RESEARCH BACKGROUND:

1.1 HISTORY:

Opioids have been used for centuries to produce euphoria, analgesia and sedation and prevent diarrhoea [Rang *et al.*, 2003]. Opium extracted from the poppy plant, *Papaver somniferum*, was the first opioid isolated and used for medicinal purposes [Gutstein & Akil, 2001; Rang *et al.*, 2003]. Assyrian medical references from 7000 B.C. refer to the use of this poppy juice while the Sumerians record usage from about 4000 B.C.. Egyptian papyri dating from 2000 B.C. report the use of opium for veterinary and gynaecological procedures and the use of the poppy is thought to have spread from here through Asia Minor and Greece. The use of this medicine then travelled with Arab traders through Persia, India and China and by the 10th and 11th centuries A.D., the opium trade was firmly established in Europe [Berridge & Edwards, 1981].

As availability increased, opium use became common and salves or elixirs containing opium were used as a sedative and anaesthetic throughout Europe in the 16th century. Even at this early time the potential for opium to cause “deepe deadly sleapes” was well known, as described by W.D. Bullein in his book “Bulwarke of defense against all sicknesse, soarnesse and woundes“ published in 1579 [Bullein, 1579]. The effectiveness of this compound, however, ensured its continued use. Over the following centuries, medicinal and recreational use continued, and despite the identification of the potential for opioid abuse and addiction, it was not openly discussed in the 1700s. While complications were known, opium was still readily available through grocer or druggist stores in England during the 1800s in the form of laudanum or tinctures, which are

mixtures of opium, alcohol and various other ingredients. As the century progressed, the number of deaths due to opium poisoning increased, but were mainly attributed to errors in dosing or inadequate medical care. By the end of the century, the legal sale of opium in England was restricted to pharmacies, but its use was still popular as it was considered a cure-all for conditions, ranging from cough to insanity. Other countries were experiencing similar problems, so restrictions on the production, importation and use of opioids were applied, but this did not curb the problems associated with opioid use, which still continue today [Berridge & Edwards, 1981].

In 1806, morphine (named after Morpheus, the Greek god of dreams) was isolated by Friedrich Serturner and shown to be the active component of opium [Hamilton & Baskett, 2000]. Over the next decades, several other alkaloids were isolated and the use of these pure compounds increased. Their use was also facilitated with the invention of the hypodermic needle and syringe in the 1850s, which rapidly rivalled smoking as the most popular form of ingestion [Gutstein & Akil, 2001; Hamilton & Baskett, 2000; Rang *et al.*, 2003]. Heroin, the first semi-synthetic opioid, was produced in 1874 and whilst it did not enter widespread medical use, it became a popular opioid for illicit use. It was over this period that the truly addictive nature and the possibility for complications and overdose from opioids was fully realised [Berridge & Edwards, 1981; Gutstein & Akil, 2001; Rang *et al.*, 2003]. These compounds continued to be used medically, but over time, their abuse became more prevalent in both the lower and middle classes of society [Berridge & Edwards, 1981]. From this period until the present, opioids have shared the reputation of being highly effective therapeutically, particularly

for the treatment of pain, and being sought after for their abuse potential, despite restrictions on the supply and use of this class of drug.

Extensive research has resulted in many distinct opioid peptides being isolated, discovered endogenously or synthetically derived, in a quest to produce an opioid with positive effects but without the addictive potential or adverse effects associated with their use [Gutstein & Akil, 2001]. Many of these compounds are still used both medically and illicitly, with illicit use surging throughout the world over recent years. The medical and scientific usage of morphine alone in Australia during 2002 was estimated to be 1789 kg, with the total opioid usage in this country being close to 20 000 kg [International Narcotics Control Board, 2002]. It is also estimated that in 1997-1998 there were approximately 74 000 dependent heroin users in Australia, while 20 000 Australians were on methadone maintenance treatment in 2000 [Darke *et al.*, 2000; Hall *et al.*, 2000]. These statistics highlight that opioid use plays both a beneficial and deleterious role in society.

1.2 EPIDEMIOLOGY:

Deaths due to opioid overdose are a significant problem around the world, and in Australia contribute to the majority of illicit drug deaths reported each year. In 1999, 960 deaths were related to heroin overdoses in Australians aged 15-44 years [Trewin, 2001]. Similar findings have also been reported around the world. A study of the number of overdose deaths in 25 cities in the United States of America (USA) reported increases from 8.7 per 100 000 population in 1988 to 13.8 per 100 000 population in 1997 [Seal *et al.*, 2003].

Not all opioid overdoses that occur are fatal, and non-fatal overdoses are often treated and reported. In the 1999 Illicit Drug Reporting System (IDRS) survey of injecting drug users in Sydney, Melbourne and Adelaide, 51% of the people surveyed had experienced an opioid overdose and 28% had overdosed in the previous 12 months [Miller & Draper, 2001]. This can be extrapolated to approximately 7000 hospital admissions for opioid related events in Australia each year [Miller & Draper, 2001]. The USA has also reported similar statistics, with the number of heroin related emergency department visits doubling from 33 900 in 1990 to 70 500 in 1996 [Sporer, 1999].

People receiving methadone for either the management of drug dependence or treatment of chronic pain also suffer complications, which can be fatal [Drummer *et al.*, 1990]. In the USA during 1996, 552 methadone related deaths were reported, the majority occurring during induction onto maintenance programs [Karch & Stephens, 2000]. In New South Wales during 1994, 38 methadone maintenance patients died, 13 of these within 2 weeks of induction onto the methadone program. In the same year, 16 people also died from the administration of methadone tablets, which are used primarily for pain relief [Caplehorn & Drummer, 1999]. Similar statistics have also been reported in South Australia, with 49 deaths (out of a total of approximately 1000 patients receiving methadone) occurring over a 10 year period (1984-1994) in patients taking methadone in both syrup and tablet form [Williamson *et al.*, 1997].

The study by Williamson *et al.* [1997] highlighted that it is not only illicit drug users or those on maintenance therapies that are at risk of complications and overdose. There is also the potential for overdose in patients prescribed opioids for the treatment of chronic pain and undesirable effects such as constipation, nausea, vomiting and urinary retention, are often unpreventable [Yuan *et al.*, 1996]. Suggestions have been made that in an attempt to reduce side effects and the possibility of accidental overdose, the dose of opioids administered therapeutically may be lower than that required for adequate pain control [Florez *et al.*, 1983; McQuay, 1999; Yuan *et al.*, 2000]. A recent study by Salvato *et al.* [2003] investigated the opioid prescribing attitudes of general practitioners in Italy. They found that 49.6% of doctors surveyed were concerned by the potential for respiratory depression to occur, and this was the most frequently indicated cause of inadequate opioid prescription. Several cases of accidental overdoses in hospitals have also been reported, as have children overdosing after ingesting methadone prescribed for adults [Binchy *et al.*, 1994; Dawson, 1995; Notcutt *et al.*, 1992; Rawal *et al.*, 1987; Tobias, 1997]. Therefore, the potential for opioid overdoses exists whenever opioids are administered, and the measures taken to reduce this threat may reduce the effectiveness of opioid treatment. This could ultimately result in inadequate patient care.

Complications or deaths due to opioid overdoses are of concern for illicit drug users, those seeking treatment to control opioid dependence and patients being administered opioids for the treatment of moderate to severe pain. Further research is this area is needed to improve our understanding of why these

adverse events occur and to develop ways to treat or prevent these complications.

1.3 OPIOID OVERDOSE AND IMPAIRED RESPIRATORY FUNCTION:

The dangers of opioid use have been known for centuries and it is now widely accepted that opioid induced respiratory depression contributes significantly to opioid related deaths [Gutstein & Akil, 2001; White & Irvine, 1999]. Respiratory depression has been defined as "a drug related increase in arterial carbon dioxide (CO_2) and decrease in oxygen (O_2) and pH which remains uncompensated by the expected increase in pulmonary ventilation" although the term is often used to encompass any changes in respiration produced by opioids [Ko *et al.*, 2003; Yeadon & Kitchen, 1989]. Opioids can affect all aspects of respiration, such as respiratory rate, minute volume, tidal exchange, and produce irregular or periodic breathing [Gutstein & Akil, 2001]. It is thought that during opioid overdose, breathing becomes shallower and the normal drive to recommence breathing or compensate for decreases in respiratory rate is reduced. This depressed breathing can progress into coma, which can eventually lead to cardiopulmonary arrest and death.

Bammer & Sengoz [1994] reported the overdosing experiences of heroin users and found that one of the main features was losing consciousness or having breathing difficulties. Autopsy studies on opioid overdose patients have shown that respiratory abnormalities, such as acute pulmonary alveolitis and oedema, are frequently present [Byers *et al.*, 1975; Cherubin *et al.*, 1972; Garriott *et al.*, 1973; Karch & Stephens, 2000; Wetli *et al.*, 1972]. Drummer *et al.* [1992]

examined the deaths of patients commencing onto methadone maintenance programs and found that half of the 10 deaths investigated could be directly attributed to respiratory complications. Investigations into fatal and non-fatal overdoses due to heroin, which is licensed in the United Kingdom for the treatment of pain, have shown that pulmonary oedema, vomiting and respiratory depression are commonly associated with these adverse events [Kendall & Latter, 2003]. Abnormalities in pulmonary function have also been illustrated in ex-injecting heroin users [Camargo & Colp, 1975]. There is also limited evidence showing that smoking heroin or "chasing the dragon" can impair lung function, but further investigation is required to determine if this is a heroin effect or a result of smoking [Buster *et al.*, 2002]. This shows that respiratory abnormalities may not just occur in overdose situations, but may be a common complication present in opioid users. These abnormalities may increase the possibility of respiratory depression occurring, resulting in fatal or non-fatal opioid overdose, and may have a negative effect on the health of these people.

In all of these studies, it is difficult to determine if the respiratory effects observed are due to the opioid administration alone or other drugs that are often used in combination [Buster *et al.*, 2002]. Tobacco and marijuana smoking has been shown to alter respiratory function, and alcohol and benzodiazepines are themselves weak respiratory depressants. The concurrent use of these compounds with opioids can, therefore, potentiate the risks involved [Buster *et al.*, 2002; Forster *et al.*, 1980; Sporer, 1999; White & Irvine, 1999; Williamson *et al.*, 1997]. This must be taken into consideration, as many illicit opioid users are poly-drug users (they administer more than one type of drug at any time)

[Williamson *et al.*, 1997]. In an investigation of heroin related deaths in Victoria during 1997-1998, benzodiazepines were detected in 44% of cases, alcohol in 37%, and drugs of any kind other than morphine detected in 86% of deaths [Gerostamoulos *et al.*, 2001]. There are also several reported overdoses in patients administered buprenorphine, a partial opioid receptor agonist, which are thought to be predominantly due to the co-administration of benzodiazepines [Boyd *et al.*, 2003; Reynaud *et al.*, 1998]. While the use of partial opioid receptor agonist/antagonists is considered relatively safe, poly-drug use appears to increase the potential for fatalities. The danger of adverse respiratory effects is, therefore, more prominent in poly-drug users and must be considered when evaluating opioid overdoses.

