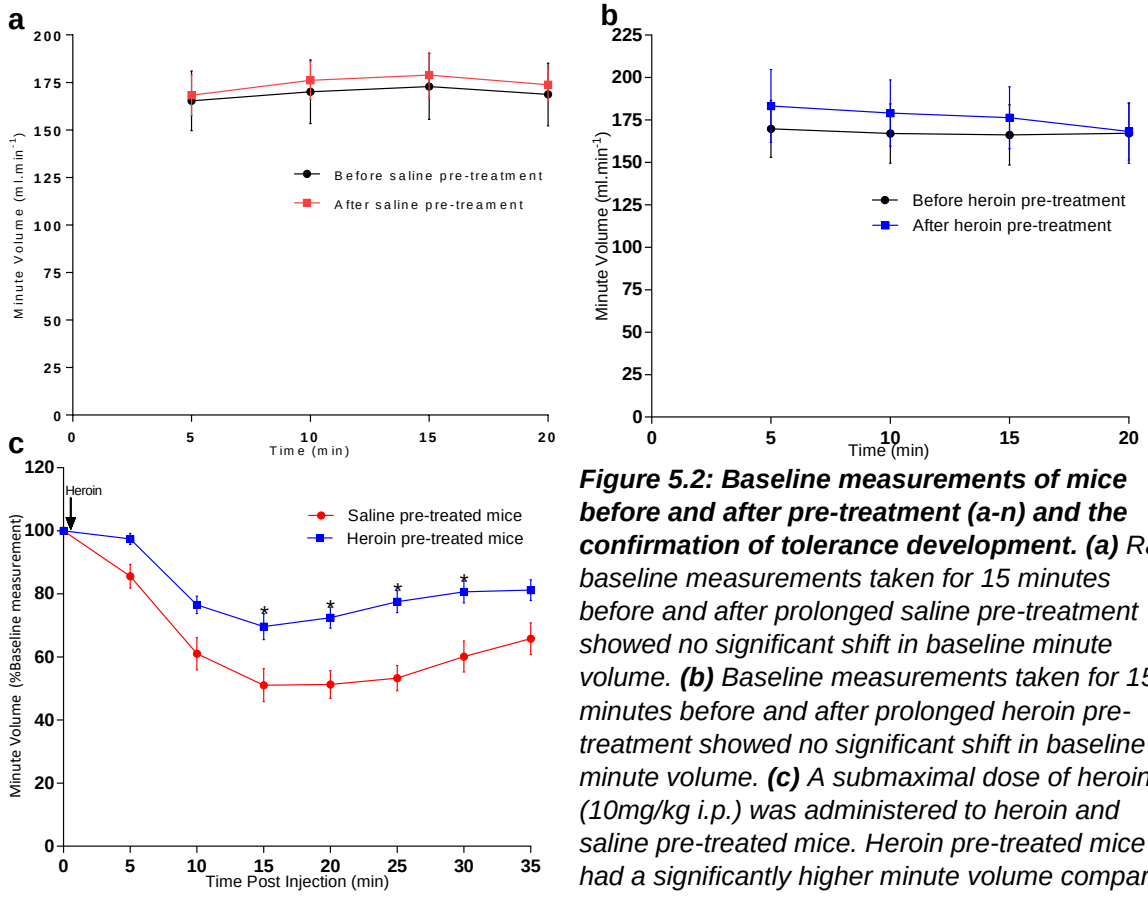

Figure 5.2: Baseline measurements of mice before and after pre-treatment (a-n) and the confirmation of tolerance development. (a) *Raw baseline measurements taken for 15 minutes before and after prolonged saline pre-treatment showed no significant shift in baseline minute volume.* ***(b)*** *Baseline measurements taken for 15 minutes before and after prolonged heroin pre-treatment showed no significant shift in baseline minute volume.* ***(c)*** *A submaximal dose of heroin (10mg/kg i.p.) was administered to heroin and saline pre-treated mice. Heroin pre-treated mice had a significantly higher minute volume compared to saline pre-treated mice. All data presented as mean ±SEM. A two-way ANOVA with Bonferroni's corrections were made for statistical comparison between heroin and saline pre-treated mice after heroin administration. *indicates p<0.05 compared to saline. n=9 for each group.*

was no significant difference between baseline measurements of MV obtained before and after pre-treatment in heroin or saline treated mice. When a challenge dose of 10mg/kg heroin was administered i.p after the pre-treatment phase there was a significant difference in response between heroin pre-treated and saline pre-treated mice (Figure 5.2c). The decreased depression of MV in heroin pre-treated mice relative to saline pre-treated mice, shows that tolerance had developed in the heroin pre-treated mice.

