PUBLICATIONS:

PUBLICATION 1:

Naloxone methiodide reverses opioid-induced respiratory depression and analgesia without withdrawal.

Lewanowitsch T & Irvine RJ

European Journal of Pharmacology (2002) 445: 61-67.

The aim of this publication was to determine if naloxone methiodide was effective in reversing opioid induced respiratory depression and analgesia without inducing withdrawal. The mice used in this study were treated either acutely or chronically with high doses of morphine to simulate an opioid overdose situation. In the acute study, male Swiss-Albino mice were injected with 300 mg/kg i.p. morphine, then 40 minutes later administered saline, naloxone or naloxone methiodide and monitored for another 40 minutes. Over the treatment period withdrawal behaviour was monitored continuously, respiratory rate determined every 10 minutes and analgesia tested at the end of the treatment period. In the chronic treatment protocol, this experimental procedure was replicated in mice treated with 300 mg/kg/day i.p. morphine for 5 days.

The primary aim of this project was to determine the effect of peripheral opioid receptor antagonist treatment on opioid induced respiratory depression, but analgesia and withdrawal measurements were taken to investigate a variety of opioid actions. Opioid induced analgesia contains a peripheral component (See Section 1.6.3.1.8 on Page 94), so it was hypothesised that naloxone methiodide would antagonise the analgesia produced by morphine through peripheral

actions. Opioid withdrawal behaviour, however, is thought to be predominantly centrally mediated (See Section 1.6.3.1.5 on Page 86), so it was hypothesised that withdrawal behavioural would only be noted if naloxone methiodide could readily enter the CNS.

Treatment with 300 mg/kg i.p. morphine depressed respiration to 62.1±1.3% of baseline (pre-treatment) respiratory rates. This depression was reversed by both naloxone and naloxone methiodide, with a complete reversal of the effects of morphine with higher doses of both opioid receptor antagonists. Larger doses of naloxone methiodide, however, were required to achieve this effect compared to naloxone, as expected from the results of previous studies (See Section 1.6.2.1 on Page 66). Both naloxone and naloxone methiodide produced a dose dependent decrease in the analgesia produced by morphine administration, but only naloxone induced significant withdrawal in these animals.

Similar results were obtained after the chronic morphine treatment, but the magnitude of withdrawal observed and reversal of the respiratory depression and analgesia by naloxone and naloxone methiodide was lower compared to the acutely treated mice. This may be explained by higher circulating morphine concentrations after this chronic treatment regime or increased opioid dependence in these animals.

These results suggest that the administration of appropriate doses of naloxone methiodide can effectively antagonise the analgesia and decreases in respiratory rate produced by acute or chronic morphine administration, without precipitating

withdrawal. Therefore, peripherally selective opioid receptor antagonists, such as naloxone methiodide, may hold advantages over the current forms of treatment for opioid induced respiratory depression.

This publication is included on pp. 121-126 in the print copy of the thesis in the Barr Smith Library.

It is also available online:

Tanya Lewanowitsch, and Rodney J. Irvine
Naloxone methiodide reverses opioid-induced respiratory depression and analgesia without withdrawal
European journal of pharmacology 445 (2002), pp. 61-67
http://dx.doi.org/10.1016/S0014-2999(02)01715-6

PUBLICATION 2:

Naloxone and its quaternary derivative, naloxone methiodide, have differing affinities for μ, δ, and κ opioid receptors in mouse brain homogenates.

Lewanowitsch T & Irvine RJ

Brain Research (2003) 964: 302-305.

The results shown in the Publication 1 provide evidence that naloxone methiodide may act in the periphery to antagonise the analgesia and decreases in respiratory rate produced by morphine. It also highlighted that larger doses of naloxone methiodide were required to produce the same effects as naloxone. This difference in potency has been shown by other researchers in rat, guinea pig and avian tissue (See Section 1.6.2.1 on Page 66) and is thought to be due to a difference in the affinity of naloxone or naloxone methiodide for opioid receptors. The results of these studies, however, are variable and the affinity of these compounds for opioid receptors have not been determined in the mouse. The aim of this second publication, therefore, was to determine the differences in the affinity of naloxone and naloxone methiodide for μ, δ and κ opioid receptors in mouse brain tissue homogenates.

In this experiment, mouse brain homogenates from untreated animals were incubated with [^{3}H]-DAMGO, [^{3}H]-DPDPE or [^{3}H]-U-69,593 to measure the binding capacity of μ, δ and κ opioid receptors, respectively. The displacement produced by naloxone and naloxone methiodide was then determined using concentrations of these opioid receptor antagonists ranging from $1x10^{-12}$ M to $1x10^{-4}$ M.

Naloxone methiodide was found to have a lower affinity for opioid receptors compared to naloxone, with concentration ratios of 15:1 for μ opioid receptors, 6:1 for κ opioid receptors and 330:1 for δ opioid receptors. This shows that the requirement for higher doses of naloxone methiodide than naloxone, as seen in Publication 1, is most likely due to a difference in the affinity of this quaternary antagonist for opioid receptors and not a result of its reduced ability to interact with centrally located opioid receptors. This study did not address the issue that naloxone methiodide could gain access to centrally located opioid receptors, which will be investigated in Publication 3, but it does confirm that much of the previous literature has used inadequate doses of naloxone methiodide to investigate peripherally mediated opioid effects.

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http://dx.doi.org/10.1016/S0006-8993(02)04117-3

PUBLICATION 3:

Reversal of morphine, methadone and heroin induced respiratory depression and analgesia by the quaternary antagonist, naloxone methiodide.

Lewanowitsch T, Miller JH, Irvine RJ

Drug and Alcohol Dependence (2004) In Review.

The first two publications have demonstrated the efficacy of naloxone methiodide to reverse morphine induced decreases in respiratory rate when administered in doses that take into consideration its lowered affinity for opioid receptors. This next publication sought to determine if the effects observed with naloxone methiodide treatment were due to the entry of naloxone methiodide into the CNS. This has not been clearly defined in the previous literature, and is of particular concern given the high doses of naloxone methiodide administered in these experiments (See Section 1.6.2.3 on Page 69). It is also unclear if the actions of naloxone methiodide are due to the activity of metabolites, which could gain access to central opioid receptor binding sites. It has been suggested that quaternary opioid receptor antagonists are metabolised to their tertiary counterparts (i.e. methylnaltrexone is metabolised to naltrexone), which would result in the antagonism of opioid effects through central mechanisms [Misra *et al.*, 1987].

This first aim was examined by determining the [^{3}H]-DAMGO binding in mouse brain homogenates from animals pre-treated with saline, naloxone or naloxone methiodide for 20 minutes. A decrease in the [^{3}H]-DAMGO binding would signify that the opioid receptor antagonist gained entry into the brain and could compete

with this radioligand for μ opioid receptor binding sites. This was observed after naloxone pre-treatment, but naloxone methiodide binding was not significantly different to that of saline controls. This confirms that naloxone methiodide cannot readily cross the blood brain barrier to produce centrally mediated effects through μ opioid receptors. It also shows that centrally acting metabolites are not responsible for the effects produced over the first 20 minutes after naloxone methiodide administration, as their central activity would have been detected by this method. This is also confirmed by the lack of withdrawal observed in the naloxone methiodide treated animals in Publication 1. Therefore, the respiratory and analgesic effects observed with naloxone methiodide treatment are due to the reversal of peripheral components of these opioid actions.

Morphine was used in Publication 1 as it is considered the 'gold standard' by which all other opioid receptor agonists are compared, and morphine has been used in much of the previous research examining opioid induced respiratory alterations [Hamilton & Baskett, 2000]. This study sought to extend these findings to other opioid receptor agonists to determine if the respiratory and analgesic actions of naloxone methiodide are a general μ opioid receptor effect or are morphine specific. Methadone and heroin were chosen as they are both clinically relevant and differ from morphine in their pharmacokinetics and metabolism.

The metabolism of opioid receptor agonists differs between species, which should be taken into consideration when comparing data between humans and rodents. Mice and rats only produce trace concentrations of M-6-G compared to

humans, which may alter the respiratory and analgesic effects of this compound [Milne *et al.*, 1996]. This also affects the actions of heroin in these animals, as heroin is metabolised rapidly to morphine, which is then glucuronidated [Borg & Kreek, 1998]. Methadone is metabolised in a similar manner in humans and rodents, but the half life of this opioid receptor agonist is much shorter, being approximately 89 minutes compared to 24 hours in humans [Borg & Kreek, 1998; Ling *et al.*, 1981]. Therefore, it would be expected that the duration of effects of this compound would differ between rodents and humans.

There is a lack of information in the current literature comparing the respiratory effects of these three opioid receptor agonists in rodents, so in this study dose response curves were determined and ED_{80} respiratory depressive doses of each opioid receptor agonist calculated. These doses were then used to examine the effects of naloxone and naloxone methiodide on respiratory rate, analgesia and withdrawal. The respiratory depressive ED_{80} doses of each opioid receptor agonist were 9 mg/kg i.p. for morphine, 7 mg/kg i.p. for methadone and 17 mg/kg i.p. for heroin. This indicates that at this dose range, heroin has less respiratory effects compared to morphine and methadone. At these doses, however, heroin produced greater analgesia (89% of the maximum possible effect (MPE)) compared to morphine (44% of MPE) and methadone (46% of MPE). This shows that there are differences between the respiratory depressive and analgesic effects of opioid receptor agonists, and highlights the need for further investigation into the differences between these compounds.