Another interesting finding is that high blood opioid concentrations are not always observed after opioid overdoses. Many deaths occur several hours after opioid administration, when blood opioid concentrations are falling [Monforte, 1977; Richards *et al.*, 1976; Zador *et al.*, 1996]. Illicit opioid users that undergo a period of abstinence appear to be at a greater risk of respiratory depression and overdose, even if they use smaller doses than they would have previously administered [Tagliaro *et al.*, 1998]. It is known that opioids readily collect in tissues, the lungs in particular, which accumulates the largest concentrations of methadone in humans followed by the liver, kidney and brain, but the impact this may have on respiratory function is not known [Garriott *et al.*, 1973]. Therefore, it appears that a number of mechanisms can contribute to opioid overdoses and it is not a simple dose related depression of respiratory control.

1.3.1 Current treatments for opioid overdose:

The most common treatment for respiratory depression produced by opioid overdose is the administration of the opioid receptor antagonist, naloxone hydrochloride (Narcan®; commonly referred to as naloxone). Whilst this is an effective treatment, naloxone can act on all opioid receptor types in both central and peripheral locations of the body. This means it will not only reverse respiratory depression but antagonise all other opioid effects, such as analgesia, and will precipitate withdrawal in opioid dependent patients [Martin, 1976].

This lack of specificity can limit the suitability of naloxone in many situations. Opioid overdoses can occur in clinical settings and whilst the administration of naloxone will reverse the respiratory depression present and prevent overdose, it will also reverse the analgesic effects of the opioid, which can compromise patient care [Dawson, 1995; Notcutt *et al.*, 1992]. This highlights that an alternative for either the treatment or prevention of this respiratory effect would be advantageous. In illicit opioid overdose situations, Seal *et al.* [2003] reported that 82% of opioid dependent users treated with naloxone during opioid overdoses described the experience as unpleasant and intolerable. These disagreeable withdrawal symptoms may hamper treatment being sought in the future. There is also concern that patients may re-administer opioids to reverse the withdrawal symptoms produced by naloxone after an opioid overdose, which could increase the potential for subsequent overdose to occur [Seal *et al.*, 2003].

Another problem associated with the use of naloxone to treat opioid overdose is that it is short acting, with a serum half life of approximately 60 minutes in

humans [Ngai *et al.*, 1976]. This may result in some patients returning to a respiratory depressed state after the naloxone treatment, particularly if the half life of the opioid receptor agonist administered is longer than that of naloxone. This would require further naloxone treatment, which could produce greater withdrawal [Strang *et al.*, 1996]. The balance between a dose of naloxone that will prevent overdose but not induce substantial withdrawal can often be difficult to determine, particularly when the doses or types of opioids administered are unknown.

Other potential treatments have been tested, for example, the use of nalbuphine an opioid receptor agonist/antagonist, but they have been rendered unacceptable due to the adverse effects they produce. These include agitation, nausea, vomiting and cardiac dysrhythmias [Blaise *et al.*, 1990]. Currently the use of opioid receptor antagonists, such as naloxone, are the best treatments available, but alternatives should be sought that can target the respiratory effects of opioids, without altering other opioid effects. One example of this is the use of peripherally acting opioid receptor antagonists, such as naloxone methiodide, that appear to alter peripheral opioid actions, but do not affect those that are mediated centrally. The main aim of this research project was to determine if naloxone methiodide would provide a suitable alternative to prevent respiratory depression in opioid overdose situations.

1.4 OPIOID INDUCED RESPIRATORY DEPRESSION:

Whilst the most dramatic effects of opioids on respiration occur in overdose situations, respiratory depression can occur whenever opioids are administered.

This has resulted in much research being conducted examining the effects of acute and chronic opioid administration in both animals and humans. The general findings of this research and the limitations that have been identified are discussed in the following sections.

1.4.1 Human studies:

It is well established that opioids affect respiration in humans and research has identified that this effect can be altered by a number of factors. These include the age, sex and state of health of patients, the type of opioid receptor agonist being administered, its dose and route of administration, and the duration of treatment. These factors will be discussed below in relation to acute and chronic opioid administration.

1.4.1.1 ACUTE ADMINISTRATION OF OPIOIDS:

The respiratory effects of acute opioid administration have been the subject of much published research. Camporesi *et al.* [1983] investigated the differences between intravenous (i.v.) and epidural administration of 10 mg morphine in healthy male subjects. End tidal pCO_2 (partial pressure of CO_2 at the end of a breath) increased significantly above control values 30 minutes after i.v. administration and 1 hour after epidural administration. The duration of this elevation was also greater with epidural administration, peaking at 10 hours and remaining until 22 hours. The elevation after i.v. administration returned to control values between 1 and 3 hours, peaked again at 6 hours, and subsided after 16 hours. The peak effect, however, was the same for both routes of morphine administration. Epidural administration produced a greater decrease in

tidal volume, while i.v. administration produced a lower tidal volume compared to control values. Respiratory rate was also further decreased after epidural administration. The authors suggested that these variations may be due to differences in serum opioid concentrations, which are dependent on the route of administration. Another contributing factor may be the diffusion of opioids throughout the body, which occurs later with epidural administration. This study, therefore, indicates that while respiratory depression is produced by morphine, the route of administration may alter the duration and severity of the effects observed.

While morphine is considered the 'gold standard' by which other opioids are compared, not all opioids produce the same respiratory effects. This may be due to factors such as different binding affinities for opioid receptors, alterations in the distribution of opioid receptor agonists around the body, or whether active or inactive metabolites are produced [Chen *et al.*, 1991; White & Irvine, 1999]. Certain opioid receptor agonists can also affect non-opioid systems. Methadone, for example, can bind with low affinity to N-methyl-D-aspartate (NMDA) receptors, which may also modulate respiration [Ebert *et al.*, 1995; Gorman *et al.*, 1997]. Some opioids have predominant analgesic actions without producing considerable respiratory effects, while others affect respiration at the same doses that produce analgesia. Ling *et al.* [1985] observed that morphine was more potent in producing antinociception than respiratory depression, while Yang *et al.* [1992] found that alfentanil caused fewer respiratory effects while still producing analgesic effects similar to morphine. The effect of a variety of opioids on

respiratory parameters must, therefore, be examined to obtain a complete knowledge of the effects of opioids on respiration.

Olsen *et al.* [1981] administered an oral methadone dose of 15 mg/1.79 m^2 body surface area to healthy, opioid naïve women and measured various respiratory and ventilatory parameters for 48 hours. Compared to the placebo treatments these women experienced significant decreases in minute ventilation, respiratory rate, alveolar ventilation and significant increases in arterial pCO_2, which continued for up to 48 hours. Methadone was also shown to have a longer duration of action compared to other opioids, such as morphine, which is due to its slower elimination and metabolism.

Bailey *et al.* [1990] investigated the effect of two opioids, sufentanil and fentanyl, on respiration in healthy males. These opioid receptor agonists are both pure μ receptor agonists but differ in their potencies, receptor affinities, lipophilicity and pharmacokinetics. 1, 2 and 4 μg/kg i.v. fentanyl and 0.1, 0.2 and 0.4 μg/kg i.v. sufentanil were shown to depress ventilation, but the magnitude and duration of depression was greater for fentanyl. This may have resulted from the doses of sufentanil and fentanyl not being equipotent, despite the administration of a 10:1 ratio. This highlights that opioids that would be expected to act in a similar manner can have varied effects on respiration. Another comparison study between remifentanil and alfentanil was conducted by Glass *et al.* [1999], who found that the i.v. EC_{50} for the depression of minute ventilation in healthy males was approximately 40 times lower for remifentanil (1.17 ng/ml) than alfentanil (49.4 ng/ml). These studies show that the type and dose of opioid receptor

agonist administered can alter the degree of respiratory depression that can occur.

Partial opioid receptor agonist/antagonists have also been shown to alter respiration. Healthy adult males were orally administered 0 to 32 mg of buprenorphine and the effects compared to 0 to 60 mg of methadone [Walsh *et al.*, 1994]. With buprenorphine doses greater than 4 mg, respiratory rate decreased from 16 breaths/minute to 12 breaths/minute. Arterial O_2 saturation (SpO_2) also decreased from 98% to 95%. Methadone also produced a reduction in SpO_2, but the decrease in respiratory rate was not significant. This study indicates that although partial opioid receptor agonist/antagonists have a ceiling effect, they are still capable of decreasing respiration at therapeutically administered doses. At present, the implications of this are unknown.

1.4.1.2 CHRONIC ADMINISTRATION OF OPIOIDS:

Acute opioid administration can alter respiration, but the effect of chronic opioid administration is also of interest as opioids are often administered repeatedly. The development of tolerance and dependence must be taken into consideration in many different opioid effects, including respiration.

People with a history of opioid use become tolerant to many of the effects, so are able to tolerate much larger opioid doses with fewer complications. This tolerance, however, is reversible, as chronic users that remain abstinent and then resume opioid use can be particularly susceptible to overdose, even if they administer lower doses [Tagliaro *et al.*, 1998]. How this tolerance develops is not

clear, but several suggestions have been made. These include the up regulation of the cyclic adenosine 3'5'-monophosphate (cAMP) pathway due to supersensitisation of adenylate cyclase, and altered coupling of opioid receptors and G-proteins. Changes in signal transduction leading to receptor desensitisation and receptor internalisation are also thought to occur from the receptor phosphorylation [Borgland, 2001; Liu & Anand, 2001; Nestler & Aghajanian, 1997]. Major alterations can occur with the development of tolerance, so it would be expected that many opioid effects, such as respiration, would change with the chronic administration of these drugs. How this tolerance alters the respiratory effects of opioids, and whether or not this tolerance is complete, is still a contentious area of research.

Respiratory rate was measured by Dyer *et al.* [1999] in men and women stabilised on a methadone maintenance program for at least 6 months (doses ranging from 0.12 to 1.9 mg/kg). Tolerance to the respiratory effects of methadone did not appear to occur, as these subjects still experienced a significant decrease in respiratory rate after methadone dosing, which returned to pre-dose values approximately 12 hours after methadone administration. Gritz *et al.* [1975] also examined the respiratory rates of normal controls, abstinent heroin users and methadone maintenance patients and observed that methadone maintained patients had significantly lower respiratory rates compared to the other two groups. Therefore, even under controlled dosing, respiratory depression occurs in humans, and tolerance does not appear to develop, even after extended periods of stabilisation.