5.2.2 The redevelopment of tolerance to heroin following a period of abstinence.

Pre-treatment of heroin with osmotic mini-pumps in which the doses of heroin were chosen based on previously obtained data using morphine. In non-pre-treated mice the comparable dose of did not induce tolerance (Rob Hill, personal communication). Once the 8 total days of the pre-treatment phase had ended (Figure 2.2), pumps were removed, the period of abstinence from active opioids had started. The brain equilibration half-lives (s.c. administration of mice) of heroin (5 min) and subsequent metabolites 6-mam (26 min), morphine (120 min) (Andersen et al., 2009). Furthermore, the morphine metabolite M3G (morphine-3-glucuronide) inactive at the MOPr and the lack of the metabolite M6G (morphine-6-glucuronide) being produced in mice (Kuo et al., 1991; Milne et al., 1996; Hill et al., 2016; Xie et al., 2017). From this, it is evident that morphine accurately predicts the loss of active opioid, and thus the 6-day abstinence period was opioid free. After the period of abstinence had ended a twice daily heroin 10mg/kg i.p. injection protocol was used, to redevelop tolerance.

The data in Figures 5.3a-b highlight the pattern of MV across the twice daily injection protocol in heroin and saline pre-treated/detoxed mice respectively. Figure 5.3c shows that in heroin pre-treated mice across the 5 days, AUC generally trends to decrease, however, we only observed a significant decrease in AUC at day 5 (Figure 5.3c). This indicates that some tolerance had developed. In contrast, in saline pre-treated mice, there was no statistically significant change in AUC over the 5 day period indicating that tolerance had not developed (Figure 5.3d) although there was a tendency for the AUC to decrease on successive days.