In the next experiment, both naloxone and naloxone methiodide were shown to be effective in reversing the decrease in respiratory rate and analgesia produced by the administration of the ED_{80} doses of morphine, methadone and heroin. As seen with the high doses of morphine in Publication 1, withdrawal was precipitated after naloxone administration, but not with the administration of naloxone methiodide. This confirms that naloxone methiodide is effective in reversing the peripheral effects produced by various μ opioid receptor agonists.

The final aim of this publication was to further characterise the respiratory alterations produced by methadone and their reversal by naloxone and naloxone methiodide. While respiratory rate is a good indicator of respiratory depression, the use of whole body barometric plethysmography enables the measurement of other respiratory parameters, such as tidal volume and minute volume, to fully characterize the changes that occur. In this set of experiments, female C57BL/6J mice were used to confirm that naloxone methiodide was still effective in a different strain and sex of mouse.

The administration of 20 mg/kg i.p. methadone again produced a significant decrease in respiratory rate, but this was compensated by an increase in tidal volume, such that minute volume was unchanged. These alterations were reversed by the administration of naloxone and naloxone methiodide 40 minutes later, as previously shown in this research project. Therefore, naloxone methiodide appears to antagonise the respiratory changes produced by various μ opioid receptor agonists, irrespective of the strain of mouse or sex of animal used.

Publication 3:

This publication extends the current literature to confirm that the actions of naloxone methiodide occur through peripheral, and not central, mechanisms. It also shows that naloxone methiodide is not rapidly metabolised to centrally acting compounds that are responsible for the effects observed. Naloxone methiodide can also reverse the respiratory and analgesic effects of morphine, methadone and heroin without inducing withdrawal. Finally, the use of whole body barometric plethysmography has confirmed that naloxone methiodide is effective in reversing all respiratory alterations produced by μ opioid receptor agonists.

Reversal of morphine, methadone and heroin induced respiratory depression and analgesia by the quaternary antagonist, naloxone methiodide

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Abstract:

Overdose due to opioid induced respiratory depression is a problem with therapeutic or illicit opioid use. This study aimed to determine if the quaternary derivative of naloxone, naloxone methiodide, could reverse morphine, methadone and heroin induced respiratory depression and analgesia. Firstly, brain homogenates from saline, naloxone or naloxone methiodide treated mice were incubated with [^{3}H]-DAMGO. Significantly lower [^{3}H]-DAMGO binding was observed after naloxone treatment, but not after naloxone methiodide treatment, confirming that this opioid antagonist has limited access to central mu opioid binding sites. The effect of naloxone and naloxone methiodide on opioid induced changes in respiratory rate, analgesia and withdrawal were also examined. Respiratory depressive ED_{80} doses of morphine (9 mg/kg i.p.), methadone (7 mg/kg i.p.) and heroin (17 mg/kg i.p.) were determined and used to show that naloxone and naloxone methiodide reversed the respiratory and analgesic effects of these opioid receptor agonists, but only naloxone induced withdrawal. Further investigation using barometric plethysmography found that methadone decreased respiratory rate and increased tidal volume, both of which were reversed by naloxone and naloxone methiodide. Therefore, naloxone methiodide antagonises the respiratory and analgesic effects of a variety of opioid receptor agonists through actions in the periphery. We conclude that naloxone methiodide is able to reverse the respiratory and analgesic actions of opioid agonists without affecting opioid induced withdrawal.

Key words:

naloxone methiodide, respiratory depression, analgesia, opioid withdrawal

1. Introduction:

The potential for opioid overdose in illicit and therapeutic situations is well known, with the primary mechanism being opioid induced respiratory depression (White and Irvine, 1999, Yeadon and Kitchen, 1989). It has also been suggested that due to the potential for adverse effects, in particular respiratory depression, opioid doses administered may be lower than those required to produce adequate analgesia (McNicol et al., 2003, Salvato et al., 2003). Opioids are known to interact with the ventral and dorsal respiratory groups in the medulla region of the brain that are vital to respiration (Ballanyi et al., 1997, Miyawaki et al., 2002). A lack of sensitivity and response to changing physiological conditions, in particular CO_2 concentrations, in the brain regions may also result from the administration of opioids (Florez and Hurle, 1993, Yeadon and Kitchen, 1989).

Our previous investigations into opioid induced respiratory depression and analgesia have shown that, in addition to these well described central mechanisms, opioid effects may also have a peripheral component. The respiratory depressant and analgesic actions produced by high doses of morphine administered acutely or chronically were not only reversed by naloxone hydrochloride (NAL) in a dose dependent manner, but were also reversed by its peripherally selective quaternary derivative, naloxone methiodide (NAL-M) (Lewanowitsch and Irvine, 2002). The effects of NAL-M may possibly be explained by the existence of peripherally located opioid receptors associated with chemoreceptors, stretch receptors or J receptors in the lungs (Willette and Sapru, 1982, Yeadon and Kitchen, 1989, Zebraski et al., 2000). The importance of peripheral actions have already been shown for opioid induced gastrointestinal

and antitussive effects as well as ischaemic pre-conditioning and analgesia (Adcock, 1991, Schultz et al., 1997, Stein, 1995, Yuan et al., 2000).

Many studies support the view that the actions of NAL-M are confined to the periphery (Czapla et al., 2000, Negri et al., 1998, Rohde et al., 1997, Russell et al., 1982, Schultz et al., 1997). A few studies have challenged this view as small concentrations of quaternary opioid antagonists have been detected in brain samples after their peripheral administration in rats, using high performance liquid chromatography (HPLC) and [^{3}H]-labelled antagonist (Kim et al., 1989, Misra et al., 1987). However, methodological limitations in these studies, such as blood contamination of the brain samples, were not considered. It is also not clear from these studies if the metabolites of quaternary opioid antagonists, such as NAL, can enter the central nervous system in high enough concentrations to produce effects (Kotake et al., 1989, Misra et al., 1987). These issues were addressed in this study by the measurement of [^{3}H]-DAMGO binding in brain homogenates from animals pre-treated with NAL or NAL-M. Reduced binding would indicate access of these antagonists, or their metabolites, to central mu opioid receptors at pharmacologically effective concentrations. We hypothesise that this would occur with the administration of NAL, but not NAL-M.

This study also aimed to extend our previous findings to determine if the NAL-M effects observed are morphine specific or occur with different opioid agonists. Methadone and heroin were chosen because of their direct relevance to opioid overdoses and their varied pharmacokinetics and metabolism (Borg and Kreek, 1998, Pacifici et al., 1994). Fatal and non-fatal overdoses have been reported

with the illicit use of heroin and therapeutic use of methadone in opioid maintenance therapies or the treatment of moderate to severe pain (Oliver and Keen, 2003, Walder et al., 2001, Williamson et al., 1997).

The respiratory depression produced by heroin, morphine and methadone in humans is well known, but the respiratory effects of these drugs in rodent models have not been fully characterised. It is, therefore, difficult to determine equipotent respiratory depressive doses for each opioid agonist from the existing literature. This study aimed to compare the respiratory depressant effects of morphine, methadone and heroin to determine a dose of each opioid that produces a similar decrease in respiratory rate (ED_{80} = 80% of the maximum effect). This approach differs from our previous experiments that used high doses of morphine to simulate an opioid overdose situation (Lewanowitsch and Irvine, 2002). These ED_{80} test doses were then used to determine whether NAL or NAL-M administration could reverse the opioid agonist effects on respiratory rate, analgesia and withdrawal.

The final aim of this study was to investigate the changes in respiratory parameters that occur with methadone, and subsequent NAL or NAL-M administration, using whole body barometric plethysmography. Our previous studies have measured changes in respiratory rate in male Swiss Albino mice, but the determination of tidal volume and minute volume can provide a better understanding of the underlying physiological responses. We also extended our observations to female C57BL/6J mice to confirm that NAL-M still is effective, since strain and sex have been shown to influence respiratory and

cardiovascular effects in rodents (Cruz and Rodriguez-Manzo, 2000, Muraki and Kato, 1986).

2. Methods:

2.1 Animals

For Parts 2.2, 2.3 and 2.4, male Swiss Albino mice (27 ± 0.3 g) obtained from Laboratory Animal Services, University of Adelaide, were kept under constant environmental conditions in a 12 hour light-dark cycle with food and water *ad libitum*. For Part 2.5, female C57BL/6J mice (23 ± 0.5 g) were obtained from the Victoria University of Wellington Animal Facility and maintained under the same environmental conditions. Procedures were approved by the University of Adelaide Animal Ethics Committee or the Victoria University of Wellington Animal Ethics Committee, and all observations were conducted in a blind fashion.