Doverty [2002], compared methadone maintained men and women to non-opioid dependent controls. They showed that methadone maintained subjects had significantly lower respiratory rates both prior to and 3 hours after methadone dosing and also experienced a decrease in respiratory rate after dosing. The SpO_2 of the methadone maintained subjects was also depressed compared to controls. This study again suggests that tolerance to the respiratory effects of methadone is not complete.

Mitchell *et al.* [2003] measured the respiratory rates of opioid dependent patients who had been stabilised for at least 4 weeks on either methadone or slow-release oral morphine. They found that whilst there were no statistically significant changes over the 24 hour monitoring period, respiratory rate decreased from 15 breaths/minute to 12 breaths/minute or 13 breaths/minute 2 hours after methadone or morphine administration, respectively. They also observed that after 12 hours, the respiratory rate had returned to normal in the methadone treated patients, but was still lower than pre-treatment levels after morphine treatment. This study shows that differences exist between the respiratory effects of opioid receptor agonists and that while the respiratory alterations observed may not be significant, they are still present after chronic treatment.

Martin *et al.* [1968] investigated the changes in respiration with the chronic administration of morphine. Male prisoners were administered increasing doses of morphine, up to 60 mg of morphine four times a day for 8 months, and respiratory function monitored by measuring alveolar ventilation in the presence of CO_2. When compared to the results obtained before morphine treatment

commenced, these subjects were less responsive to the CO_2 exposure after morphine administration. After 8 months, this decrease in CO_2 sensitivity persisted, so little tolerance to the respiratory depressant effects of morphine appeared to develop over time. This indicates that if chronic opioid users continue to increase the dose of opioids consumed they do not appear to become completely tolerant to the respiratory effects of these drugs.

Sapira [1968] also conducted a more intensive investigation into the respiratory effects that occur before, 1 and 5 months after the initiation of 240 mg/day morphine treatment. Alveolar ventilation was significantly reduced 1 month after the initiation of morphine treatment, but returned to normal levels after 5 months. SpO_2 decreased and remained lower than control levels, while arterial pCO_2 was significantly higher with prolonged treatment. Arterial pO_2 (partial pressure of O_2), CO_2 production and arterial hydrogen ion (H^+) concentrations did not differ over the treatment period. From this study, it appears that tolerance develops to opioid induced changes in respiratory rate, but other respiratory parameters do not return to normal, and even compensate for these alterations. This highlights the difficulties in understanding the respiratory effects that occur during chronic opioid use.

Research into patients commencing methadone maintenance programs compared to those who have been on the program for extended periods has also been conducted. As previously mentioned (See Section 1.2 on Page 3), people being inducted onto methadone maintenance programs appear to be particularly susceptible to the respiratory depressant effects of methadone. Santiago *et al.*

[1977] showed that subjects who had been on the maintenance program for less than 2 months experienced greater changes in respiration than those who had been on the program for over 8 months. In particular, they noted that before their daily methadone dose, the inducting subjects had higher arterial CO_2 tensions and lower ventilatory responses to hypoxia. After their methadone dose, the recently inducted subjects experienced significant reductions in ventilation and arterial O_2 tension and significant increases in arterial CO_2 tensions. They also experienced significant depression in ventilatory responses to both hypercapnia and hypoxia. In contrast, the subjects that had remained on the program for 8 months only experienced a significant decrease in ventilatory response to hypoxia. This indicates that people on the same maintenance dose of methadone become tolerant to certain respiratory changes induced by opioids, but this tolerance does not appear to be complete. It also confirms that people being inducted onto methadone maintenance programs are at a greater risk of respiratory complications.

The effect of methadone on lung function in patients being inducted onto methadone maintenance programs was examined by Marks & Goldring [1973]. The patients chosen all had forms of lung or extrapulmonary disease but the effects observed were not attributed to these conditions, nor were these conditions exacerbated with methadone treatment. Chronic hypercapnia developed upon the administration of methadone, and in some cases persisted after 8 months of methadone treatment. This hypercapnia along with other parameters, such as decreased tidal volume and arterial blood O_2 concentration (PaO_2), was reversed upon the discontinuation of methadone treatment.

Hypercapnia was not observed with chronic morphine treatment in the study by Sapira *et al.* [1968], which again indicates that different opioid receptor agonists can produce varied respiratory effects. This is not unexpected, given that the pharmacological characteristics of opioid receptor agonists can be diverse.

Stoermer *et al.* [2003] have recently published work comparing the respiratory alterations that occur with i.v. methadone or heroin administration. The subjects were maintained for at least 7 days on the same dose of either heroin or methadone, and the effects monitored for 30 minutes after the administration of the opioid receptor agonist or saline. Over this short period of maintenance, tolerance did not develop to the respiratory effects produced by the i.v. injected heroin or methadone, as the mean SpO_2 levels significantly decreased after opioid receptor agonist administration and respiratory rates were also reduced from baseline measures. Mild or greater hypoxemia (a decrease in SpO_2 to 86-90%) was observed in all subjects receiving heroin and 7 out of 9 patients receiving methadone. Severe hypoxemia (a decrease in SpO_2 of less than 81%) developed in 3 out of the 9 patients administered heroin. These opioid receptor agonists also produced a Cheyne-Stokes breathing pattern, a form of periodic breathing in which apneas and hyponeas alternate to produce a waxing-waning pattern of tidal volume [Leung & Bradley, 2001]. This resulted in disturbances in respiratory rhythm that had subsided 30 minutes after methadone administration, but persisted after heroin treatment. These findings indicate that initial respiratory alterations still occur in opioid dependent patients that have been maintained for a short time on either i.v. methadone or heroin. This suggests that the route of

administration may also alter the development of tolerance to the respiratory effects of opioid receptor agonists.

From these studies, it would appear that incomplete tolerance develops to the respiratory effects of chronically administered opioid receptor agonists. While the greatest risk of respiratory complications is known to occur during the initiation of opioid use, respiratory parameters do not appear to return to normal after chronic opioid treatment. This is an area of particular concern, as the long term implications of this depressed respiration have not been investigated.

1.4.1.3 LIMITATIONS WHEN EVALUATING DATA FROM PREVIOUS HUMAN STUDIES:

The studies described above indicate that opioids can produce respiratory depression in humans, but a number of considerations and limitations must be taken into account when comparing these previous data.

The sex of subjects used in clinical studies has been shown to produce differences in the respiratory effects of opioid receptor agonists. These differences have been observed with other opioid effects, such as analgesia and subjective measures [Sarton *et al.*, 2000; Zacny, 2001]. Dahan *et al.* [1998] administered an i.v. dose of morphine (100 μg/kg bolus then 30 μg/kg/hr) to healthy men and women and found morphine affected ventilatory control in both sexes, but women were more sensitive in their responses to CO_2 and O_2. The authors suggested that these sex differences may be due to varied morphine pharmacokinetics, as males have lower concentrations of morphine and its

metabolite, morphine-6-glucuronide (M-6-G), in regions of the body, such as the brain stem. The same research group also investigated if these respiratory differences were due to opioid actions within the peripheral chemoreflex loop or the central chemoreflex loop [Sarton *et al.*, 1999]. They found an equal reduction in central ventilatory CO_2 sensitivity in men and women, but peripheral ventilatory CO_2 sensitivity was greater in women, indicating a sex difference in the peripheral chemoreflex pathway after opioid administration. Therefore, the respiratory actions of opioid receptor agonists differ between the sexes, and are mediated by a number of different factors. This should be taken into consideration when evaluating studies that use men, women or both.

Another factor that should be considered when evaluating previous studies is the influence of age on the respiratory alterations observed after opioid receptor agonist administration. Hamunen [1993] investigated the effect of equipotent i.v. doses of morphine, pethidine and methadone on ventilation during ophthalmic surgery in children aged 3 to 10 years. Methadone produced slightly greater and more prolonged respiratory depression than morphine and pethidine, which is consistent with the results obtained in adults. The onset of the decreases in end-tidal O_2 concentrations and SpO_2, however, were more rapid in children than in adults. This may be a result of the shorter circulation time in children. Cepeda *et al.* [2003] conducted a cohort study of a variety of patients receiving short term parenteral morphine, pethidine or fentanyl, and found that the risk of life threatening respiratory depression increased substantially in patients over the age of 60. This was not found to be a dose-related effect, but may be due to pharmacokinetic differences, in the elderly, such as reduced opioid clearance,

half life and smaller volume of distribution. Therefore, age can alter the respiratory depressant effects of opioid receptor agonists.

Variations in results can also occur with the type of patient tested. For example, patients with underlying pain may respond differently to opioid administration than healthy volunteers, as investigated by Borgbjerg *et al.* [1996]. In this study, healthy males were administered 0.2 mg/kg i.v. morphine and the ventilatory response to CO_2 measured during periods of experimental pain. As expected, morphine decreased tidal volume and minute ventilation and reduced the sensation of pain produced by 300 mmHg in the modified tourniquet pain technique [Hagenouw *et al.*, 1986]. With an increase in pressure of between 400 and 450 mmHg, which increases the pain sensation, morphine was no longer effective in attenuating the painful stimuli and the respiratory effects of morphine were completely reversed. These findings have important implications in the clinical setting, as the effectiveness of opioid doses may alter depending on the severity of the pain experienced. Patients in severe pain may also tolerate larger doses of opioids without the risk of respiratory depression occurring. This study also highlights that there are limitations in the data collected in controlled clinical trials as the effectiveness of opioids can be altered by additional factors, such as the degree of pain experienced by patients in the therapeutic setting.

Whilst the studies described above have shown that opioids can alter respiratory parameters, high doses of opioids have not been administered to simulate overdose situations. This means that there are limited data regarding the mechanisms involved in opioid overdoses and severe respiratory depression.

This has led to the use of CO_2 to enhance these respiratory alterations, but this does not replicate the administration of high doses of opioids and may activate mechanisms that are not normally affected.

While some patient groups do receive high doses of opioids, such as the terminally ill and those on opioid maintenance programs, the studies that can be conducted are limited, as patient care must not be compromised. These patients are often being administered other medications, which may alter the results collected, and invasive procedures, such as inducing respiratory difficulties, must also be avoided. It is also likely that these patients will be administered opioids chronically, and the development of tolerance (as seen in Section 1.4.1.2 on Page 14), can further complicate the results obtained. Finally, the effects of opioid receptor antagonists cannot be tested as this will reverse the opioid induced analgesia required and induce withdrawal in opioid dependent patients. Therefore, the studies that can be conducted in patient groups that are receiving high opioid doses are limited.

It can be seen from the above studies that opioids do have the potential to produce respiratory depression in humans, but the information that can be obtained from these human studies is often limited. These limitations must be taken into consideration when evaluating these previous studies and relating them to therapeutic situations.