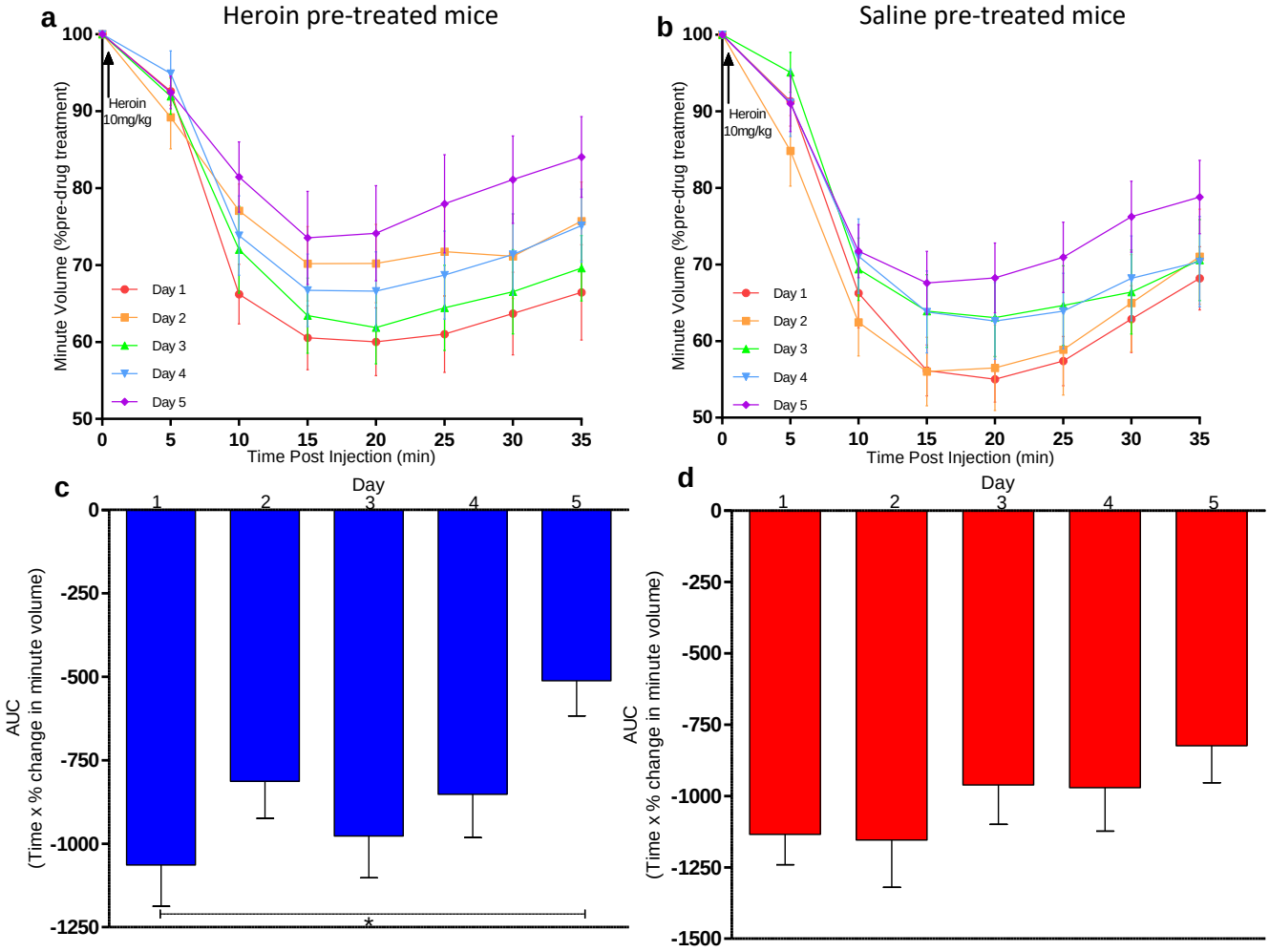

Figure 5.3: The Redevelopment of tolerance to heroin after a period of abstinence. a) *Normalised minute volume measurements taken after 6 days of heroin abstinence, daily challenge with heroin (10mg/kg i.p.) administration using a twice daily injection protocol in heroin pre-treated mice (blue).* ***b)*** *Normalised minute volume measurements taken after 6 days of abstinence, daily challenge heroin (10mg/kg i.p.) administration during a a twice daily injection protocol in saline pre-treated mice (red).* ***c)*** *The overall depression of minute volume across each of the twice daily injection protocol in heroin pre-treated mice , illustrated by the area under the curve (AUC) analysis of data presented in* ***(a)****.* ***(d)*** *The overall depression of minute volume across each of the twice daily injection protocol in saline pre-treated mice, illustrated by the area under the curve (AUC) analysis of datum presented in* ***(b)****. All data presented as mean ±SEM.* ***(c-d)*** *A one-way ANOVA with Bonferroni's corrections were made for statistical comparison of each day during the twice daily injection protocol. *indicates p<0.05 compared to saline. n=9 for each group.*

5.3 The development of tolerance to heroin after a period of abstinence following methadone or buprenorphine pre-treatment

Following 6 days of methadone or buprenorphine pre-treatment, a 10mg/kg heroin challenge dose was administered, and respiration monitored. Figure 5.4a illustrates the response to i.p. 10mg/kg heroin challenge after pre-treatment. Both methadone and buprenorphine pre-treatment groups displayed significantly smaller depression of MV compared to saline pre-