2.2 Competition binding of NAL and NAL-M for central mu opioid binding sites:

Mice were injected with NAL (3 mg/kg) or NAL-M (100 mg/kg) and sacrificed by cervical dislocation 20 min later to allow for the peak pharmacodynamic effects of these antagonists to occur. The doses of NAL and NAL-M chosen have previously been shown to produce a similar reversal of opioid effects *in vivo*, and were based on the differences in opioid receptor affinity observed in *in vitro* binding studies (Lewanowitsch and Irvine, 2002, Lewanowitsch and Irvine, 2003, Lewanowitsch et al., 2004). To remove contaminating blood from the brain vasculature the heart was exposed and the animal perfused with saline (50 ml at 5 ml/min) via the left ventricle, with the right atrium cut to allow for drainage (Ovadia et al., 2001). The brain was then removed, homogenised in 10 ml/100 gm 50 mM Tris-HCl buffer (pH 7.4), placed on ice and assayed soon after, using a modified [^{3}H]-DAMGO binding technique described previously (Lewanowitsch

and Irvine, 2003). The brain homogenates (500 □l) were incubated on an orbital shaker at room temperature for 90 min with 100 µl [^{3}H]-DAMGO (final concentration 5.2x10^{-8} M, two times the K_d concentration (Chan et al., 1995)), 100 µl Tris-HCl with 100 µM $MgSO_4$ and 1.3 ml Tris-HCl. After incubation, the solutions were vacuum-filtered through dry glass filter discs (GF/B, Whatman International, England) pre-soaked with 0.5 % polyethylamine and rinsed twice with 4 ml cold Tris-HCl. The filters were then transferred to scintillation vials, 10 ml of scintillation fluid (CytoScint, ICN, Australia) added and the vials left overnight before counting. Protein concentrations in the brain homogenates were determined using the bicinchoninic acid assay and binding data expressed as fmol/mg protein (Smith et al., 1985).

2.3 Effects of morphine, methadone and heroin on respiratory rate and analgesia, and reversal with NAL and NAL-M

During respiratory and withdrawal behaviour monitoring, mice were placed into 20 cm^3 Plexiglass monitoring cages and experimental procedures conducted as described previously (Lewanowitsch and Irvine, 2002). Briefly, the respiratory rate of each animal was determined every 10 min by counting the number of breaths over a 5 s period. Withdrawal symptoms were recorded continuously using the rating of Blasig et al. (1973) and the results from 10 min periods grouped for analysis. To test the effect of treatment on analgesia, animals were placed on a 50 °C hot plate until jumping, paw shaking or paw licking behaviour was observed or until the maximum latency time of 60 s (Suzuki et al., 1997). The % maximum possible effect (% MPE) was determined using the equation % MPE = [(test latency-control latency)/(60-control latency)]x100 (Carmody,

1995). The control latency (17 ± 1 s (*n*=6)) was determined in untreated animals.

In order to determine the doses of morphine, methadone and heroin that produce equivalent effects on respiration, dose response curves were produced using 5 doses of each opioid agonist. These doses ranged from 5-300 mg/kg for morphine, 3-50 mg/kg for methadone and 5-70 mg/kg for heroin. After injection of each opioid, respiratory rate was monitored, % inhibition of respiratory rate was calculated every 10 min and the data obtained 40 min after drug administration used for further analysis. Dose response curves were derived using GraphPad Prism 4.0 non-linear regression and ED_{80} doses (the dose that produced 80 % of the maximum possible effect) determined.

To test the effectiveness of NAL and NAL-M, mice were administered the respiratory depressive ED_{80} dose of morphine, methadone or heroin, then 40 min later treated with 3 mg/kg NAL, 100 mg/kg NAL-M or saline. The respiratory, analgesia and withdrawal data presented were taken 10 min after antagonist administration. Data are expressed as % of baseline (pre-treatment) respiration, % maximum possible effect (MPE) for analgesia or total withdrawal grading over the 10 min period.

2.4 Measurement of respiratory alterations produced by methadone and reversal by NAL and NAL-M using whole body barometric plethysmography

Whole body barometric plethysmography measurements in mice were conducted as previously described using rats (Colman and Miller, 2001). Briefly, the mice

were placed into a sealed plexiglass container (220 ml capacity) connected to an identical reference chamber. Air was heated in a water bath, passed through a humidifier and pumped into the chamber at a rate of 1.5 L/min. Chamber temperature was maintained at 31 °C. Pressure changes between the chambers were detected using a differential pressure transducer (Validyne DP45, USA), the signal passed through a chart recorder (Gould, USA) and collected using MacLab Software (Chart 4v3.1.1, ADInstruments Ltd, New Zealand). During the respiratory measurements, airflow was temporarily stopped (30-60 s) and recordings were calibrated by injecting 0.2 ml of air into the chamber.

Animals were placed into the chamber at least 30 min prior to the initiation of the experiment. They were then injected with methadone (20 mg/kg) to produce maximum respiratory depression then 40 min later administered saline, NAL (3 mg/kg) or NAL-M (30 mg/kg). Rectal temperatures (Cole-Parmer Thermistor Thermometer 8402-20) were taken from these animals prior to methadone administration, 40 min later, but before opioid antagonist injection, and a subsequent 40 min later at the end of the experimental period.

Respiratory measurements were taken every 5 min with respiratory rate being determined from the duration of three consecutive breaths. Respiratory peak height was determined by averaging the peak heights of these three consecutive breaths, and tidal volume was calculated using the Drorbaugh-Fenn equation (Colman and Miller, 2001). All measurements are expressed as % change from baseline (pre-treatment) values.

2.5 Drugs

[^{3}H]-DAMGO ([^{3}H][d-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin) was purchased from PerkinElmer Life Sciences, Australia. Morphine hydrochloride and methadone hydrochloride were purchased from GlaxoSmithKline (Australia) and heroin hydrochloride (diacetylmorphine) was obtained from Australia Government Analytical Laboratories (Australia). NAL and NAL-M were purchased from Mayne Pharma (Australia) and Sigma-Aldrich (Australia), respectively. Morphine, methadone, NAL and NAL-M were all dissolved in saline (0.9 % NaCl), while heroin was dissolved in 2.5 mM HCl in saline (to increase its solubility), and used immediately to reduce the extent of degradation. All injections were administered i.p. in a volume of 5 ml/kg.

2.6 Statistics

All results are presented as mean ± S.E.M. with significance set at $P<0.05$. Data were analysed with GraphPad Prism 4.0 for Windows using a one-way ANOVA with Tukey's Post-Hoc test, except for the withdrawal gradings, which were analysed using a Kruskal-Wallis test with Dunn's Post-Hoc test.

3. Results:

3.1 Competition binding of NAL and NAL-M for mu opioid binding sites in brain homogenates

Figure 1 shows the results of [^{3}H]-DAMGO binding in mouse brain homogenates prepared from animals pre-treated with saline, NAL or NAL-M. NAL pre-treatment produced a significant decrease in binding compared to saline controls, indicating that NAL was present in the brain tissue and could compete with the [^{3}H]-DAMGO for mu opioid receptor binding sites. Binding in the NAL-M brain homogenates was not significantly different from that of the saline treated animals, despite the NAL-M dose administered producing a similar antagonism of opioid induced respiratory depression and analgesia as the NAL dose. These results confirm that NAL can readily enter the brain and bind to mu opioid receptors after peripheral administration, whereas NAL-M, or its metabolites, cannot.

3.2 Dose response effects of morphine, methadone and heroin and reversal with NAL and NAL-M

Table 1 shows the data obtained from the dose response curves produced for morphine, methadone and heroin. At the highest doses tested, all opioid agonists produced 100% of the maximum possible analgesic effect (MPE), whereas the maximum respiratory depression was approximately 50% for each opioid agonist. The ED_{50} values for respiratory depression were found to be similar for morphine, methadone and heroin, while the Hill slope for heroin was lower than for morphine or methadone. This difference was not significant, however, due to the high variability associated with the morphine dose response curve. The

respiratory depressive ED_{80} doses determined from these data were 9 mg/kg for morphine, 7 mg/kg for methadone and 17 mg/kg for heroin.

These ED_{80} doses were then administered and their respiratory depressive and analgesic effects measured 40 min later. Each of the opioid agonists produced a decrease in respiratory rate to approximately 60% of baseline (pre-treatment = 100%), while the analgesia was significantly greater after heroin administration (80% of MPE) than morphine or methadone treatment (approx. 45% of MPE). This shows that these opioid agonists differ in their ability to reduce respiratory rate, since higher concentrations of heroin were required to produce the same effect. The analgesic potential at the ED_{80} doses also differed for the three agonists.

Once the ED_{80} values for respiratory depression for morphine, methadone or heroin were determined, these equipotent doses were administered to measure the effect of NAL and NAL-M treatment on respiratory rate, withdrawal and analgesia. Respiratory rate, measured 40 min after injection, was decreased in the presence of each opioid agonist ($P<0.001$ for morphine, methadone and heroin). Respiratory rate remained depressed after 10 min in the saline treated animals, but was significantly increased by both NAL and NAL-M administration (Figure 2a).

Figure 2b) shows the effect of opioid antagonist treatment on the analgesia produced by morphine, methadone and heroin. After 10 min, saline treatment did not significantly alter the analgesia produced by these opioid agonists (See Table

1 for opioid agonist % MPE values); whereas, both NAL and NAL-M treatment significantly reversed the opioid induced analgesia.

Figure 2c) shows the withdrawal behaviour over the 10 min of antagonist treatment. Significant withdrawal was noted in the NAL treated animals, while the behavioural signs observed with NAL-M treatment were not significantly different to the saline treated controls. This would again suggest that NAL-M has limited access to the central nervous system, as withdrawal behaviour has been shown to be mediated predominantly by direct effects on the brain (Rohde et al., 1997, Russell et al., 1982).

3.3 Measurements of respiratory alterations produced by methadone and reversal by NAL and NAL-M using whole body barometric plethysmography

We further investigated the changes in respiratory parameters that occur with opioid agonist and subsequent antagonist administration using whole body barometric plethysmography (Figure 3). In these animals, respiratory rate was decreased to 51 ± 3 % of baseline 40 min after the administration of 20 mg/kg methadone. NAL (3 mg/kg) and NAL-M (30 mg/kg) completely reversed the depression of respiratory rate back to untreated values, while respiratory rate remained depressed after saline treatment (Figure 3a).