1.4.2 Animal studies:

Human research has provided us with a good background regarding how opioids

affect respiration, but different subject groups can produce variations in results and there are restraints on the experiments that can be conducted. Consequently, animal studies are required to further explore the respiratory depressant effects of opioids, and how changing conditions can affect these actions. This section will concentrate on the respiratory alterations observed in animal studies and the factors that can limit these results, while the mechanisms responsible for these effects will be discussed later (See Section 1.5 on Page 34).

1.4.2.1 ACUTE ADMINISTRATION OF OPIOIDS:

One of the earliest studies investigating the respiratory effects of opioids in rats was conducted by Kokka *et al.* [1965], who measured the effect of subcutaneous (s.c.) morphine (0 to 160 mg/kg) on respiratory rate, minute volume and O_2 consumption (VO_2). 5 mg/kg morphine was shown to produce a slight decrease in minute volume, which lasted for approximately 2 hours, but the higher doses of morphine exhibited much greater effects. 10 and 20 mg/kg produced the greatest depression of ventilation, along with a depression of minute volume of between 39 to 45% of control animals. The higher doses of morphine produced slightly less depression of minute volume compared to the 10 and 20 mg/kg doses, but respiratory rate continued to decrease with increasing morphine doses. VO_2 decreased with 10 and 20 mg/kg morphine but increased over time with higher morphine doses. This study, therefore, suggests that a maximal degree of respiratory depression occurs with increasing doses of opioid receptor agonists.

Isom *et al.* [1969] also investigated the effect of similar doses of morphine on Sprague-Dawley and Long-Evans rats. As expected, respiratory rate, tidal volume, minute volume and VO_2 decreased in both strains. A maximum respiratory depression of approximately 50%, however, was noted with doses higher than 80 mg/kg, indicating that the respiratory depressant effects of morphine are not linear. Muraki & Kato [1986] investigated the change in respiratory rate produced by up to 20 mg/kg morphine in male DBA/2N, C57BL/6N, Balb/c, C3H/HeN and ICR mice. The degree of respiratory rate decreases differed between the mouse strains, but all strains reached an apparent maximal decrease in respiratory rate between 50 and 70% of baseline respiratory rate.

White & Zagon [1979] investigated the effects of methadone administration in rats by measuring arterial blood pH and gas concentrations. pO_2 and pH decreased while pCO_2 increased in a dose dependent fashion with intraperitoneal (i.p.) methadone doses ranging from 2.5 to 7.5 mg/kg. These effects persisted for up to 240 minutes in the animals treated with the highest dose of methadone.

Changing the environment in which these respiratory measurements are taken highlights the complexity of opioid induced respiratory depression. Ho *et al.* [1986] treated male Sprague-Dawley rats with 32 mg/kg i.p. morphine and observed significant decreases in pO_2 and pH and significant increases in pCO_2. Under hypoxic conditions, pO_2 was increased while hypercapnic conditions surprisingly produced a significant increase in pCO_2 and pO_2, and decrease in pH. van den Hoogen & Colpaert [1986] also investigated the effects of acute

morphine administration on awake, unrestrained rats. They measured the effect of 0.16 to 160 mg/kg s.c. morphine on tidal volume, minute volume and ventilatory frequency using a modified whole body plethysmographic technique with increasing CO_2 concentrations (Normal or 0%, 4%, 6% and 8% CO_2). Under normal CO_2 conditions, morphine decreased the frequency of breathing, though not significantly, tidal volume was significantly decreased with doses higher than 40 mg/kg s.c., and minute volume was significantly decreased after the administration of 160 mg/kg s.c.. These depressant effects were enhanced with increasing CO_2 concentrations whereas in non-opioid treated animals, higher CO_2 concentrations increased tidal volume, minute volume and respiratory rate. These studies indicate that the effects of administered opioids can be altered according to the breathing environment the animals are placed into during treatment.

1.4.2.2 CHRONIC ADMINISTRATION OF OPIOIDS:

The respiratory effects of chronic opioid administration in animals have not been comprehensively researched. This is surprising, given the inconsistencies in the development of tolerance to the respiratory effects of opioids observed in human studies (See Section 1.4.1.2 on Page 14). There are, however, difficulties in obtaining these data, which will be discussed in the following section.

van den Hoogen & Colpaert [1986] have provided one of the most comprehensive studies investigating both the acute and chronic effects of morphine on respiration in rats (See Section 1.4.2.1 on Page 24 for acute treatment results). In the chronic section of this study, rats were injected with 10

mg/kg s.c. morphine twice daily or saline for 5 days. On the final day, respiratory measurements were taken upon the administration of the last morphine dose in the presence of increasing CO_2 concentrations. No differences were observed between the morphine and saline treated animals under normal breathing conditions (no CO_2), nor were any differences noted in breathing frequency in the presence of CO_2. Increasing CO_2 concentrations, however, significantly increased tidal volume and minute volume in the chronically morphine treated animals compared to those pre-treated with saline. The authors suggest that this indicates tolerance to the respiratory effects of morphine, but this is difficult to determine as 10 mg/kg s.c. morphine administered acutely did not significantly alter respiratory parameters in rats under normal breathing conditions.

Kokka *et al.* [1965] pre-treated rats with 10 mg/kg s.c. morphine for 4, 8, or 12 days prior to measuring respiratory parameters in the presence of varying test doses of morphine. On the first day of treatment, minute volume decreased to 41% of control (pre-treatment) levels but after 4, 8 and 12 days this increased to 67%, 91% and 88% of control values, respectively. In the animals administered a 20 mg/kg test dose of morphine partial tolerance developed, with minute volume only decreasing to 70% of controls. When animals were treated with 10 mg/kg morphine for 4 or 12 days, respiratory rate was not depressed and even increased slightly with the 5, 10 and 20 mg/kg test doses. This is in contrast to the significant respiratory depression produced upon the administration of 20 mg/kg morphine to opioid naïve animals. The decrease in VO_2 normally observed was also abolished in the animals pre-treated for 8 and 12 days, and an increase was observed after the administration of the 5 and 10 mg/kg test doses.

Animals pre-treated with morphine also exhibited partial tolerance to the effect of 10% CO_2 after 4 days, which was almost complete after 8 days. This study, therefore, indicates that tolerance does develop to the respiratory depressant effects of morphine but the parameters measured were not affected simultaneously, so periods of partial tolerance do exist.

A limited number of studies have investigated the effect of chronic morphine treatment on respiration in mice. McGilliard & Takemori [1978b] administered 50 mg morphine pellets to mice and observed a maximum depression within 1 hour that only partially recovered over 24 to 72 hours. They also found that after this pre-treatment the ED_{50} for respiratory depression was approximately 3 fold less than in control mice, which was much lower than the tolerance that had developed to the analgesic effects of morphine. Roerig *et al.* [1987] found that mice administered 75 mg morphine pellets over 72 hours still had lowered respiratory rates, but tolerance had developed as the ED_{50} concentrations of morphine and etorphine were higher than those observed in placebo treated controls.

In two separate studies, White & Zagon [1979] and McCormick *et al.* [1984] injected animals with 2.5, 5.0 or 7.5 mg/kg methadone for 14 days and investigated changes in blood gas concentrations and pH compared to acutely treated animals. Only the pO_2 concentrations were significantly decreased compared to saline treated animals while the decreases in pH and increases in pCO_2 were much smaller than those observed in acutely treated animals. This study suggests that tolerance to the respiratory depressant effects of methadone

can develop, but it appears to be both time and dose dependent. A degree of tolerance to the respiratory effects of chronic methadone has also been observed in pygmy goats administered 3 mg/kg s.c. methadone twice daily for 8 days [Neal & Olsen, 1980].

These chronic studies indicate that repeated opioid administration does alter the respiratory effects produced in animals. Determining the exact mechanisms involved and how they contribute to the effects observed will require further research, but will be valuable in our understanding of the respiratory actions of opioid receptor agonists.

1.4.2.3 LIMITATIONS WHEN EVALUATING DATA FROM PREVIOUS ANIMAL STUDIES:

Generally, rodent studies indicate that opioids produce respiratory depression, but several factors can alter the results obtained. Firstly, Isom *et al.* [1969] identified that the respiratory effects of morphine can differ depending on the rodent strain used, with Long-Evans rats being more sensitive to the depressant effects of morphine than Sprague-Dawley rats. Conversely, lethality studies showed that Long-Evans rats were more resistant to the lethal effects of morphine than Sprague-Dawley rats, with LD_{50} values of 2040 mg/kg and 650 mg/kg respectively [Isom *et al.*, 1969]. Differences in opioid induced respiratory depression have also been seen in different mouse strains and for other opioid effects, including analgesia [Muraki & Kato, 1986; Pick *et al.*, 1991]. Therefore, the potency of opioids and the responses reported may be dependent on the strain of animal used.

Species differences have also been reported. In rodent, dog and monkey studies opioids produce respiratory depression, as seen in humans, but in other animals, such as cats, goats, sheep and cows, opioids appear to stimulate respiration [Neal & Olsen, 1980; Santiago & Edelman, 1985; Simon & Hiller, 1978; Szeto *et al.*, 1991]. For example, Neal & Olsen [1980] observed decreases in arterial blood CO_2 concentrations ($PaCO_2$) and PaO_2, but increases in arterial blood pH, respiratory frequency, V_E (minute volume), V_A (alveolar volume) and VO_2, in pygmy goats administered i.v. methadone. Respiratory stimulation has also been noted in anaesthetised and conscious cats, particularly upon morphine administration directly into the third ventricle of the brain [Florez *et al.*, 1968]. There are several explanations for these opposing effects. It may be due to suppression of the suprapontine influences that normally inhibit breathing, as endogenous opioid production in the medulla region of all species produces respiratory depression. It has also been suggested that the number of opioid receptors in the amygdala and frontal cortex may play a role, as higher proportions of opioid receptors in these regions are found in animals whose respiration is stimulated by opioids [Santiago & Edelman, 1985]. Regardless of the mechanisms that contribute to these differences, the species used in experiments investigating the respiratory effects of opioids must be chosen with care, and should be considered when comparing studies using different species.

As observed in humans (See Section 1.4.1.3 on Page 20), sex and age can alter the respiratory depressant effect of opioids in animals. Sex differences have not been intensively investigated in animals, and no studies have compared opioid

induced respiratory changes between the sexes. Cruz & Rodriguez-Manzo [2000] investigated the gender differences that exist with cardiovascular responses to 30 mg/kg i.v. morphine and found that male spinalised rats experienced a longer bradycardic effect compared to females. Craft *et al.* [1999] found that during chronic morphine dosing (10 mg/kg/day for 5 days), 7 out of the 31 male rats tested died (4 within 24 hours of the initiation of treatment) while no female rats died. Although this is not conclusive evidence, sex differences have been shown to occur for opioid induced analgesia, sedation and locomotor activity, so it can be predicted that this will also be the case for opioid induced respiratory effects [Kest *et al.*, 2000].