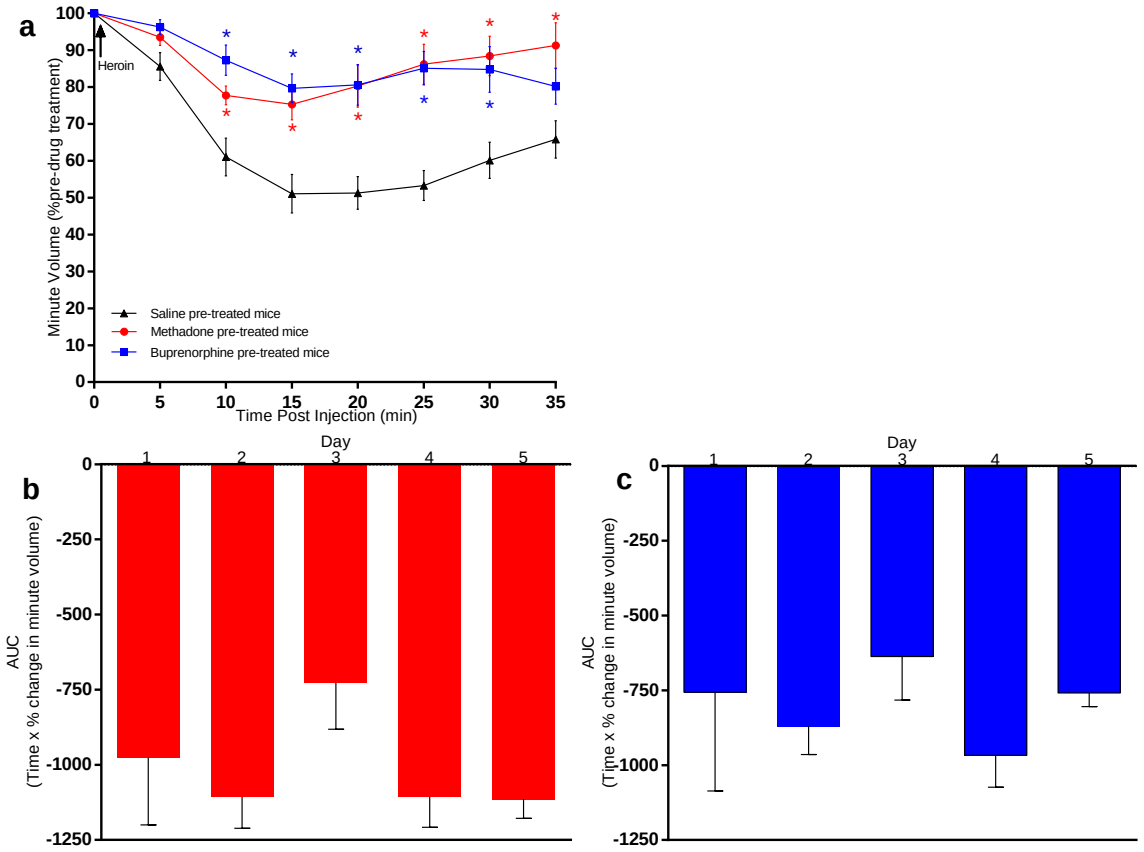

Figure 5.4: Methadone and Buprenorphine confirmation of tolerance during pre-treatment and the development of tolerance to heroin after a 6-day period of abstinence. a) *Submaximal doses of heroin (10mg/kg i.p.) were administered to saline, methadone (red), and buprenorphine (blue) pre-treated mice. Methadone and buprenorphine pre-treated mice had a significantly higher minute volume compared to saline pre-treated mice.* ***b)*** *Area under the curve of normalised minute volume measurements taken after 6 days of heroin abstinence followed by daily challenge heroin (10mg/kg i.p.) administration during a 5 day twice daily injection protocol in methadone pre-treated mice. The overall depression of minute volume across the twice daily injection protocol in methadone pre-treated mice* ***c)*** *Area under the curve of normalised minute volume measurements taken after 6 days of abstinence, daily challenge heroin (10mg/kg i.p.) administration during a 5 day twice daily injection protocol in buprenorphine pre-treated mice. The overall depression of minute volume across each of the twice daily injection protocol in methadone pre-treated mice. All data presented as mean ±SEM.* ***(a)*** *A two-way ANOVA with Bonerroni's corrections was used for statistical comparison.* ***(b-c)*** *A one-way ANOVA with Bonferroni's corrections were made for statistical comparison of each day during the twice daily injection protocol. *indicates p<0.05 compared to saline. n=7 for methadone and buprenorphine pre-treated mice groups and n=9 for saline groups.*

treatment mice (note, this is the same saline pre-treated group as described Figure 5.3), suggesting that tolerance had been induced during the methadone pre-treatment. Whilst we can conclude that the lowered response in the methadone pre-treated group indicates that tolerance has been induced, with buprenorphine pre-treatment the reduced response to the heroin challenge could be due to buprenorphine acting as an antagonist. Buprenorphine is a partial agonist and has a slow dissociation rate from the MOPr meaning that no discrimination can be made as to whether there is cross tolerance between heroin and buprenorphine or whether buprenorphine acts as an antagonist and reduces the heroin response.

Mice were then withdrawn from methadone and buprenorphine (osmotic mini-pumps removed) over a 6 day period. Thereafter they were administered heroin (10 mg/kg i.p.) to determine whether tolerance could be re-established. The data for responses to heroin challenge following withdrawal and abstinence from methadone and buprenorphine are illustrated as AUC in Figure 5.4d and Figure 5.4e respectively. There was no significant difference in AUC across the 5 days for both groups, showing that tolerance to heroin was not induced during the 5 days of twice daily heroin injections.

5.4 Discussion

After a 6 day abstinence break from heroin, mice appeared to redevelop some tolerance during the 5 day twice daily injection protocol, whereas saline pre-treated mice did not. Showing signs of an enhanced rate of tolerance redevelopment. However, the level of tolerance reached was quite small. Also, tolerance did seem to be developing in the saline pre-treated mice, although the level of reduction in the response to the heroin challenges never reached statistical significance. The redevelopment of tolerance is needed to be robust to induce a larger degree of tolerance by the end of the 5-day twice daily injections. This could be achieved by increasing the pre-treatment dose of heroin to induce higher initial tolerance before the period of abstinence. Unpublished work by Rob Hill (Figure 2.2**)** shows that a 75mg/kg morphine pellet had shown a higher degree of tolerance than osmotic mini-pumps. It has been shown that morphine pellets deliver a significantly higher plasma drug concentrations than osmotic mini-pumps (McLane et al., 2017). Osmotic mini-pumps were limited to deliver 45mg/kg/day due to heroin solubility issues and the lack of availability of heroin, buprenorphine and methadone pellets. Since heroin pellets are currently unavailable, preliminary studies could be carried out to identify methods to induce robust heroin induced tolerance. Possible preliminary studies could include, increasing the three initial 100mg/kg

i.p. doses (frequency of doses and/or dose size) at the start of pre-treatment and comparing degrees of tolerance after the pre-treatment phase. Once a higher degree of tolerance can consistently be reached, redoing the experiments again to see whether a more discernible enhanced rate of redevelopment of tolerance to heroin can be seen, as that of Rob Hill's preliminary studies with morphine (Figure 5.1).

A possible mechanism for the observation of enhanced tolerance development in morphine and heroin pre-treated mice could be speculated to be due to an upregulation of PKC. A study by Bailey *et al* (2009) shows that PKC is involved in desensitisation to morphine in LC neurones, demonstrated by the lack of desensitisation in PKCα knockout mice (Bailey et al., 2009). Furthermore, another group increased the activity of PKCα within the pre-Bötzinger complex using lentivirus, which increased morphine induced respiratory depression tolerance (Lin et al., 2012). This may occur from the initiation of drug exposure and maintained elevated levels of PKC could possibly persist throughout the abstinence period thus enhancing the development of tolerance on re-exposure. Evidence in support of this hypothesis comes from the work of Xu *et al* who showed an upregulation of PKC within the pons with chronic morphine treatment (3 weeks), which could be blocked with the coadministration of naloxone (Xu et al., 2015). Enhanced levels of PKC expression would cause increased MOPr desensitisation on agonist exposure. In whole-cell patch-clamp recordings of GIRK conductance in mature rat LC neurons, the administration of phorbol-12-myristate-13-acetate (PMA), a PKC activator, showed to enhance morphine induced MOPr desensitisation. Morphine elevating PKC activity did not decrease the initial peak response but enhances subsequent desensitisation. Showing that the receptor only desensitises once morphine had activated it (Bailey et al., 2004).