Although respiratory rate was decreased 51% with methadone administration, tidal volume increased 54 ± 11% 40 min after methadone treatment ($P<0.001$; 0 min vs. 40 min)(Figure 3b). NAL and NAL-M were again effective in reversing this increase in tidal volume, while saline treatment produced no alterations. NAL

reversed this methadone induced increase in tidal volume for the 80 min period; whereas, the tidal volume in the NAL-M administered animals began to increase after 20 min, but did not differ significantly from the pre-methadone tidal volume values. The reciprocal changes in respiratory rate and tidal volume resulted in no significant changes in minute volume either over the treatment period or between the antagonist treatments (Figure 3c).

4. Discussion:

In this study, NAL-M reversed the decrease in respiratory rate and increase in analgesia produced by different opioid agonists, without the withdrawal observed with NAL administration. Methadone administration produced a decrease in respiratory rate and corresponding increase in tidal volume, which were antagonised by both NAL and NAL-M. The inability of peripherally administered NAL-M to compete with [^{3}H]-DAMGO binding to brain homogenates suggests that NAL-M cannot cross the blood-brain barrier in significant concentrations to block binding to mu opioid receptors, thus indicating that it can act at peripheral sites to mediate its effects on respiration and analgesia. It is likely that the peripheral action of NAL-M occurs via peripheral chemoreceptors and pulmonary stretch receptors or J receptors to alter central respiratory mechanisms (Willette and Sapru, 1982, Yeadon and Kitchen, 1989, Zebraski et al., 2000). Our binding experiments with [^{3}H]-DAMGO also show that NAL-M is not metabolised to other centrally active compounds, such as NAL, since these metabolites would have reduced [^{3}H]-DAMGO binding in a manner similar to NAL.

In the dose response studies, the maximum decrease in respiratory rate observed for morphine, methadone and heroin was approximately 50%. Several other researchers have also reported an upper limit on the inhibition of respiration in rats, and that this limit is maintained until the opioid dose becomes high enough to cause generalised toxicity, with convulsions and sudden death (Isom et al., 1969, Kokka et al., 1965). This toxicity associated with high opioid doses may also be present in humans, but no data exist to confirm this, partly because opioids are not administered in doses high enough to produce severe respiratory

depression, and treatment is rapidly initiated in overdose situations without the measurement of these parameters. Despite all of the opioids producing a similar level of maximum respiratory depression, the ED_{80} doses were not the same. Heroin had a 2-fold higher ED_{80} for respiratory rate, as reflected in its lower Hill slope compared with morphine and methadone. The greater variation of morphine responses may be due in part to the generalised toxicity and severe excitation associated with higher doses of morphine (Pazos and Florez, 1984).

Although the respiratory ED_{80} doses of morphine, methadone and heroin produced a similar depression of respiration, their analgesic effects were different. After administration of the respiratory ED_{80} doses, heroin produced a much greater analgesic effect compared to morphine and methadone. These results support the concept that the respiratory depression and analgesia produced by opioid agonists are mediated differently, and highlight that the results obtained from analgesia studies should not be used to predict the respiratory depressive effects of opioids (Czapla et al., 2000, Ling et al., 1983, Stott and Pleuvry, 1991).

Both the centrally acting NAL and the peripherally acting NAL-M reversed the decreases in respiratory rate produced by the ED_{80} doses of the three opioid agonists. The reversal produced by NAL-M, however, occurred without the precipitated withdrawal associated with NAL, regardless of the opioid agonist administered. Therefore, it appears that NAL-M can effectively reverse opioid induced respiratory depression with reduced incidence of withdrawal symptoms, presumably because of its peripheral rather than central action. NAL and NAL-M

were also effective in reversing the analgesia produced by morphine, methadone and heroin. This is in line with previous evidence, which indicates that peripheral mechanisms contribute significantly to the antinociceptive effects of opioids (Junien and Wettstein, 1992, Stein, 1993, 1995). Taken together, our results show that NAL-M is effective in reversing the respiratory depression and analgesia produced by equipotent doses of opioids with unique pharmacokinetic and metabolism characteristics.

This reversal of analgesia makes NAL-M inappropriate for the prevention or treatment of adverse effects in patients receiving opioids for pain relief. The possibility of its use, or that of other peripherally acting opioid antagonists, still exists in illicit overdose situations or in patients using opioids for non-analgesic purposes, such as those on opioid maintenance therapies. This is particularly relevant given that the reversal of opioid effects by NAL-M occurs without the induction of withdrawal, an unwanted side effect that accompanies the use of NAL in overdose situations (Seal et al., 2003).

In the final part of the present study, we used barometric plethysmography to show that opioid doses sufficient to inhibit respiratory frequency by 50% did not alter ventilation due to a compensatory increase in tidal volume. A similar compensatory increase in tidal volume has been seen with i.c.v. administration of dermorphin in rats, i.v. administration of morphine and oxycodone in humans and s.c. administration of opioids in neonatal rat pups (Colman and Miller, 2002, Leino et al., 1999, Vonhof and Siren, 1991). In these studies, the increased tidal volume was unable to fully compensate for the frequency decrease, and thus

minute volume was generally depressed after opioid treatment, as reported for most opioid studies on respiration (Isom et al., 1969, van den Hoogen and Colpaert, 1986). Several other studies have, however, observed either no effect or an increase in minute volume with the administration of various doses of opioid agonists, highlighting the fact that some respiratory effects of opioids remain unresolved (Czapla et al., 2000, Kokka et al., 1965, Negri et al., 1998). The results produced after methadone administration may be due to its ability to act on both opioid and non-opioid (NMDA receptor) sites and will require further investigation to elucidate the mechanisms involved (Ebert et al., 1995, Gorman et al., 1997).

The administration of 3 mg/kg NAL and 30 mg/kg NAL-M reversed both the decrease in respiratory rate and the compensatory increase in tidal volume, resulting in no overall change in minute ventilation. The dose of NAL-M required to produce this reversal in the female C57BL/6J mice (30 mg/kg)(Figure 3) was lower than that administered to male Swiss Albino mice (100 mg/kg)(Figure 2), indicating that the strain or sex of mouse used may influence the peripheral effects of opioid antagonists. This has not been previously reported and should be investigated more thoroughly. A difference in the duration of the effects on tidal volume was also observed between NAL and NAL-M. NAL continued to decrease tidal volume for the full 80 min of treatment, whereas, after 60 min, tidal volume began to increase in the NAL-M treated animals. This may be explained by differences in the pharmacokinetics of these two drugs, as well as differences in response between central and peripheral sites of action.

We conclude that NAL-M is a peripherally acting opioid antagonist that is capable of reversing opioid effects, such as respiratory depression and analgesia, without entering the central nervous system in concentrations high enough to significantly bind to mu opioid receptor sites. The peripheral mechanism involved in opioid induced respiratory effects is currently unconfirmed, but is most likely due to interactions with opioid receptors associated with peripheral nerve endings in the lungs, which could transmit signals to the brain regions to alter respiration. This interaction with peripheral nerve terminals has previously been shown to contribute to opioid induced effects on analgesia and the cough reflex (Adcock, 1991, Stein, 1995). By interacting with these peripheral sites, NAL-M could antagonise the effects of respiration and analgesia as effectively as NAL without having to enter the brain, thus reducing its ability to antagonise opioid effects that require direct interaction with sites in the brain, such as those involved in withdrawal. It is also surprising that NAL-M can completely reverse the respiratory and analgesic effects of opioid agonists through peripheral mechanisms. However, similar results that suggest a major role for peripheral mechanisms in the actions of opioids has been demonstrated in other analgesic tests (Binder et al., 2001, Binder and Walker, 1998, Hong and Abbott, 1995, Shannon and Lutz, 2002). It is clear that these peripheral mechanisms are currently not well understood, but further research will clarify their role in opioid modulation of breathing and analgesia.

Our study has shown that morphine, methadone and heroin have different potencies with regard to their analgesic and respiratory effects, but NAL-M is capable of antagonising these effects without inducing withdrawal. The whole

body barometric plethysmography results indicate that methadone decreases respiratory rate with a corresponding increase in tidal volume, and these responses are reversed by NAL and NAL-M. Overall, these results extend our previous studies and indicate that NAL-M is an effective drug for reversing opioid induced respiratory depression and analgesia without inducing withdrawal in mice of different strains and sexes.

Publication 3:

Acknowledgements:

The authors are grateful to Dr Anne La Flamme for the supply of C57BL/6J mice. Tanya Lewanowitsch was funded by an NHMRC Dora Lush Postgraduate Scholarship. These experiments were supported by a University of Adelaide Small Grant, the Victoria University of Wellington, the Wellington Medical Research Foundation and the Lottery Health Board of New Zealand.

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Figure 1:
Change in [^{3}H]-DAMGO binding to saline perfused brain homogenates after treatment with saline (SAL), 3 mg/kg naloxone hydrochloride (NAL) or 100 mg/kg naloxone methiodide (NAL-M). ***P<0.001 compared to SAL, $^{\#\#}$P<0.01 compared to NAL (*n*=4) using one-way ANOVA with Tukey's Post Hoc Test.

Table 1:

Summary of data from morphine, methadone and heroin dose response curves 40 minutes after administration.