With regard to age, opioid receptors continue to develop during and after birth and their distributions change over the lifetime of the animal, which could alter the effects of opioid receptor agonists [Greer *et al.*, 1995; Simon & Hiller, 1978; Ueno *et al.*, 1988]. Older, mature rats experience a progressive decline in metabolic activity with age, which can alter the activities and duration of action of compounds, such as opioids [Spratto & Dorio, 1978]. Of particular interest was one study performed by Spratto & Dorio [1978], which found that rats aged 6 to 10 months were more sensitive to the respiratory depressive effects of morphine when compared to animals of 1 to 5 months of age. Another study has also shown that the antinociception produced by morphine doses up to 40 mg/kg s.c. was significantly less in aged mice (24 to 27 months) than mature adult mice (3 to 6 months), and locomotor activity was also reduced in the older animals [Hoskins *et al.*, 1986]. These age related differences were not attributed to changes in the concentrations of morphine in the brain or its distribution throughout the brain

regions, but rather alterations in the affinity, number or responsiveness of the centrally located opioid receptors. Further investigation has identified that while renal function is reduced in aged rats, the pharmacokinetics of morphine are not altered, which is consistent with renal clearance not contributing greatly to the clearance of morphine in rats [Van Crugten *et al.*, 1997]. Morphine-3-glucuronide (M-3-G) concentrations, however, were significantly higher in the aged rats, as was the concentration time curve after the administration of M-6-G. These age related effects are most likely due to the diminished renal function and lowered biliary excretion of these metabolites in the aged animals [Van Crugten *et al.*, 2000]. Therefore, age is another confounding factor that may affect the results obtained in studies examining the effects of opioids on respiratory depression.

The method of measuring respiration in animals is also a source of great variation, and can limit the information obtained from the experiments performed. Anaesthetised animals are often used in studies measuring respiratory alterations after opioid administration. Anaesthetics can depress respiration, which can complicate the results obtained, and anaesthetised animals cannot respond to drug induced changes in the same manner as conscious animals. Florez & Pazos [1982] compared the respiratory effect several opioid peptides after intracerebroventricular (i.c.v.) administration and found higher ED_{50} doses were required for awake, rather than anaesthetised, animals. Studies have also shown that naloxone is able to partially block the action of anaesthetics, such as halothane, nitrous oxide and barbiturates. This has led to the suggestion that some anaesthetics may produce their actions by inducing endogenous opioid release [Santiago & Edelman, 1985]. Therefore, the use of anaesthetic agents

may have significant effects on the respiratory alterations observed in opioid receptor agonist studies.

In experiments involving conscious animals, the impact of stress on respiration is an important factor that cannot be overlooked. Many techniques, such as whole body barometric plethysmography and pneumotachography, require the placement of animals into small chambers. This can produce stress on the animals and affect their behaviour, as can the handling of animals throughout these procedures. Stress alone has been shown to produce changes in respiration, which makes the evaluation of respiratory data from opioid receptor agonist studies difficult. This stress can also induce endogenous opioid release, which may complicate the opioid receptor agonist effects observed [Adler *et al.*, 1988; Akil *et al.*, 1976; Bodnar & Hadjimarkou, 2002; Joris *et al.*, 1987; Kelly & Franklin, 1987; Pierce & Raper, 1995; Yates *et al.*, 2001]. Whilst this is an unavoidable outcome in the experimental manipulation of animals, it should be considered when comparing the results from studies using different methodology. Future advances in technology may lead to the development of techniques that allow the measurement of respiratory parameters in conscious, unrestrained and unstressed animals to allow us to measure the respiratory effects of opioids without other confounding factors. The use of radiotelemetry implanted animals is one such technique that will be investigated in this research project (See Publication 4 on Page 171).

Whilst there are many limitations in the information that can be obtained from human and animal studies investigating the effect of opioids on respiration,

continued research is vital in order to determine the mechanisms that are involved. Whilst we have a good understanding of the effect of opioids on the respiratory system, the mechanisms that may contribute to these effects are complex and will be discussed in the next section.

1.5 MECHANISMS CONTRIBUTING TO OPIOID INDUCED RESPIRATORY DEPRESSION:

Whilst it is clear that opioids can alter respiration, the exact mechanisms involved are difficult to elucidate. Control of respiration is complex and involves many different pathways. It must be both voluntary so that breathing can adapt to different situations, such as talking and swimming, and involuntary so that breathing will continue without conscious effort, such as during sleep [Boyle, 1991]. Both peripheral and central areas of the body control respiration and those that are altered by opioids are described below.

1.5.1 Central mechanisms involved in opioid induced changes to respiration:

1.5.1.1 CENTRAL CONTROL OF RESPIRATION:

The medulla oblongata region of the brain generates the basic rhythm of breathing. This region has been split into two sections, the ventral respiratory group (VRG) and the dorsal respiratory group (DRG). The cells in the DRG are mainly active during inspiration, whilst those in the VRG are involved in both inspiration and expiration [Bianchi *et al.*, 1995; Long & Duffin, 1984]. The exact functions of these regions are not well defined, but it is thought that the DRG is involved in signalling to the spinal inspiratory motor neurons, while the VRG

controls rhythm generation [Bianchi *et al.*, 1995; Long & Duffin, 1984]. There appear to be slight differences in the activity of these regions between different species, but the overall anatomical and functional organisation of this network is consistent, indicating their actions are conserved in all species [Bianchi *et al.*, 1995].

Although the VRG and DRG are considered to be the main respiratory centres, they are not exclusive and rely on other regions to receive or relay signals. The DRG and VRG are affected by activity in the reticular activating system, the cerebral cortex and the vagus, glossopharyngeal and somatic nerves [Boyle, 1991; Hurle *et al.*, 1983; Rutherfurd & Gundlach, 1993; Willette & Sapru, 1982]. The pontine region of the brain contains a high proportion of respiratory neurons, and is often called the pneumotaxic centre as it is involved in controlling the duration of respiration [Bianchi *et al.*, 1995; Long & Duffin, 1984]. This brain region may also be involved in adjusting respiration in response to inputs from the periphery, and conveying information from the hypothalamus to the respiratory centres in the medulla [Bianchi *et al.*, 1995]. Regions of the spinal cord (in particular the upper cervical level) have inspiratory modulated neuronal activity, and receive inputs from inspiratory neurons of the DRG and VRG. They appear to act as a relay station between the respiratory motoneurons and peripheral receptors or other regions of the central nervous system (CNS) [Bianchi *et al.*, 1995]. Together all of these areas work in combination to allow normal breathing and adapt respiration to changing conditions.

1.5.1.1.1 Effect of opioids on the central control of respiration:

With so many regions being involved in the modulation of respiration, opioids can

act at various sites to produce their respiratory effects. Endogenous opioids do participate in respiratory control, but their effects appear to be limited. Endogenous μ opioid receptor agonists depress tidal volume and slow respiratory timing but this only appears to be important in stressful situations or lung disease, and not in healthy animals [Moss & Laferriere, 2002]. The endogenous opioid system is also involved in respiratory and sleep-wake state control during development [Moss & Laferriere, 2002]. Therefore, the role of endogenous opioid mechanisms in normal respiration is still largely unknown.

Exogenously administered opioids produce more pronounced effects on respiration. Significant depression of respiration occurs when opioid receptor agonists, such as D-Ala^2-N-Me-Phe^4-Gly-ol^5-enkephalin (DAMGO) and D-Ala^2-D-Leu^5-enkephalin (DADLE), are injected into the nucleus ambiguus in spontaneously and artificially breathing anaesthetised rats [Hassen *et al.*, 1984]. Injection of D-Pen^{2,5}-enkephalin (DPDPE) and DAMGO into the rostral ventrolateral medulla activates δ and μ opioid receptors and may alter the somato-sympathetic, baroreceptor and chemoreceptor reflexes in this brain region [Miyawaki *et al.*, 2002]. The infusion of morphine in the cisterna magna alters respiration in anaesthetised dogs, which suggests that opioids are active in the superficial and deeper brainstem and suprapontine structures [Pelligrino *et al.*, 1989]. Microinjection of opioid receptor agonists into the hypothalamus has shown that μ opioid receptor agonists depress respiration more than δ or κ opioid receptor agonists [Faden & Feuerstein, 1983; Pfeiffer *et al.*, 1983]. The pontine region is also directly influenced by the presence of μ and δ opioid receptor agonists, and may be more sensitive to opioid effects than the medulla regions of

the brain [Hurle *et al.*, 1983]. All of these studies indicate that opioids can act centrally to control respiration and these actions are predominantly due to the presence of opioid receptors in these regions.

1.5.1.2 CENTRAL DISTRIBUTION OF OPIOID RECEPTORS:

Whilst there have been difficulties in determining which opioid receptor types are involved in opioid induced respiratory depression, the distribution of opioid receptors does provide us with evidence regarding their sites of action. Numerous researchers have found opioid receptors in high concentrations in the areas of the brain that affect respiration in a variety of species. These include the nucleus solitarius tractus, nucleus ambiguus, raphe nuclei, septal nuclei, pontine regions, the hypothalamus and mid brain regions, such as the periaqueductal gray [Delfs *et al.*, 1994; Mansour *et al.*, 1995; Moskowitz & Goodman, 1984; Tempel & Zukin, 1987]. Opioid receptors that are not of the typical μ, δ and κ types have also been identified in brain regions that are involved in regulating respiration, indicating that atypical receptors may also mediate the respiratory effects produced by opioids [Bunzow *et al.*, 1994]. In order to clarify these differences an explanation of the different types of opioid receptors identified is detailed below.

1.5.1.2.1 <u>Identification and classification of opioid receptors:</u>

While the effects of opioids on respiration and other parameters have been known for centuries, the mechanisms by which these effects occur, such as through activation of opioid receptors, have only recently been determined. In the late 1960s, opioids were shown to produce a lack of responsiveness to CO_2 so it

was proposed that opioids were somehow able to alter central sympathetic centres [Martin *et al.*, 1968; Martin & Jasinski, 1969]. In 1973, several research groups reported the use of radiolabelled opioid ligands to indicate the presence of specific opioid receptors in the brain [Pert & Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973]. Several different opioid receptors have since been identified and have assisted in explaining the various opioid effects observed. There is still controversy regarding the different receptor types that exist and their different functions. Confirmation of different receptor types with separate effects has remained elusive, but research is continuing to determine the exact functions of each of the receptor types identified.

It is generally accepted that there are three main opioid receptor classes: μ (mu), δ (delta) and κ (kappa). There appears to be approximately 60% amino acid sequence homology between the three opioid receptor sequences, but they are present on different chromosomes in mice and humans [Satoh & Minami, 1995; Zaki *et al.*, 1996]. All identified opioid receptors have the seven putative transmembrane helices, which are characteristic of G-protein coupled receptors. A proposed model of the μ-receptor structure is illustrated in Figure 1.