In contrast, methadone is a high efficacy MOPr agonist (Rodriguez-Martin et al., 2008) which desensitizes MOPr through GRK phosphorylation and arrestin binding (McPherson et al., 2010; Lowe et al., 2015). It has been previously reported that a PKC inhibitor, calphostin c, reverses morphine-induced tolerance to respiratory depression but not methadone-induced tolerance (Withey et al., 2017). This indicates that methadone induced tolerance, like MOPr desensitisation is not PKC dependent. Therefore, we can hypothesise that methadone pre-treatment is unlikely to upregulate PKC activity and thus in the pre-treatment/abstinence/re-induction experiments tolerance did not develop to the heroin injections in methadone-pre-treated mice. Thus, the lack of PKC upregulation during methadone pre-treatment could explain why tolerance did not develop after abstinence and subsequent heroin injections.

Buprenorphine binds to the MOPr with a high affinity and a slow dissociation rate, thus other agonists are ineffective at displacing buprenorphine, giving it antagonistic properties (Lewis,

1985). Due to this it is difficult to distinguish between this effect of buprenorphine and the induction of tolerance after buprenorphine pre-treatment. However, we would predict that following buprenorphine pre-treatment and abstinence then on exposure to heroin tolerance would not develop to the heroin injections. This was what was observed.

There are other potential mechanisms that might underly the rapid redevelopment of tolerance following pre-treatment with heroin. Previous work on potential receptor-based changes has been conducted, showing an upregulation of the mRNA for MOPr splice variants after prolonged morphine treatment (Verzillo et al., 2014) . This may suggest that a change in the structure of the MOPr could have an impact on downstream desensitisation, internalisation and recycling pathways and thus could affect the onset of the redevelopment of tolerance. For example, a splice variant with altered ability to be recycled could cause a faster onset of tolerance (Chakrabarti et al., 2016). The literature shows a phenomena whereby chronic morphine use causes adaptive changes which upregulates mRNA for MOPr-1B2 and MOPr-1C1 splice variants (Verzillo et al., 2014; Chakrabarti et al., 2016)**.** Another study suggests that these splice variants can associate with G_s associated proteins, instead of G_i associated proteins (Chakrabarti et al., 2020). This has been shown to be sexually dimorphic, where it occurs within male mice but not with female mice (Chakrabarti et al., 2012, 2016, 2020). These splice variants contain phosphorylation sites which are not present in normal MOPr-1 receptors. PKA inhibition showed to inhibit MOPr-1C1 G_s signalling but not MOPr-1, suggesting that these C-terminal phosphorylation sites are unique to MOPr-1C1 splice variants (Chakrabarti et al., 2020). Superactivation and upregulation of adenylyl cyclase has been shown to occur as an adaptive change as a result of prolonged morphine use (Beavo et al., 1974). A subsequent downstream effect of this would be increased levels of cAMP within cells and thus cause the activation of protein kinases such as PKA (Jiang and Bajpayee, 2009; Sassone-Corsi, 2012). With elevated activated PKA within cells during chronic opioid pre-treatment in combination with the upregulation of MOPr-1C1, an increased population of G_s coupled MOPr could be established. This could explain why a significant degree of tolerance was seen following abstinence in heroin pre-treated mice but not with saline pre-treated mice. With the redevelopment of tolerance in heroin pre-treated mice, a higher proportion of G_s MOPr-1C1 receptors may oppose the effects G_i coupled MOPr, with phenotypic tolerance being observed. Saline pre-treated animals would have a smaller concentration of G_s coupled MOPr-1C1 receptors and thus no significant tolerance was developed during the twice daily injection protocol. Since it has been suggested that this is a sexually dimorphic male bias event, repeating the experiment with female mice would help to establish this theory. If a significant difference of the degree

of tolerance is seen between female and male heroin pre-treated mice, this would suggest that this dimorphic mechanism is a possibility.

5.5 Conclusion

In conclusion, this chapter contributes to the unexplored topic of whether there is a biological component to the perceived enhanced rate of redevelopment of heroin tolerance after a period of abstinence. The data in this chapter suggest that heroin pre-treated mice redeveloped a significant level of tolerance whereas saline pre-treated mice did when mice were re-exposed to heroin. However, the protocol for the heroin pre-treatment and for the re-exposure to heroin following a period of abstinence need to be optimised further to ensure that hard conclusions can be drawn from the data obtained. This chapter has shown that the pre-treatment of methadone and buprenorphine did not enhance the development of heroin tolerance, indicating that the enhanced rate of redevelopment of heroin tolerance is agonist dependent. It will be important to determine whether any enhanced redevelopment of heroin tolerance is PKC mediated.

6.0 General Discussion

The main aims of the work described within this thesis, were to investigate acute heroin and fentanyl respiratory depression, the effect of heroin and fentanyl mixtures during intravenous administration. Also, whether ketamine and metabolites can reverse heroin induced respiratory depression and to characterise tianeptine's effect on respiratory depression. Finally, whether there is an enhanced rate of redevelopment of tolerance to heroin after a period of abstinence and the mechanisms behind this potential phenomenon. A variety of techniques used in this thesis allowed for this study to replicate many real-world scenarios involved in opioid induced respiratory depression.