The table shows the maximum analgesia and inhibition of respiratory rate at the highest doses of opioid agonists administered, the ED_{50}, Hill Slope and ED_{80} calculated from the respiratory rate dose response curves and the decrease in respiratory rate and analgesia produced by the ED_{80} doses of each opioid agonist. Comparisons between each opioid agonist were performed using a one-way ANOVA with Tukey's Post Hoc Test (*n*=4 for dose response curves and *n*=18 for ED_{80} induced changes in respiratory rate and analgesia). ***P<0.001 compared to heroin.

Figure 2:

Changes in respiratory rate (a), analgesia (b) and withdrawal (c) 10 min after naloxone (NAL) or naloxone methiodide (NAL-M) administration. Morphine (9 mg/kg), methadone (7 mg/kg) and heroin (17 mg/kg) were administered 40 min prior to antagonist administration. *P<0.05, **P<0.01, ***P<0.001 compared to saline (*n*=6 for each group) using one-way ANOVA with Tukey's Post Hoc Test for respiratory rate and analgesia and Kruskal-Wallis Test with Dunn's Post Hoc Test for withdrawal behaviour.

Figure 3:

Effect of 20 mg/kg methadone (METH) then saline (SAL, ■), 3 mg/kg naloxone (NAL, ▼) or 30 mg/kg naloxone methiodide (NAL-M, ○) treatment on a) respiratory rate, b) tidal volume and c) minute volume. *P<0.05, **P<0.01, ***P<0.001 compared to SAL (*n*=4 for each group) using one-way ANOVA with Tukey's Post Hoc Test.

FIGURE 1:

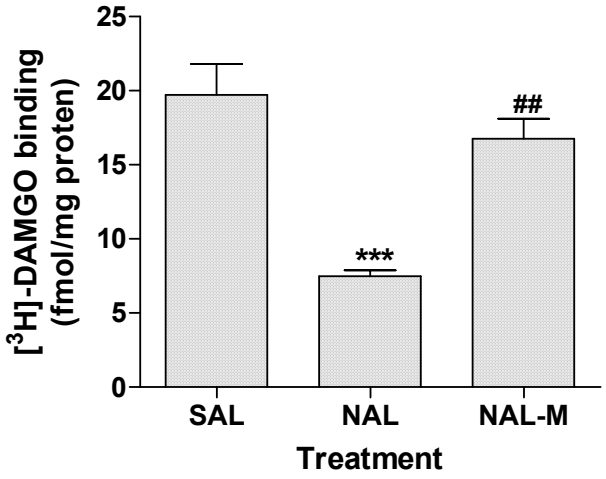

[3H]-DAMGO binding
(fmol/mg proten)
25
20
15
10
5
0
***
##
SAL
NAL
NAL-M
Treatment

TABLE 1:

	Morphine	Methadone	Heroin
Maximum analgesia (% MPE)	100 ± 0	100 ± 0	100 ± 0
Maximum ↓ respiratory rate (% baseline)	45.4 ± 2.9	50.4 ± 3.6	52.1 ± 1.3
Respiratory curve ED_{50} (mg/kg)	4.6 ± 0.8	3.6 ± 0.4	5.3 ± 0.6
Respiratory curve Hill Slope	2.8 ± 1.1	2.1 ± 0.4	1.2 ± 0.1
Respiratory curve ED_{80} (mg/kg)	9 mg/kg	7 mg/kg	17 mg/kg
ED_{80} ↓ respiratory rate (% baseline)	62.0 ± 0.6	61.8 ± 0.6	60.4 ± 0.6
ED_{80} induced analgesia (% MPE)	44.2 ± 5.8***	45.5 ± 5.1***	89.0 ± 4.4

FIGURE 2:

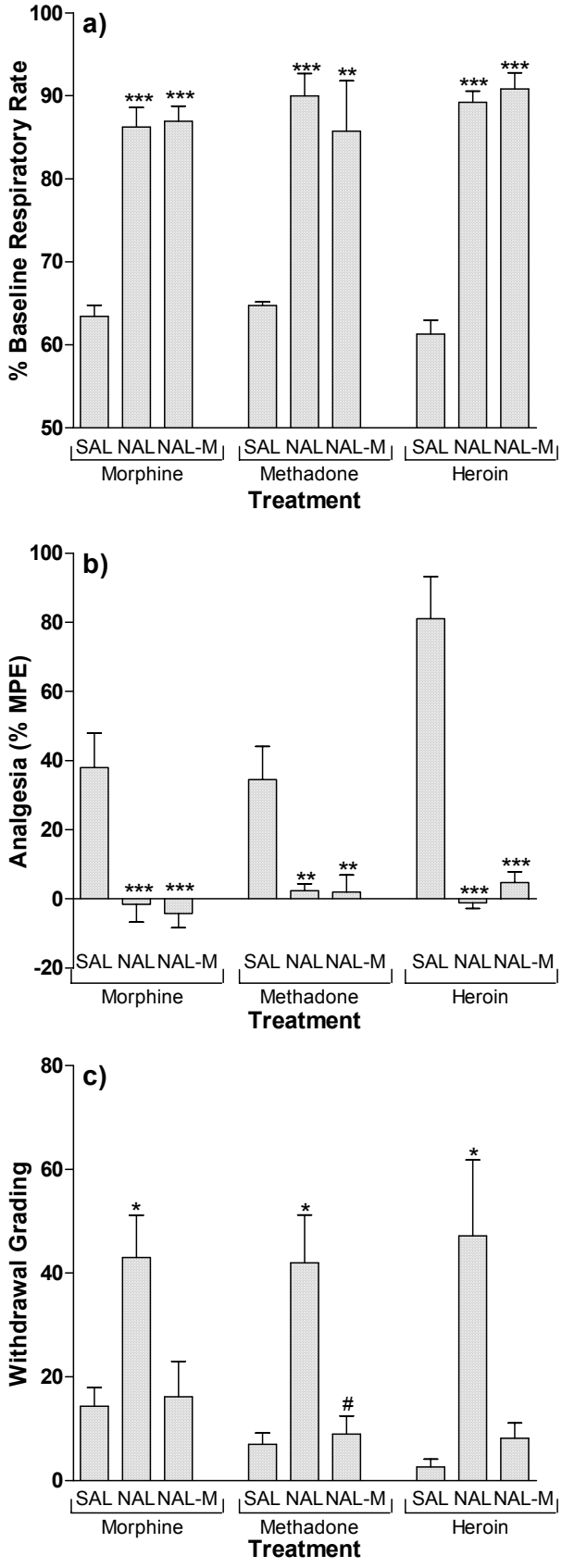
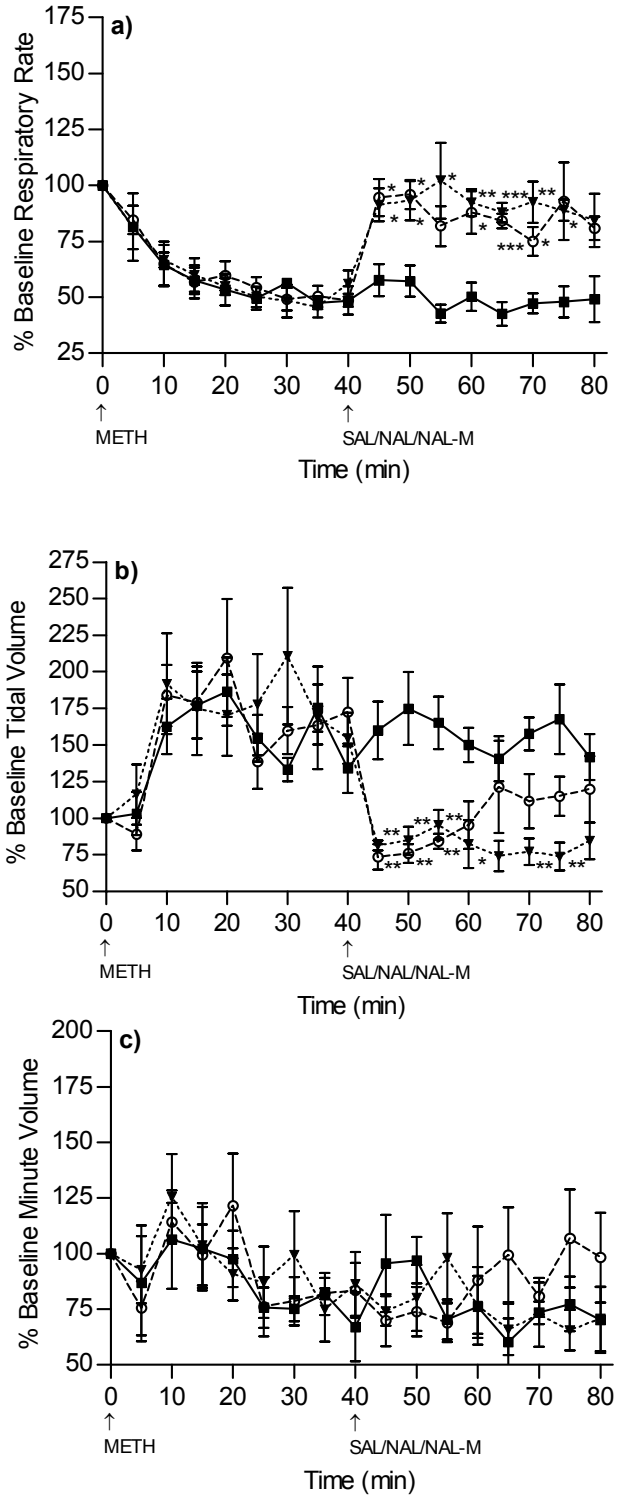

a)
% Baseline Respiratory Rate
100
90
80
70
60
50
***
***
***
**
***
***
SAL NAL NAL-M
Morphine
SAL NAL NAL-M
Methadone
SAL NAL NAL-M
Heroin
Treatment
b)
Analgesia (% MPE)
100
80
60
40
20
0
-20
***
***
**
**
***
***
SAL NAL NAL-M
Morphine
SAL NAL NAL-M
Methadone
SAL NAL NAL-M
Heroin
Treatment
c)
Withdrawal Grading
80
60
40
20
0
*
*
*
#
SAL NAL NAL-M
Morphine
SAL NAL NAL-M
Methadone
SAL NAL NAL-M
Heroin
Treatment