Numerous subtypes of each of these receptors classes have been suggested (at least three μ subtypes, two δ subtypes and four κ subtypes) but conclusive evidence to separate each subtype is still lacking [Connor & Christie, 1999]. Various opioid actions have been attributed to the same receptor type, which has strengthened the hypothesis that different receptor subtypes exist. Different opioid receptor agonists and antagonists for the same receptor type also have

different binding affinities and produce different effects, again suggesting subtle differences between receptors in the same class [Pasternak & Wood, 1986; Zaki *et al.*, 1996]. The various opioid receptors and subtypes that have so far been identified have been, or are being, cloned in many species [Bare *et al.*, 1994; Evans *et al.*, 1992; Li *et al.*, 1993; Thompson *et al.*, 1993; Zhu *et al.*, 1995].

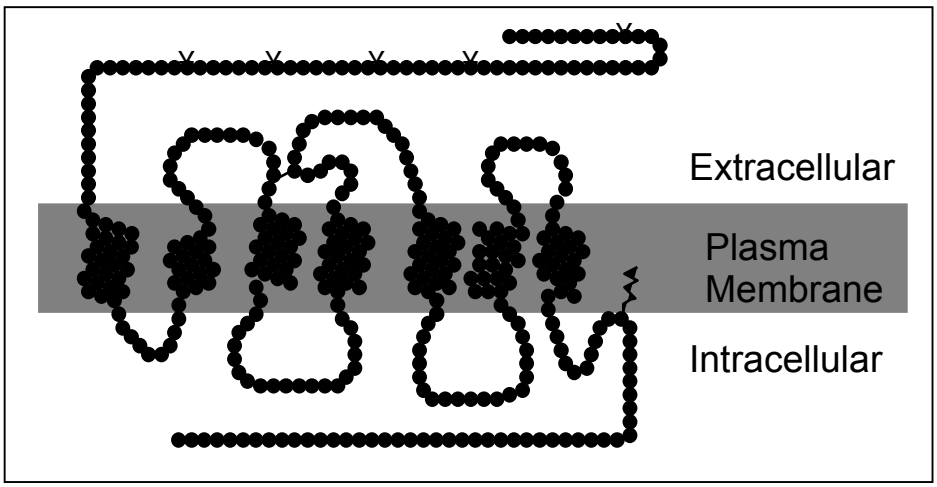

Figure 1: *Proposed model for the membrane topography of the rat μ opioid receptor.*
From Satoh & Minami [1995].

Different genes for each subclass may exist or these receptor subtypes may be produced from alternative splicing of existing genes, post-translational modifications of the receptor proteins, or receptor proteins interacting with different G-protein complexes. The location of these receptors and different cellular environments may also alter their functions, while the accessibility of substrates for different receptor populations throughout the body may also contribute to the various effects produced [Zaki *et al.*, 1996].

Other opioid receptor types, such as sigma (σ), epsilon (ε), iota (ι), and zeta(ξ), have also been identified [Zaki *et al.*, 1996]. This has complicated the classification of opioid receptors as it is not known if these receptor types are subtypes of the μ, δ and κ opioid receptor classes or are distinct opioid receptor classes. In recent years another opioid receptor class was identified, which exhibits a high degree of structural homology to conventional opioid receptors but displays distinct pharmacological characteristics, such as not responding to opioid receptor agonists and antagonists in a similar fashion to classical opioid receptors [Connor & Christie, 1999]. These have been called 'orphan' receptors, 'opioid related' or 'opioid like' receptors or are identified according to the compounds that bind to them, for example, nociceptin/orphanin FQ (N/OFQ) [Alexander & Peters, 2000]. It is likely that these receptors are a unique class of opioid receptors, but they may also be a subtype of the classical opioid receptors. While the roles of these N/OFQ receptors (NOP receptors) and their specific receptor agonists are not well defined, they have been shown to produce effects in the airways, as described in further detail below (See Section 1.5.1.3 on Page 42) [Corboz *et al.*, 2001].

It should also be noted that in 1996 the nomenclature of opioid receptors was revised by the International Union of Pharmacology (IUPHAR), which suggested the use of OP3, OP1 and OP2 for μ, δ and κ opioid receptors, respectively [Dhawan *et al.*, 1996]. This nomenclature was not readily accepted by researchers and several other classifications were developed (See Table 1), so in 1999 an IUPHAR subcommittee recommended that the nomenclature return to the Greek classification of these receptors [Cox *et al.*, 2003]. This

original nomenclature will be used throughout this dissertation as shown in Table 1.

Original Nomenclature	Other Nomenclature
Mu (μ)	MOP, OP3, MOR
Delta (δ)	DOP, OP1, DOR
Kappa (κ)	KOP, OP2, KOR
NOP	N/OFQ, ORL1, NOR, OP4

Table 1: *Original and other nomenclature for opioid receptors. From Alexander & Peters [2000].*

1.5.1.2.2 Opioid receptor signalling mechanisms:

Opioid receptors have the characteristic 7 putative transmembrane domains that are associated with G-protein coupled receptors and are mainly coupled to pertussis toxin-sensitive $G_{i/o}$-proteins. The activation of these receptors produces inhibition of adenylate cyclase activity and modulates a number of membrane conductances, such as rectifying potassium ion (K^+) currents and inhibiting calcium ion (Ca^{2+}) currents to reduce membrane excitability. The inhibition of adenylate cyclase activity may contribute to the regulation of cellular mechanisms by opioids, which include the control of gene expression and modulation of cellular phosphatases and kinases. The coupling of opioid receptors to the mitogen-activated protein kinase (MAPK) pathway has also been recently characterised, and is thought to occur via three mechanisms. Firstly, the release of G-protein βγ-subunits following activation of opioid receptors can activate phosphatidylinositol-3 kinase (PI3K) which, after several phosphorylation steps,

can activate MAPK. Secondly, phosphorylation and internalisation of receptors can activate the MAPK signalling pathway. Finally, MAPK can be activated by protein kinase (PK)A in the CNS, which is produced when opioid receptors are activated. While this may not be significant in acute opioid administration, it may contribute to changes in the gene regulation of various transcription factors during chronic opioid treatment [Borgland, 2001; Connor & Christie, 1999; Satoh & Minami, 1995; Simon & Hiller, 1978; Zaki *et al.*, 1996].

1.5.1.3 INVOLVEMENT OF OPIOID RECEPTORS IN OPIOID INDUCED RESPIRATORY ALTERATIONS:

Research has identified that μ receptors, and in particular μ_2 receptors, are predominantly responsible for opioid induced respiratory depression, as shown by Ling *et al.* [1985]. They observed that the administration of naloxonazine (an irreversible μ_1 receptor antagonist) antagonised morphine induced analgesia, but did not affect respiratory depression. This suggested that μ_1 receptors are involved in mediating analgesia but do not contribute significantly to respiratory depression. Equipotent analgesic doses of morphine and metkephamid ((Tyr-D-Ala-Gly-Phe-N(Me)Met-NH_2) an opioid receptor agonist that binds to μ_2 and δ receptors with equal potency) were then tested and found to produce similar respiratory depressant effects. This again suggested that μ_2 receptors were involved but did not discount δ receptor involvement. A comparison of equi-analgesic doses of DADLE (an opioid receptor agonist that preferentially binds to δ rather than μ_2 receptors) and metkephamid then found that DADLE was less potent in producing respiratory depression, indicating that δ receptors were not the main receptor type contributing to the effects observed. Therefore, it was

concluded that μ_2 opioid receptors were the main receptor type involved in producing opioid induced respiratory depression.

δ receptors are thought to play a role in the respiratory effects of opioids, but the significance of this contribution has not been fully elucidated [Morin-Surun *et al.*, 1984; Pazos & Florez, 1984]. These observations may be because compounds such as D-Pen-L-Pen-enkephalin (DPLPE), DPDPE and DADLE have been used as specific δ receptor agonists. Despite these agonists binding with greater affinity to δ opioid receptors than other opioid receptor types, the effects reported may still be due to μ opioid receptor actions [Shook *et al.*, 1990]. δ receptors have been found in regions of the brain that are thought to control respiration, which suggests that they are involved in the respiratory effects of opioids but this is minor compared to that of μ opioid receptors [Florez & Hurle, 1993].

σ receptors have not been well characterised, so their effects on opioid induced respiratory effects are largely unknown. Limited studies have shown that σ opioid receptor antagonists, such as SKF 10,047 and N-allylnormetazocine, can produce both respiratory stimulation and depression in spinalised dogs, but other opioid receptor types may contribute to these actions [Martin *et al.*, 1976; Wu & Martin, 1989]. Further investigation using specific σ receptor agonists is required to clarify the function of this receptor type in opioid induced respiratory effects [Shook *et al.*, 1990].

κ opioid receptors do not appear to be involved in respiratory depression to the same degree as δ or μ opioid receptors. Several studies have shown that κ

receptor agonists produce no effect on respiration, while others have shown dose dependent increases in respiratory rate with subsequent decreases in tidal volume [Castillo *et al.*, 1986; Pfeiffer *et al.*, 1983; Yeadon & Kitchen, 1990]. This, however, has not been examined in detail, and confirmation will require continued investigation.

The role of NOP receptors in opioid effects is largely uncharacterised, with most research concentrating on its involvement in analgesia. A recent *in vitro* study has shown that nociceptin (the hypothesised endogenous ligand for NOP receptors) can act as a neuromodulator to reduce respiratory frequency in the ventral region of the medulla oblongata, which is responsible for respiratory rhythm generation [Takita *et al.*, 2003]. Nociceptin, like DAMGO, can modulate cholinergic and nonadrenergic-noncholinergic transmission in guinea pig tracheal preparations, but naloxone did not alter this effect, which suggests that nociceptin does not act at classical opioid receptors [Patel *et al.*, 1997; Shah *et al.*, 1998]. Studies in anaesthetised guinea pigs have also shown that nociceptin can inhibit capsaicin induced bronchoconstriction, which can only be blocked by J-113397, a specific NOP receptor antagonist, and not naloxone [Corboz *et al.*, 2001]. Nociceptin can attenuate baroreflex sensitivity and, therefore, increase blood pressure and heart rate when injected into the nucleus tractus solitarius [Mao & Wang, 2000]. NOP receptors may also play a role in the mediation of asthma and in the cough reflex [Groneberg & Fischer, 2001]. This suggests that NOP receptors are present in the lungs and may play a role in respiration, but the effect of this receptor type on opioid induced respiratory depression in still unknown [Peiser *et al.*, 2000].