6.1 Acute Effects of Heroin and Fentanyl on Respiration

Chapter 3 has shown that heroin induced respiratory depression in a dose dependent manner. Furthermore, it was also found that it was respiratory rate decreased, in a dose dependent manner, as opposed to tidal volume, thought to be due to prolongation of inspiration via apneustic compensation (Hill et al., 2016, 2018). Heroin at a dose of 10mg/kg i.p. was shown to induce significant respiratory depression. It was presumed that this dose of heroin would not cause hyperlocomotion, which would increase respiration, based on the fact that morphine 10mg/kg i.p. has previously been found not to cause significant hyperlocomotion (Hill et al., 2016). Based on this thesis' findings and previous locomotor studies, the 10mg/kg i.p dose of heroin was chosen as a suitable challenge dose for further experiments. Given more time, locomotion studies on heroin 10mg/kg i.p. could have been undertaken to study whether significant hyperlocomotion occurs. If not, it would be useful to characterise the effect of a higher dose of heroin (e.g. 30mg/kg i.p.) on respiratory depression and on locomotion. If heroin 30mg/kg i.p. was identified as a submaximal dose and did not induce significant hyperlocomotion, this would be useful in tolerance studies. This is because a higher dose of heroin are able to induce a higher degree of tolerance more quickly (Collett, 1998; Tobias, 2000), which could provide useful in further tolerance experiments described in Chapter 6.3.

6-MAM was shown be more potent than heroin. However, 6-MAM was supplied in solution with acetonitrile. Acetonitrile alone caused respiratory depression and thus the comparison between the two drugs was bias towards the potency of 6-MAM. Future experiments would involve obtaining 6-MAM in powder form to dilute in a solution that does not have an effect on respiratory depression to accurately compare its effects versus those of heroin. Morphine, a metabolite of 6-MAM, was once thought to cause heroin's subsequent analgesic and

respiratory depressant actions (Halbsguth et al., 2008). The characterisation of morphine has been conducted and published previously by our lab using the same equipment, showing similar potencies at the doses used (Hill et al., 2016). It is now realised that response to heroin is due to 6-MAM activating the MOPr (Andersen et al., 2009; Boix et al, 2013; Gottås et al., 2013)

To mimic the situation in which someone might inject heroin contaminated with fentanyl responses of each opioid dose was compared and found that when the heroin and fentanyl mixture was injected intravenously in mice it produced a sub-additive response. This could be explained by a ceiling effect, as seen with nalbuphine (Romagnoli et al., 1980). If a ceiling effect is not seen with these mixtures, then the data seen in this thesis disagrees with the current literature. It has been shown the heroin and fentanyl mixtures act synergistically in inducing brain hypoxia (Solis et al., 2017). The sub-additivity of heroin and fentanyl mixtures seen in this thesis does not support this finding. Solis et al investigated brain hypoxia whereas in this thesis, respiratory depression was measured directly. A strength of the study conducted in this thesis is that respiratory depression was directly measured instead of using brain hypoxia to determine whether respiratory depression occurs. Further experiments using higher doses of heroin and fentanyl could be performed to investigate the presence of a ceiling effect, through administering higher doses of mixtures to induce a response greater than the degree of respiratory depression seen in this thesis. Furthermore, another way to address the limitation that a possible ceiling effect constraining the data would be to use smaller doses of heroin and fentanyl mixtures, with an estimated additive response that would be within the already characterised mixture response used in this thesis.

6.2 Ketamine and tianeptine.

Chapter 4 of this thesis investigated the possibility that ketamine or its metabolite 2R,6R-hydroxynorketamine was able to reverse heroin-induced respiratory depression. Previous studies have shown that (S)-ketamine, an NMDA receptor blocker is able to reverse remifentanil induced respiratory depression (Jonkman et al., 2018), and that AMPAkines that potentiate the AMPAr can reverse fentanyl and alfentanil induced respiratory depression (Ren et al., 2006; Oertel et al., 2010). What these studies have in common is the use of fentanyls in inducing opioid induced respiratory depression. It was found that neither ketamine nor its metabolite 2R,6R-hydroxynorketamine reversed heroin-induced respiratory depression. It will be important to test whether using fentanyl to induce respiratory depression whether individual isomers of ketamine (S-ketamine, R-ketamine) or 2S,6S-hydroxynorketamine (the metabolite of S-ketamine) reverses fentanyl induced respiratory

depression. This would indicate whether reversing opioid induced respiratory depression, as seen in the studies mentioned above, is a phenomenon seen only with the fentanyls. Furthermore, by isolating each enantiomer of ketamine and their respective metabolites, this would provide a more thorough understanding of their mechanism as an overdose reversal agent without reducing their analgesic properties. Clinically, used in conjunction with opioid pain therapy, the analgesic and anaesthetic properties of ketamine combined with a possible respiratory stimulating property could provide useful in reducing opioid consumption (Halbsguth et al., 2008). Although ketamine is a drug of potential abuse, it shows little physical addiction unlike opioids (Pal et al., 2002).