FIGURE 3:


a)
% Baseline Respiratory Rate
175
150
125
100
75
50
25
0
10
20
30
40
50
60
70
80
METH
SAL/NAL/NAL-M
Time (min)
b)
% Baseline Tidal Volume
275
250
225
200
175
150
125
100
75
50
0
10
20
30
40
50
60
70
80
METH
SAL/NAL/NAL-M
Time (min)
c)
% Baseline Minute Volume
200
175
150
125
100
75
50
0
10
20
30
40
50
60
70
80
METH
SAL/NAL/NAL-M
Time (min)

PUBLICATION 4:

Use of radiotelemetry to evaluate respiratory depression produced by chronic methadone administration.

Lewanowitsch T, White JM, Irvine RJ

European Journal of Pharmacology (2004) 484: 303-310.

The previous publications presented have shown that the peripherally acting opioid receptor antagonist, naloxone methiodide, can reverse the respiratory and analgesic effects of μ opioid receptor agonists without inducing centrally mediated adverse effects, such as withdrawal. The two aims of this final publication were to extend the current knowledge of opioid induced respiratory depression by utilising new radiotelemetry technology and to test the efficacy of naloxone methiodide using a chronic opioid administration regime in rats.

Until now there have been several limitations in obtaining respiratory data in small animals during chronic opioid treatment regimes, which have limited the number of publications and knowledge of the alterations that occur (See Section 1.4.2.2 on Page 26). The techniques required to measure respiration, such as plethysmography or pneumotachography, involve moving animals from their natural environments into potentially stressful environments. As stress can affect respiration and endogenous opioid systems, this may influence the respiratory changes observed with exogenous opioid administration [Vaccarino & Kastin, 2001]. These methods are also limiting as they do not facilitate the long term measurement of respiratory parameters, as the animals cannot remain in the required apparatus for extended periods of time.

The first aim of this publication was to explore the potential of measuring changes in respiration using radiotelemetry implants with newly designed software to determine respiratory rate from blood pressure signals [Kramer *et al.*, 1999]. Our laboratory has extensive experience with the use of radiotelemetry to measure cardiovascular changes and this advancement in technology now allows for the continuous (every minute) measurement of respiratory and cardiovascular parameters of animals in their normal environment and without stress artefacts [Chan *et al.*, 1999; Irvine *et al.*, 2000]. This has not been possible with the hitherto techniques available.

After insertion of the radiotelemetry implants and baseline measurements, the rats were implanted with osmotic mini-pumps filled with methadone such that 30 mg/kg was continuously delivered over each day of treatment. This model was designed to replicate a methadone maintenance or pain treatment protocol in humans, where steady state concentrations are desired. Over the next 4 days, the animals were left undisturbed and cardiorespiratory parameters recorded continuously. Over the subsequent 3 days, the animals were injected with either saline, naloxone or naloxone methiodide and the average response determined after 40 minutes.

This novel radiotelemetry technique showed that differences in the average respiratory rate and heart rate occurred between night and day periods and that while these parameters were depressed during methadone treatment, tolerance to these effects occurred more rapidly over the night periods. Locomotor activity

and mean arterial blood pressure were greater at night than during the day, which highlights that the normal circadian rhythms were not affected by the methadone administration.

The administration of naloxone and naloxone methiodide produced the expected increase in respiratory rate and heart rate also significantly increased with opioid receptor antagonist treatment. Locomotor activity was only significantly increased after naloxone administration, while mean arterial blood pressure was unaffected by these treatments.

These results again confirm that naloxone methiodide can effectively reverse the decreases in respiratory rate produced by opioid receptor agonists, such as methadone, particularly when used in a treatment regime similar to that employed during therapeutic opioid administration in humans. The use of this radiotelemetry technique has identified, for the first time, a difference in the development of tolerance to the respiratory and cardiovascular alterations that occur with chronic opioid receptor agonist administration between day and night periods. This highlights the usefulness of radiotelemetry as a technique to further investigate opioid induced respiratory depression and its treatment or prevention.

This publication is included on pp. 174-181 in the print copy of the thesis in the Barr Smith Library.

It is also available online:

Tanya Lewanowitsch, Jason M. White and Rodney J. Irvine
Use of radiotelemetry to evaluate respiratory depression produced by chronic methadone administration
European journal of pharmacology 484 (2004), pp. 303-310
http://dx.doi.org/10.1016/j.ejphar.2003.11.025

DISCUSSION:

The main aim of the four publications presented in this thesis was to determine if naloxone methiodide could effectively reverse opioid induced respiratory depression. This research has shown that this opioid receptor antagonist can reverse the respiratory changes induced by morphine, methadone and heroin after acute and chronic treatment regimes in male Swiss-Albino mice, female C57BL/6J mice and male Sprague-Dawley rats. These experiments have also identified that naloxone methiodide can reverse the analgesia produced by these opioid receptor agonists and, unlike naloxone, does not precipitate significant withdrawal. Further investigation has shown that whilst naloxone methiodide is an effective opioid receptor antagonist it does require the administration of larger doses than its tertiary counterpart. This is due to its lowered affinity for opioid receptors. All of the effects observed, however, occur without naloxone methiodide, or its metabolites, interacting with centrally located μ opioid receptors.

The concept that some opioid actions involve peripheral mechanisms is not new, and naloxone methiodide has been previously used to examine this. An example is research that has shown the effectiveness of quaternary opioid receptor antagonists in reversing opioid induced constipation [Schmidt, 2001; Yuan *et al.*, 1996; Yuan *et al.*, 2000]. There are, however, deficiencies in much of the previous data, which have often led to incorrect conclusions regarding the efficacy of these quaternary opioid receptor antagonists and their selectivity for peripheral sites of action. The findings produced from the publications presented in this thesis will be discussed in relation to these limitations.

1. Little consideration has been given to the potency differences between naloxone and naloxone methiodide.

The differences in potency between naloxone and naloxone methiodide were addressed in each publication as higher doses of naloxone methiodide compared to naloxone were administered in these experiments. These studies have highlighted that adequate dosing of naloxone methiodide results in significant antagonism of opioid induced respiratory depression and analgesia.

Publication 2 is the first to show that naloxone methiodide has a lower affinity for μ, δ and κ opioid receptors in murine brain homogenates. Previous studies have identified that naloxone methiodide has less potency than naloxone due to its lack of affinity for opioid receptors [Deviche, 1997; Magnan *et al.*, 1982]. The results from these studies, however, have been variable and have not used tissue derived from the mouse. It was important to determine these affinities in mouse tissue as species differences have been noted and the use of mice is increasing with the development of knockout strains in the 'post-genomic era' of research. The majority of the research conducted in this project has also used mice, so quantification of the affinity differences of naloxone and naloxone methiodide for opioid receptors in murine tissue was required.

The results obtained in this publication confirmed those of Deviche [1997] and Magnan *et al.* [1982] who found that naloxone methiodide had less affinity for avian and guinea pig opioid receptors than naloxone. The K_i binding ratios for naloxone compared to naloxone methiodide, however, differed for each opioid

receptor investigated. No comparison could be made for the κ opioid receptor ratios, as this is the first study that used the specific κ opioid receptor radiolabel [^{3}H]-U-69,593. For the μ opioid receptor binding, a similar binding ratio of 15:1 was observed in the mice tissue, compared to 12:1 in avian tissue and 10:1 in guinea pig tissue. The variation in the δ opioid receptor ratios differed greatly between these studies, with a ratio of 330:1 in mouse tissue, compared to the results of Deviche [1997] who observed a difference of 27:1 in avian tissue and Magnan *et al.* [1982] who reported a ratio of 1078:1 in guinea pig brain homogenates. These differences may be due to experimental variation, but are more likely to be due to species differences in the concentrations and distributions of these opioid receptors [Robson *et al.*, 1985]. Therefore, this publication has shown that the affinity of naloxone and naloxone methiodide for μ, δ and κ opioid receptors differ, as does the binding of these compounds to opioid receptors, in different species.

2. Lack of confirmation that naloxone methiodide is acting solely at peripheral sites.

Without definitive evidence regarding the site of action of naloxone methiodide, conclusions cannot be drawn from the results that have so far been obtained using this compound. If naloxone methiodide can enter the CNS, the conclusions of the studies that have used naloxone methiodide to confirm that some opioid actions involve peripheral components would be incorrect. Likewise, if naloxone methiodide can enter the brain, its usefulness in the treatment of peripheral opioid actions without affecting central opioid effects would be limited. While it is generally thought that naloxone methiodide does not have ready access to the

CNS, this has been based on inconclusive results and required further investigation.