1.5.2 Peripheral mechanisms involved in opioid induced changes to respiration:

Whilst central mechanisms are commonly thought to control the respiratory effects of opioids, there is increasing evidence that peripheral mechanisms contribute to these effects (See Figure 2 & 3). The presence of opioid receptors in the lungs is one such example and several other mechanisms exist, as described below. The concept that peripheral mechanisms play a role in opioid effects is not unusual as opioid effects, such as analgesia and gastrointestinal alterations, are thought to contain peripheral components, presumably due to opioid receptors located in the periphery [Manara *et al.*, 1986; Pol *et al.*, 1996b; Schulz *et al.*, 1979; Stein, 1995; Yuan *et al.*, 1997].

1.5.2.1 PERIPHERALLY LOCATED OPIOID RECEPTORS:

1.5.2.1.1 Peripheral distribution of opioid receptors:

Opioid receptors have been found in many regions outside of the brain, which suggests that opioids can produce responses in the periphery as well as in the CNS. They are distributed in the spinal cord, particularly in the dorsal horn in layers I (marginal cell zone) and II (substantia gelatinosa) and the spinal trigeminal nucleus. These receptors are mainly responsible for the analgesic effects of opioids at the spinal level [Simon & Hiller, 1978]. The central control of opioid induced analgesia is well known, but the hypothesis that peripheral opioid receptors may also influence analgesia has gained wider acceptance over recent years. It is now thought that analgesia is both peripherally and centrally mediated and that the peripheral effects may act alone or in conjunction with central effects [Junien & Wettstein, 1992; Stein, 1995].

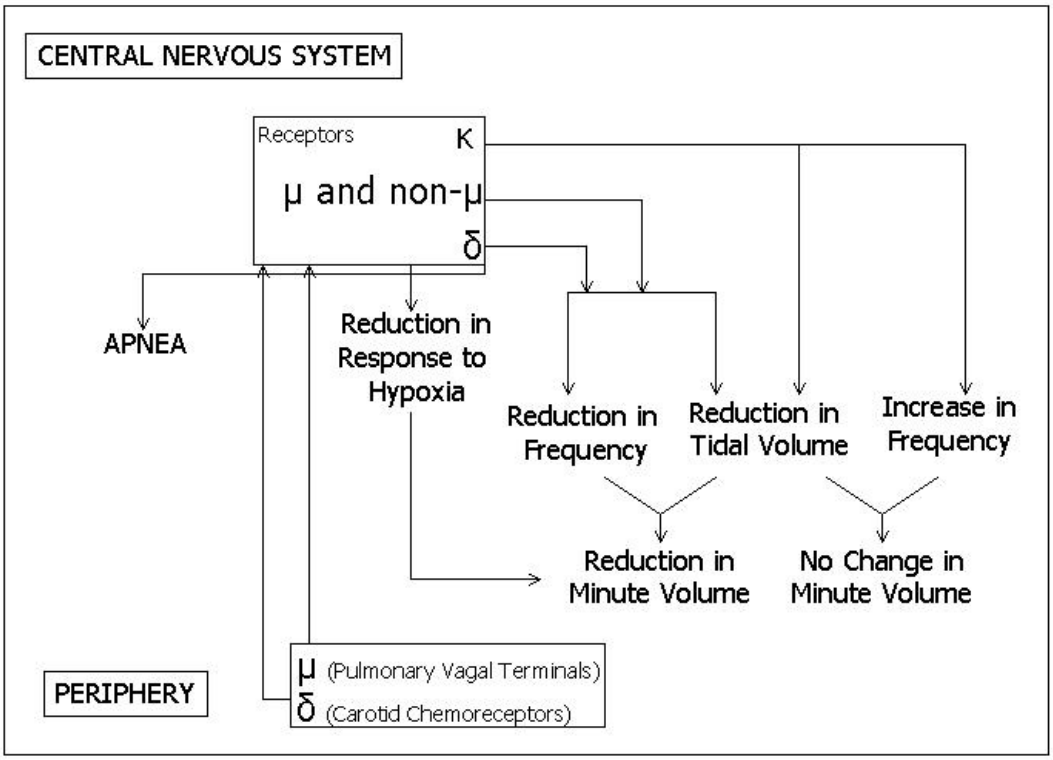

Figure 2: *Location of and respiratory effects resulting from stimulation of multiple opioid receptors.*
From Yeadon & Kitchen [1989].

Opioid receptors, in particular δ receptors, are also present in cardiac tissue and are involved in ischemic pre-conditioning, the protection of the myocardium for brief periods of ischemia or hypoxia [Barron, 2000; Bell *et al.*, 2000; Schultz *et al.*, 1997; Wittert *et al.*, 1996]. This opioid effect does not require signalling from the CNS, indicating that it is a peripherally mediated opioid action [Milanes *et al.*, 2001; Schultz *et al.*, 1997]. It has also been suggested that opioid receptors are present in blood vessels, which may contribute to these opioid actions [Peroutka *et al.*, 1980].

Another region where opioid receptors are present and produce peripherally mediated effects is the gastrointestinal tract. μ and κ opioids have been identified in different proportions throughout the gastrointestinal system and act to reduce gut motility [Fickel *et al.*, 1997]. This is thought to occur mainly through the control of neuronal communication, with little effect on smooth muscle cells [Fickel *et al.*, 1997]. As with the cardiac effects of opioids, the gastrointestinal opioid receptors are thought to produce peripheral effects independent of centrally mediated mechanisms [Gmerek *et al.*, 1986].

Other regions where opioid receptors are present are the adrenal medulla, kidneys, liver, spleen, testes, ovary and uterus [Quirion *et al.*, 1983; Wittert *et al.*, 1996]. Research investigating the exact function of the opioid receptors in these regions is continuing.

1.5.2.1.2 Identification of opioid receptors in lung tissue:

When investigating the mechanisms involved in opioid induced respiratory depression, the presence of opioid receptors in lung tissue is of particular interest. Tang *et al.* [1983] provided the first evidence that opioid binding sites may exist in the lungs. They isolated the endogenous opioid MET^5-enkephalin-ARG^6-PHE^7 from rat lung and showed that [^{3}H]-etorphine could bind to lung tissue and be displaced by this enkephalin. Sato *et al.* [1988, 1990] also showed that [^{3}H]-dynorphin (a κ opioid receptor agonist) could bind to specific binding sites in rat lung, which led to the proposal that these binding sites were κ opioid receptors.

There is much controversy regarding the presence of opioid receptors in lung tissue. Bhargava *et al.* [1997] positively identified μ, δ and κ opioid receptor binding in rodent lungs using radioligand binding. mRNA from each of these receptor types has also been isolated in rat lung tissue by Wittert *et al.* [1996]. Delfs *et al.* [1994], however, attempted to isolate μ receptor mRNA from rat lung homogenates using mRNA blotting techniques and were unable to confirm the presence of a specific μ receptor mRNA sequence in the lung.

Sunday *et al.* [2001] investigated the presence of μ, δ and κ opioid receptors in lung tissue in mice from 11 days after gestation to 14 days after birth using reverse transcriptase polymerase chain reaction (RT-PCR) techniques. They found that δ opioid receptor mRNA was present at all time points, κ opioid receptor mRNA was detected up to 16 days after gestation and again, briefly, after birth. μ opioid receptor mRNA was expressed from days 11 to 13 and 15 to 16 after gestation, from day 18 until birth and was then absent until day 14 after birth.

Various radioligand binding experiments have been conducted and have confirmed that opioid receptor agonists and antagonists bind to lung tissue slices and homogenates, but they do not behave like those receptors found in brain tissue. A study by Cabot *et al.* [1994] found that the density of opioid binding sites in rat lungs was 100 times greater in the bronchioles and lobes than in the main bronchi and trachea. They then showed that two main binding regions exist, in the alveolar wall and within the trachea and main bronchi, but the exact cellular location of these receptors remains to be determined [Bhargava *et al.*,

1997; Cabot *et al.*, 1996]. Cabot *et al.* [1994, 1996] also found that [^{3}H]-morphine and [^{3}H]-naloxone could bind to both lung homogenates and tissue slices and that naloxone could displace [^{3}H]-morphine bound to the lung, but neither of the [^{3}H]-ligands could be displaced by DAMGO, DPDPE or U50,488H. These studies suggest that the opioid receptors present in the lung are not of a typical nature.

Work conducted by Maneckjee & Minna [1990] on opioid receptors present in human lung cancer cell lines has yielded interesting data. Specific binding of [^{3}H]-etorphine, [^{3}H]-DAMGO, [^{3}H]-DPDPE and [^{3}H]-U50,488H was observed in small-cell lung cancer and non-small-cell lung cancer cell lines. They also showed that these cell lines produced β-endorphin, enkephalin and dynorphin. Further experimentation found that exposure of these cancer cells to methadone inhibited cell growth and produced cell death that could be inhibited by the addition of naltrexone or naloxone to the culture medium. This would suggest that the growth inhibition produced by methadone was mediated by opioid binding sites. It was also interesting to note that the pertussis toxin was able to reverse the effects of morphine on these cells, but not the effects of methadone. In further research, they found that in rat brain membranes, [^{3}H]-methadone could be displaced by DAMGO, DPDPE, U-50,488H, SKF-10,047 (a specific σ receptor agonist), and MK-801 (specific for phencyclidine (PCP)/NMDA receptors). In lung cancer cell lines, U-50,488H, MK-801 and naloxone could displace the [^{3}H]-methadone, while no effect was observed with DAMGO and DPDPE [Maneckjee & Minna, 1992].

The subsequent paper published by this research group showed that these specific methadone binding sites were present in non-cancerous brain and lung tissue [Maneckjee & Minna, 1997]. U-50,488H, SKF 10,047 and MK-801 could displace the methadone binding in human brain membranes, while binding in lung tissue could only be displaced by U-50,488H and MK-801. They also showed that PCP/NMDA receptors were not responsible for the actions of methadone observed in this study. The endogenous opioids beta-endorphin, enkephalin and dynorphin could not displace the methadone binding in either of these tissue types, but naloxone was successful. This research again suggests that although opioid receptor binding sites are present in the lungs, they may not be of a typical nature.

Fimiani *et al.* [1999] investigated the displacement of [^{3}H]-dihydromorphine binding with μ, δ and κ ligands in normal human lung and non-small cell lung carcinoma. They found that the opioid receptors present had little affinity for any of the opioid peptides or analogues tested, but had high affinity for the opiate alkaloids dihydromorphine and morphine. They concluded that these receptors are of the μ_3 subtype because they behave similarly to those receptors present in granulocytes.

Investigation of opioid receptors in human lung tissue during development has also been conducted [Gomez-Roman *et al.*, 2002]. μ, δ and κ opioid receptors were identified in the canalicular stage and peak distributions were noted at birth. In the adult samples analysed, only δ opioid receptors were identified, indicating that significant changes in opioid receptor expression and distribution occur

throughout development. Positron emission topography (PET) imaging using [^{11}C]-carfentanil and [^{11}C]-N-methyl-naltrindole to identify μ and δ opioid receptors has, however, identified these receptors in lung tissue, but naloxone administration only blocked δ opioid receptor binding [Villemagne *et al.*, 2002].