The literature suggests that S-ketamine and ketamine can reverse opioid induced respiratory depression (Ren et al., 2006; Oertel et al., 2010; Jonkman et al., 2018). Yet these studies use fentanyl, remifentanil and alfentanil. The literature also shows evidence for muscle stiffness to be induced by the fentanyls (Benthuysen et al., 1986; Weinger et al., 1991; Streisand et al., 1993). Ketamine has been shown to stabilise breathing through genioglossus nerve activity, airway dilation and end-expiratory lung volume (Jordan et al., 2010). An interaction of these effects of ketamine and the muscle rigidity of respiratory muscles induced by fentanyl could explain the claimed reversal seen in the literature. This thesis contributes the use of a non-fentanyl opioid, heroin to this field, where no reversal of respiratory depression was seen. Suggesting that this could be a fentanyl specific phenomenon. Repeating this experiment using fentanyl induced respiratory depression would show to be more conclusive.

The aim of investigating the use of ketamine and 2R,6R-hydroxyketamine was to investigate its effect on heroin induced respiratory depression. In this study ketamine and 2R,6R-hydroxyketamine was administered to heroin and saline pre-treated mice. A limitation is that ketamine and 2R,6R-hydroxynorketamine sole effect was not investigated to see potential respiratory effects. If this experiment was repeated including control animals of heroin and saline pre-treated mice with saline administration would provide useful in investigating the sole effects of these drugs on respiration.

Tianeptine is an atypical antidepressant with AMPAr potentiating at lower doses and MOPr agonism activity at higher doses (Gassaway et al., 2014). Tianeptine was found to induce antinociception and respiratory depression in mice, which could be reversed with the administration of naloxone. This indicates the presence of MOPr agonism activity. For respiratory depression, tianeptine was found to be at least 3-fold less potent than heroin. Due to MOPr activity, this makes tianeptine a potential drug of abuse, especially dangerous for those initiating tianeptine use for its antidepressant effects (Springer and Cubała, 2018).

A limitation faced during studies on tianeptine was the availability of the drug. Due to this, more extensive characterisation was not carried out. A study in the literature showed that a tianeptine intraperitoneal dose of 2mg/kg prevented respiratory depression in morphine pre-treated rats (Cavalla et al., 2015). Ideally, future experiments would include expanding the range of doses used in order to produce a dose-response relationship This would give more of an insight in the low dose AMPAr potentiating and high dose MOPr activation described by Gassaway et al (2014). Furthermore, it would be interesting to investigate whether a sub-additive effect would be seen with tianeptine and heroin mixtures. Perhaps due to more MOPr effect at higher tianeptine doses (relative to AMPAr potentiation), a potential sub-additive effect would become smaller as the dose of tianeptine increases.

6.3 Investigating the redevelopment of heroin tolerance after a period of abstinence.

It is reported by heroin users from the Bristol Drugs Project (BDP); that there is a perceived enhanced rate of tolerance development after a period of abstinence from heroin (Kesten et al., 2020). Qualitative interviews with recovering users at the BDP carried out by Dr Joanna May Kesten, showed that many participants experienced the development of tolerance to heroin induced euphoria more quickly during relapse. Furthermore, tolerance was found to return more rapidly with each abstinence-relapse cycle. This work shows that the psychological tolerance component does play a role in perceived tolerance which encourages the user to increase doses and become even more tolerant, thus feeding into the possible biological component of tolerance. Both aspects play as positive feedback loops and thus can increase the likelihood of overdose. Once the user is comfortable using a high dose of heroin, the next abstinence-cycle with a higher starting dose could prove to be fatal (Kesten et al 2020, Addiction submitted). White and Irvine proposed that tolerance to euphoria induced by opioids developed to a greater extent than tolerance to respiratory depression (White and Irvine, 1999). Thus, by escalating the dose used by users in order to accommodate for euphoric tolerance this may be too high of a dose to accommodate for tolerance to respiratory depression, causing overdose.

It is likely that there are both biological and psychological components to the enhanced development of tolerance following abstinence and relapse. The overall aim of Chapter 5 was to determine whether biological component. Through *in vivo* studies in mice, tolerance to respiratory depression was observed to develop to a greater extent in heroin pre-treated mice compared to saline pre-treated mice. The use of methadone pre-treatment did not cause heroin tolerance to redevelop within the 5 day twice daily injection protocol, this thesis

suggests that a possible mechanism of tolerance redevelopment is via PKC upregulation. A limitation of the use of buprenorphine is that it has high affinity towards the MOPr with a slow dissociation rate (Lewis, 1985). Heroin metabolites, 6-MAM and morphine from the heroin challenge dose are ineffective at displacing buprenorphine. This makes it difficult to distinguish between the presence of tolerance and the antagonist like activity of buprenorphine.