Two separate results in this thesis have provided evidence that naloxone methiodide does not readily enter the CNS. Firstly, in Publications 1 and 3, naloxone methiodide administration to opioid treated mice did not precipitate significant withdrawal, compared to that observed after naloxone treatment. As withdrawal is predominantly centrally mediated, this does provide evidence that naloxone methiodide is either unable to enter the CNS or does not reach the brain regions that are responsible for this effect.

Further confirmation that naloxone methiodide cannot readily enter the brain was provided in Publication 3, where the changes in [^{3}H]-DAMGO binding in the brain was measured in mice pre-treated with saline, naloxone and naloxone methiodide. In this experiment, care was taken to remove the blood contamination from the brain tissue analysed. This has not been considered in previous studies and may be the cause of small traces of the quaternary antagonists being detected in brain tissue [Kim *et al.*, 1989; Misra *et al.*, 1987].

The results in this publication have provided the best evidence so far to show that naloxone methiodide, or its metabolites, cannot easily access centrally located μ opioid receptor binding sites. Therefore, it can be assumed that the effects of naloxone methiodide on respiration, analgesia and withdrawal are due to its activity in the periphery. This is a significant finding, but should be further examined by measuring the concentrations of naloxone methiodide in different

regions of the brain after perfusion, using techniques such as HPLC and autoradiography.

These results show naloxone methiodide does not readily enter the brain but an alternative site of action should be determined. A peripheral site of action has been identified for the reversal of opioid induced analgesia, as opioid receptor binding sites have been isolated on dorsal root ganglia, the central terminals of primary afferent neurons and peripheral sensory-nerve fibres and their terminals [Junien & Wettstein, 1992; Stein, 1995]. As described previously, respiration is mediated via a number of peripheral mechanisms, so naloxone methiodide could act at several sites (See Section 1.5.2 in Page 45). If opioid receptor binding sites exist in the lungs, naloxone methiodide would be able to antagonise the binding of opioid receptor agonists at this site, therefore reversing any respiratory effects. Morphine has been shown to reduce the activity of peripheral chemoreceptors, so naloxone methiodide could prevent this lack of response by displacing opioid receptor agonists from these sites [Berkenbosch *et al.*, 1997]. Morphine and naloxone can interact with J receptors and stretch receptors, so naloxone methiodide could also act at these sites to antagonise the actions of opioid receptor agonists, thereby reversing opioid induced respiratory depression [Delpierre *et al.*, 1995; Willette & Sapru, 1982].

In this research project the possibility of naloxone methiodide interacting with opioid receptor binding sites in the lung tissue was investigated. Rat and mouse lung tissue samples were examined using the radioligand binding methodology from Publication 2 and that of Bhargava *et al.* [1997] who reported the

measurement of μ, δ and κ opioid receptor binding in lung homogenates. Unfortunately, a significant difference between the total and non-specific binding for each opioid receptor radioligand was not achieved, so the binding of either naloxone or naloxone methiodide to opioid receptors in lung tissue could not be determined (unpublished results). This was not surprising as the research that has been conducted investigating lung opioid receptor binding sites has led to the hypothesis that they do not behave as classical opioid receptors and may contain a unique type or subtype of opioid receptor (See Section 1.5.2.1.2. on Page 47). The results published by Bhargava *et al.* [1997] did show a significant difference between the opioid receptor binding of normal and sensitised lung tissue but did not confirm that the binding measured was specific for each opioid receptor type and not a result of changes in non-specific binding.

This area of research, therefore, remains incomplete and requires further examination, which was beyond the scope of this project. Further investigation would most likely utilise techniques, such as western blotting to identify opioid receptor subtypes or the sequencing of sections of DNA or RNA specific for various opioid receptor subtypes. It would also be of interest to determine if other closely related receptors, such as the NOP receptors, are present in lung tissue. These receptors have been implicated in the modulation of baroreflex sensitivity, asthma and the cough reflex [Cabot *et al.*, 1994; Cabot *et al.*, 1996; Corboz *et al.*, 2001; Groneberg & Fischer, 2001; Mao & Wang, 2000]. If classical μ, δ and κ opioid receptors are not present in lung tissue, these NOP receptors may be responsible for many of the effects observed, an hypothesis that is confirmed by the non-conventional behaviour of opioid receptor agonists and antagonists in

this tissue [Cabot *et al.*, 1994; Cabot *et al.*, 1996]. This interaction, however, remains to be investigated, as does the site of action of naloxone methiodide in the periphery.

3. The doses of opioid receptor agonists administered are often below those which produce opioid related adverse effects, in particular respiratory depression.

To test the efficacy of naloxone methiodide in reversing opioid induced respiratory depression, attempts were made in Publication 1 to simulate an overdose situation by the administration of a high dose of morphine (300 mg/kg i.p.). This dose produced a maximum decrease in respiratory rate without resulting in the death of these animals. Morphine doses as high as this have not been administered to test the effect of naloxone methiodide treatment. It was vital to test this high dose, as it is not known if any additional mechanisms are involved when high doses of opioid receptor agonists are administered. It was somewhat surprising to find that this high dose of morphine only reduced respiratory rate to 62% of the baseline value, which suggests that the respiratory effects produced by opioid receptor agonists are complex and do not follow a simple dose response relationship.

The results produced from this experiment show that even with high dose morphine treatment, the administration of adequate doses of naloxone methiodide can reverse the analgesia and decreases in respiratory rate produced, without inducing withdrawal. Given the effectiveness of naloxone methiodide in this dose range of morphine, it is likely that this opioid receptor

antagonist will also be effective after the administration of lower doses of opioid receptor agonists.

4. Lack of information regarding the effects of naloxone methiodide after treatment with a range of opioid receptor agonists relevant to human overdose.

In Publication 1, the changes in respiratory rate that occur after morphine administration were examined. This opioid receptor agonist is classed as the 'gold standard' for opioid analgesics, but it was also important to test the effectiveness of naloxone methiodide after the administration of a number of opioid receptor agonists. Methadone and heroin were chosen because of their relevance to illicit opioid use and maintenance therapies in humans and their varied pharmacokinetics and metabolism. Given the lack of relative information regarding the respiratory effects of these two opioid receptor agonists, dose response curves were completed in Publication 3 to compare the respiratory effects of morphine, methadone and heroin. As seen in Publication 1, with the administration of high doses of morphine, a maximal decrease in respiratory rate of approximately 50% was observed with all three opioid receptor agonists. This change in respiratory rate was maintained with the administration of higher doses of opioid receptor agonists, until the doses produced irregular breathing patterns and rapid death. While a similar phenomenon has been noted by other researchers, the mechanisms behind this maximum effect is largely unknown [Czapla *et al.*, 2000; Isom *et al.*, 1969; Kokka *et al.*, 1965].

Despite these similar maximal decreases in respiratory rate, different respiratory depressive ED_{80} doses (the dose which produced 80% of the maximal decrease in respiratory rate) were calculated for morphine, methadone and heroin. Heroin had a smaller Hill slope than methadone and heroin, which resulted in a higher dose being required. This shows that differences exist in the steepness of the opioid receptor agonist slopes for respiratory rate, so this should be examined for a variety of opioid receptor agonists. The respiratory depressive ED_{80} doses chosen also produced different degrees of analgesia. Heroin, for example, induced 89% analgesia, while morphine and methadone only produced 45% of the maximum possible effect (MPE). This adds support to the hypothesis that the respiratory depression and analgesia produced by opioid receptor agonists are mediated by different mechanisms, and highlights that the results obtained from analgesia studies should not be used to predict the respiratory depressive effects of opioids [Ling *et al.*, 1983; Ling *et al.*, 1985].

Although differences existed between the respiratory effects of morphine, methadone and heroin, naloxone and naloxone methiodide produced similar effects when administered after these opioid receptor agonists. Respiratory rate was increased to approximately 88% of baseline for both opioid receptor antagonists, and hot plate latencies were also decreased to control values. Significant withdrawal was produced with naloxone administration, and was again absent after naloxone methiodide treatment. While the mechanisms by which naloxone methiodide produces its antagonist effects is unknown, it would appear from its ability to similarly reverse the effects of morphine, methadone and heroin that it occurs through a common mechanism, which is most likely through opioid

specific binding sites. Morita *et al*. [2002] showed that pinacidil (an ATP sensitive K^+ channel opener) and moguisteine (a non-opioid antitussive drug) could reduce capsaicin induced cough in guinea pigs, but naloxone methiodide did not antagonise the effects of these compounds, despite being effective in reversing the effects of dihydrocodeine. Therefore, it appears that the actions of naloxone methiodide in the respiratory system are mediated predominantly by an opioid receptor specific mechanism.

To further investigate the respiratory alterations that occur with opioid receptor agonist administration, whole body barometric plethysmography was used to measure the changes that occur with methadone administration and subsequent naloxone and naloxone methiodide treatment. While respiratory rate has previously been shown to be a good indicator of respiratory depression, this technique also allows the measurement of tidal volume and minute volume to obtain a full profile of the changes that occur with opioid receptor agonist and antagonist treatment [McGilliard & Takemori, 1978a]. As expected, the administration of 20 mg/kg i.p. methadone produced a significant respiratory rate decrease to 51 ± 3 % of baseline after 40 minutes. It was, however, surprising to note that a corresponding increase in tidal volume occurred, which resulted in no significant change in minute volume. As seen in the dose response experiments, unpublished data in this experiment showed that the maximal 'ceiling' decrease in respiratory rate was maintained until the dose of methadone administered produced rapid death after administration. This again suggests that a compensatory mechanism exists to maintain respiration, but if the dose of opioid receptor agonist exceeds this, adequate respiration is no longer maintained and

rapid death will occur. While the measurement of respiratory rate alone has often been criticised as being an inadequate parameter to evaluate respiratory depression, its measurement in these publications has highlighted that it is highly sensitive to changes in respiration and the compensatory actions required to maintain respiration [Ko *et al.*, 2003].