Further studies based on this work should first be aimed at improving the experimental protocol. A robust level of tolerance was not achieved within the current studies, unlike that seen in preliminary data with morphine by Rob Hill (Figure 5.1), presenting a limitation within the current study. Given more time, optimising the experimental protocol would have been done. A morphine pellet seemed to cause more profound tolerance initiation than a mini-pump (Figure 2.2) when investigated by Rob Hill. This in combination with published literature showing that morphine pellets deliver a significantly higher plasma drug concentration than osmotic mini-pumps (McLane et al., 2017), indicates that pellet implantation may be a more suitable method of pre-treating mice. Thus, by obtaining a heroin and methadone pellet in order to pre-treat the mice with would be hypothesised to initiate a more robust degree of initial tolerance.

To improve the experimental protocol used in this thesis the following experiments would aim to:

1) To enhance the initial development of tolerance during the pre-treatment period the dose of heroin in which the mice are pre-treated with should increase.
2) Determine the rate of decay of tolerance on cessation of the pre-treatment phase with heroin, methadone, and buprenorphine. This would ensure that the period of abstinence used in further studies was long enough for the initial tolerance to have been lost before the relapse protocol started, especially important for the use of buprenorphine in this model.
3) Examine different periods of abstinence from 3 -12 days to see if the enhanced tolerance on relapse decays with time during the abstinence period.
4) Increasing the number of days in which heroin was administered in the relapse period. This would enable the full progression of the redevelopment of tolerance development to be characterised, which was not the case in my work.

Further long-term research would them be aimed at characterising the mechanisms underlying the enhanced development of tolerance on relapse. This could be done by determining the effects of PKC, GRK inhibitors or C-Jun N-Terminal kinase inhibitors on the

development of tolerance upon relapse. In combination with this, the brains of mice should be collected in order to quantify levels of PKC isoforms, GRK and arrestins as well as C-Jun N-Terminal kinase at various time points during the drug pre-treatment, abstinence and tolerance redevelopment process. Such studies would be aimed at determining whether prolonged opioid drug treatment and/or subsequent abstinence resulted in the upregulation of PKC isoforms, GRKs/arrestins or C-Jun N-Terminal kinase might contribute to the enhanced development of tolerance on relapse. The hypothesis being that initial treatment of heroin (6-MAM and morphine) might enhance the rate of tolerance redevelopment through PKC upregulation but that the high efficacy agonists methadone and buprenorphine do not (Williams et al., 2013).

Alternatively, the adaptive change underlying tolerance development might involve changes in MOPr expression. Chronic morphine administration has been shown to upregulate mRNA for MOPr-1C1 splice variants which can associate with G_s proteins, which would present as tolerance (Verzillo et al., 2014; Chakrabarti, et al., 2016; Chakrabarti, et al., 2020). Chronic morphine-induced MOPr-1C1 G_s protein association was shown to be abolished by the PKA inhibitor H89.

Administering H89 to mice throughout the heroin pre-treatment is thought to reduce G_s coupling as found to occur with chronic morphine administration (Wang et al., 2016). In heroin pre-treated mice if a significant difference between H89 treated and saline treated mice occurred, this would suggest that MOPr-1C1 G_s protein association could be a factor involved in tolerance development. It would be hypothesised that heroin with H89 pre-treatment would have a slower rate of tolerance redevelopment to respiratory depression compared to saline solution with H89 pre-treated mice.

G_s coupling to MOPr-1C1 has been shown to be a sexually dimorphic event (Chakrabarti et al., 2012; Verzillo et al., 2014), showing bias towards male mice. Including females in tolerance studies would prove beneficial, as results with female mice would mimic the outcome of PKA inhibition. If female mice have a lack of enhanced heroin tolerance redevelopment, due to lower G_s coupled receptor populations, this would further indicate the involvement in splice variants in this phenomenon. This highlights a limitation in the present study which was done on male mice. Including female mice within the data would not only be of scientific benefit but would also represent the female population who are also as affected by opioid addiction.

Based on the unpublished work of Kesten et al, in heroin users for each abstinence-relapse cycle, heroin tolerance is perceived to have a more rapid rate of onset (Kesten et al 2020, Addiction submitted). This provides an interesting route for further study of biological

tolerance. In increasing the abstinence-redevelopment of tolerance phases, this phenomenon could be investigated to see whether there is biological backing to this perceived increase in rate of onset for each relapse cycle.

6.4 Conclusion

Overall, this thesis has looked at a variety of aspects involved in opioid induced respiratory depression. The main findings within this thesis, were that intravenous administration of heroin and fentanyl had a more rapid rate of onset and produced a more potent response to respiratory depression. When simulating heroin being cut with fentanyl on the street, the model used in this thesis showed that heroin and fentanyl mixture produced a sub-additive effect on respiratory depression. In addition to these finding, ketamine and 2R,6R-hydroxynorketamine did not reverse heroin induced respiratory depression and the proposed reversal seen in the literature may be a fentanyl induced respiratory depression phenomenon. Tianeptine has been shown to have MOPr activity by inducing respiratory depression and analgesia. Respiratory depression induced by tianeptine was also shown to be less potent than heroin. Finally, an enhanced rate of the redevelopment of tolerance after a period of abstinence did occur, with indications of being PKC mediated, however a more robust experimental protocol must be developed.

Though there are adjustments to be made to the current experimental design to future experiments, the studies within this thesis has shown promise in understanding opioid induced respiratory depression, whether that be acute effects or chronic tolerance studies.

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