The administration of naloxone and naloxone methiodide again produced a reversal of the decreased respiratory rate and corresponding increases in tidal volume associated with methadone administration. Of interest was the finding that 30 mg/kg i.p. naloxone methiodide maintained normal respiratory rates for the 40 minutes of recording, the tidal volumes were only decreased for approximately 20 minutes before increasing towards the pre-antagonist administration levels. Naloxone, however, fully antagonised the methadone induced changes in tidal volume. This may be due to the differences in the pharmacokinetics of the two drugs, or result from differences in response between central and peripheral sites of action. Future investigation should be conducted to fully elucidate the mechanisms responsible for these differences.

5. Lack of research investigating the effects of naloxone methiodide after chronic opioid receptor agonist treatment regimes.

Prior to Publications 1 and 4, very little research had been conducted evaluating the effect of naloxone methiodide treatment after the chronic administration of opioid receptor agonists. It is important that this is investigated as opioid use in humans, whether illicit or therapeutic, is often prolonged. This means that the effect of tolerance to these opioid receptor agonists must be considered. In

Publication 1, 300mg/kg/day i.p. morphine was administered for 5 days and respiratory rates still decreased after the final morphine treatment, indicating that tolerance does not develop rapidly to these effects. Very little tolerance was observed in this experimental regime, but this may have been due to the high circulating concentrations of morphine in these animals. While partial tolerance to the respiratory responses after chronic opioid receptor agonist administration has been observed in previous studies, they have not used doses as high as those in this study. This has often resulted in very small changes in respiratory response being detected after acute opioid receptor agonist administration, and even less with chronic opioid receptor agonist treatment [Kokka *et al.*, 1965; van den Hoogen & Colpaert, 1986]. While a small degree of tolerance may develop to the respiratory effects of morphine, this may not be enough to provide protection against the administration of high doses of this opioid receptor agonist.

After this chronic morphine treatment regime, naloxone and naloxone methiodide still antagonised the analgesia and depressed respiratory rate produced after the final morphine treatment. It did appear, however, that naloxone methiodide was slightly more effective. This may be due to the different mechanisms of action of these two opioid receptor antagonists, with chronic morphine treatment having a greater effect on central mechanisms than peripheral mechanisms.

To further investigate the effects of chronic opioid receptor agonist administration on respiration, the experiments in Publication 4 were conducted. The cardiorespiratory changes that occur with chronic methadone administration were continuously monitored using radiotelemetry, and the antagonism of these effects

by naloxone and naloxone methiodide was also investigated. This experiment was unique as the changes that occur throughout the day and night periods of opioid treatment in the rat have not been previously examined. It has highlighted that tolerance may develop differently over these periods, which supports anecdotal evidence that the clinical consequences of opioid overdose in humans appear to occur more commonly at night than during the day [Darke *et al.*, 1996; McGregor *et al.*, 2002; Wolff, 2002]. It has illustrated that respiratory rate and heart rate are altered by chronic opioid administration, while locomotor activity and mean arterial blood pressure are not. This study also confirmed that both naloxone and naloxone methiodide can reverse the decreases in respiratory rate and heart rate produced by this chronic methadone treatment, as shown after the acute administration of opioid receptor agonists [Cruz & Rodriguez-Manzo, 2000; Czapla *et al.*, 2000; Schlenker & Inamdar, 1995].

CONCLUSIONS AND FUTURE DIRECTIONS:

The overall conclusion of the publications presented in this thesis is that naloxone methiodide can effectively antagonise the peripheral effects produced by opioid receptor agonists. Throughout these experiments, different strains, species and sexes of animals have been used (male Swiss-Albino mice, female C57BL/6J mice and male Sprague-Dawley rats) to confirm that the effects observed are not species, strain or sex specific. In these studies, naloxone methiodide was effective in all animals examined, but a lower dose was required in the female C57BL/6J mice compared with the male Swiss-Albino mice and Sprague-Dawley rats. Previous studies have noted that gender and strain difference exist both with respiratory and cardiovascular opioid effects [Cruz & Rodriguez-Manzo, 2000; Muraki & Kato, 1986]. It is not known, however, if strain differences exist for the peripheral effects of opioids. These results do indicate that these strain and gender differences should be taken into consideration in this research, but naloxone methiodide was still effective in both males and females of all rodent strains utilised.

This research project has provided another example of how peripherally selective opioid receptor antagonists can be used to reduce adverse opioid effects. While the interaction of opioids in the periphery has often been considered secondary to central opioid effects, it is becoming increasing obvious that this is an important site of action of opioids, and should be considered in the development of future treatments. Other researchers have shown that in humans these compounds are effective in reversing the constipation associated with methadone maintenance programs and post-operative ileus, which can develop after surgery [Schmidt,

2001; Yuan *et al.*, 2000]. Therefore, the use of peripherally acting opioid receptor agonists and antagonist may have many advantages over the current treatments available.

The most obvious implication of this research is related to its use in the treatment of opioid overdose. While this is rare in the clinical setting, as opioid overdoses are most commonly associated with the illicit use of opioids, the suggestion has been made that opioids are under prescribed in an attempt to avoid this adverse effect [McQuay, 1999; Salvato *et al.*, 2003]. As mentioned in Publication 3, the reversal of analgesia by naloxone methiodide makes it less suitable for use in patients receiving opioids for the treatment of pain. This finding, however, is not surprising given the accumulating evidence to indicate that antinociception contains a significant peripheral component [Junien & Wettstein, 1992; Stein, 1995]. The use of peripherally acting opioid receptor antagonists, such as naloxone methiodide, for the treatment of opioid overdose is still an improvement compared to the use of naloxone, which can produce severe withdrawal. This is a major adverse effect in opioid dependent patients and may hamper treatment being sought in an opioid overdose situation (See Section 1.3.1 on Page 9).

With the development of specific targeted treatments, such as the use of peripherally acting opioid receptor antagonists, opioid overdoses could be treated more appropriately. The doses of opioid receptor agonists administered therapeutically may also be increased to provide more adequate pain control with reduced risk of potentially fatal respiratory effects. In the future, compounds that target the sites involved in adverse effects, such as respiratory depression, while

still maintaining analgesia may be developed, which would produce significant advances in pain treatment. This research may also have implications in the development of compounds with limited access across the blood brain barrier, which may be important in conditions such as severe head injury, where the blood brain barrier becomes more permeable.

This research project has highlighted the effectiveness of naloxone methiodide in antagonising the peripheral effects of opioids but further research is required. Continued investigation of the site of action of naloxone methiodide is foremost. While this study has shown that naloxone methiodide, or its metabolites, do not have ready access to centrally located opioid receptors, specific techniques, such as HPLC using perfused brain samples, should be used to quantify this. The results presented in this thesis indicate that naloxone methiodide does not interact with centrally located μ opioid receptors, so a site of action in the periphery must also be determined. While this is most likely to be in the lungs, it appears that the opioid receptor binding sites in this tissue are not of a typical nature and should be characterised in order to determine if naloxone methiodide can interact with these sites. The interaction of naloxone methiodide with other receptor systems should also be examined as this may contribute to the effects observed. At present, this has not been fully investigated, but may contribute greatly to our understanding of the effects observed with this peripherally acting opioid receptor antagonist.

Throughout this research project a number of additional findings not related to the general aims were identified. The most intriguing of these was the nature of the

respiratory alterations produced by opioid receptor agonists. The experiments conducted in this study have indicated that the decrease in respiratory rate produced by opioid receptor agonists reaches a maximum level that is not exceeded until the dose produces sudden death. This maintenance of depressed respiratory rate appears to be due to a compensatory mechanism as the decrease in respiratory rate is associated with increases in tidal volume to maintain minute volume. These two phases of respiratory depression, the first being the decrease in respiratory rate that is maintained and the second being rapid death due to respiratory complications upon the administration of higher opioid doses, has largely remained unrecognised by other researchers and may play a vital role in our understanding of opioid induced respiratory depression and subsequent death.

This compensatory effect may be rodent specific, as it is not known if these two phases occur in humans, and could be due to factors such as altered metabolism of opioid receptor agonists in rodents compared to humans. The use of an animal model, however, will be vital in the future as, due to ethical constraints in clinical trials and the need for immediate treatment in emergency opioid overdose situations, it is unlikely that this information will be easily obtained in humans. Therefore, future investigation will be required to determine the mechanisms involved in these opioid induced respiratory alterations and their impact on opioid overdoses. This research may provide insight into many anecdotal observations in humans, such as why substantial delays often exist between opioid administration and the symptoms of opioid overdose and why people experience opioid overdose symptoms on certain occasions and not others. A greater

understanding of the mechanisms that contribute to the respiratory depression associated with opioid overdoses will also aid in the prevention or treatment of this serious adverse effect.

The research conducted in this thesis indicates a strong potential for peripherally acting opioid receptor antagonists, such as naloxone methiodide, to be effective in the treatment or prevention of opioid induced respiratory effects, and highlights the need for continued research in this area. It demonstrates that the development of peripherally acting opioid drugs is likely to lead to improved and novel applications in the treatment of pain, addiction and opioid overdose.