These studies, therefore, indicate that opioid receptors appear to be present during development but their distribution and expression can alter with maturity. It also appears that the opioid receptors present in the lungs may not be of a typical nature due to their non-classical responses to opioid receptor agonists and antagonists.

1.5.2.1.3 Action of opioid receptors in lung tissue:

Regardless of whether the opioid binding sites in the lungs are of a typical or atypical nature, μ opioid receptor agonists have been shown to inhibit the increased peripheral terminal excitability of afferent nerves induced by mediators, such as prostaglandin E2 [Gold & Levine, 1996; Undem & Carr, 2001]. Opioids also inhibit cholinergic neurotransmission in isolated human and guinea pig airway preparations, demonstrating that they can produce effects on the respiratory system that are exclusive of opioid mediated pathways [Belvisi *et al.*, 1990; Belvisi *et al.*, 1992]. More specifically, μ, δ and κ opioid receptor agonists can act in the airways to inhibit respiratory reflexes [Adcock, 1991; Kotzer *et al.*, 2000]. Therefore, opioids can produce direct effects in the lungs, through what appear to be opioid receptors, so peripheral mechanisms may contribute to opioid induced respiratory depression.

1.5.2.2 CHEMORECEPTORS:

The most well known and studied of the peripheral mechanisms involved in respiration are the effects of chemoreceptors. Chemoreceptors are present both peripherally and centrally and are involved in monitoring changes in blood pH and gas concentrations. Of particular importance is the modulation of CO_2 concentrations in the blood. Chemoreceptors are able to relay information regarding these changes from the periphery to the brain via the vagal and glossopharyngeal nerves [Florez & Hurle, 1993]. This is a vital mechanism as it allows the CNS to detect hypoxia and initiate responses to maintain homeostasis.

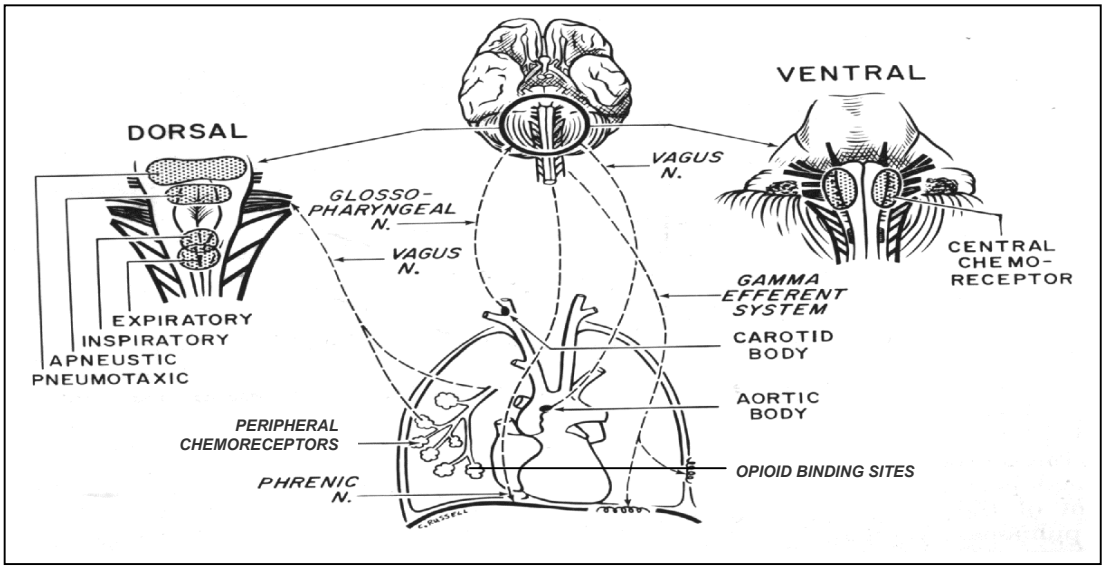

Figure 3: *The interaction of peripheral and central nerves involved in regulating respiration.*

Adapted from Slonim & Hamilton [1971].

Morphine has been shown to depress the function of peripheral chemoreceptors so they do not respond to hypoxia, but whether this plays a significant role in opioid induced respiratory depression has not been determined [Berkenbosch *et*

al., 1997]. If opioids do significantly affect the function of chemoreceptors it would explain why compensatory mechanisms are not initiated to counter the increases in CO_2 concentrations and respiratory depression that can occur in opioid overdoses [McQueen, 1983; White & Irvine, 1999].

1.5.2.3 JUXTA-CAPILLARY RECEPTORS:

Juxta-capillary receptors (J receptors) have been identified in the alveoli, close to pulmonary capillaries, and are supplied by the vagal C fibres [Sapru *et al.*, 1981]. They are often thought to be pulmonary chemoreceptors due to their similar effects. These receptors can alter respiration in both intact and vagotomised animals. This suggests that they not only relay nerve signals to the brain, but may also have a direct action on the lungs. J receptor activation has been linked to apnea, bradycardia and biphasic changes in blood pressure and can produce rapid breathing. Therefore, J receptors may be involved in altering respiration through these changes in cardiovascular parameters [Negri *et al.*, 1998]. Morphine has been shown to stimulate these effects and naloxone can antagonise them, indicating that opioids directly alter the function of J receptors [Willette & Sapru, 1982; Yeadon & Kitchen, 1989].

1.5.2.4 STRETCH RECEPTORS:

Stretch receptors, or mechanoreceptors, are found in the lungs and control the degree of inflation of the lungs. These receptors cause a discharge of impulses in the afferent fibres, which connect to the vagus nerves and input to the DRG. They are thought to be involved in important reflexes including the Hering-Breuer reflex, which terminates inspiration so that the lungs do not over-inflate, and

control the depth and rhythm of respiration [Florez & Hurle, 1993; Haji *et al.*, 2000]. It is also known that if the vagi are cut, mechanoreceptors do not function, resulting in deeper inspiration and reduced respiratory rates, while stimulation of these cut vagi inhibits inspiratory movements [Berger & Dick, 1987]. The effect of opioids on stretch receptors has not been directly investigated, but Delpierre *et al.* [1995] showed that i.v. naloxone administered to anaesthetised rabbits could increase respiratory rate through actions on stretch receptors. Therefore, opioids can act on pulmonary stretch receptors, which can then send signals to the CNS through large vagal afferent fibres.

1.5.2.5 PULMONARY SURFACTANT:

Alterations to pulmonary surfactant synthesis and function can alter respiration, and opioids may be directly involved or contribute to the complications that result from respiratory depression. Pulmonary surfactant plays a number of roles in respiration. It reduces the work of breathing by reducing the surface tension in the lung and increases lung compliance, the ease with which the lung can be inflated. It also prevents alveolar collapse and pulmonary oedema and stabilises the gas exchange area available in the lungs [Robertson *et al.*, 1992].

Pulmonary surfactant is composed of 90% lipid, the most important being disaturated phosphatidylcholine (DPPC), which is the major surface tension lowering agent in surfactant. The remaining 10% of pulmonary surfactant consists of surfactant specific proteins, namely SP-A, SP-B, SP-C and SP-D, and while the exact function of these proteins is unclear, they are vital in maintaining surfactant activity. The major stimulus for surfactant release is mechanical

stretch of the type II cells in the lungs, which occurs during deep breathing. The components released then form a three-dimensional latex-like structure, called tubular myelin, which is adsorbed into the surfactant monolayer at the air-water interface of the lungs. During inhalation and exhalation, the components of surfactant expand or compress to alter surface tension and assist lung function during breathing [Robertson *et al.*, 1992].

Opioids may also indirectly affect surfactant composition and function. Respiratory depression results in a decrease in tidal volume and the number of spontaneous breaths, which results in less surfactant release by type II cells [Berkenbosch *et al.*, 1997; Nicholas & Barr, 1981]. This could lead to increased surface tension, making breathing more difficult when respiration is already laboured. This could result in alveolar collapse, an overall endpoint in respiratory depression and opioid overdose.

Few researchers have investigated the effects of opioids on pulmonary surfactant synthesis. Gewolb *et al.* [1999] found that in foetal rat lungs, morphine, heroin and methadone significantly increased the amount of choline incorporated into the phosphatidylcholine (PC) surfactant lipids, suggesting that more surfactant is produced. An increase in the proportion of type II cells to other lung cells in culture and an increase in the number of lamellar bodies, the surfactant storage vesicles, inside these type II cells was also observed. This would suggest that opioids can affect surfactant synthesis and may directly contribute to opioid induced changes in respiration.

The role of endogenous deltorphins has also been investigated during foetal lung development in the mouse. In lung organ cultures, Sunday *et al.* [2001] observed that [D-Ala^2]deltorphin II (DADTII), a soluble δ opioid receptor specific deltorphin analogue, inhibited [^{3}H]-choline incorporation into PC lipids and stimulated cell proliferation. Dermorphin, a μ opioid receptor specific opioid receptor agonist, stimulated PC lipid production and inhibited cell proliferation. Opioids play a role in the regulation of cell proliferation and type II cell differentiation during development and may, therefore, affect surfactant synthesis and function in later life.

Studies in adult rat lungs by Lopatko & White [2000, 2001] have also produced promising data showing interactions between opioids and pulmonary surfactant. If the tidal volume of isolated perfused rat lungs is lowered, which occurs during respiratory depression, a decrease in surfactant synthesis and release will result. The addition of morphine under these conditions can lead to the exacerbation of these effects. Changes in the lipid composition of microsomes, lamellar bodies, tubular myelin and surfactant film are also observed in these morphine treated animals. Morphine also produced alterations in surfactant synthesis and turnover, which could impair the surface activity of pulmonary surfactant. This may lead to a reduction in the alveolar surface area available for gas exchange, resulting in hypoxemia during an opioid overdose. These alterations also occur within two hours of morphine administration, so may play a vital role in opioid induced respiratory depression and subsequent overdose.

There is evidence that several peripheral mechanisms are involved in respiration and that opioids are capable of altering these effects [Willette & Sapru, 1982]. The exact contribution of these peripheral mechanisms in opioid induced respiratory depression is still being investigated and may be assisted by the use of peripherally acting opioid receptor antagonists to determine the role of these peripheral actions in opioid effects.

1.6 PERIPHERALLY ACTING OPIOID RECEPTOR ANTAGONISTS:

Opioid receptor antagonists are defined as compounds that bind to opioid receptors to prevent other compounds from binding and, therefore, prevent their effects [Katzung, 1995]. Naloxone and naltrexone are two opioid receptor antagonists commonly used both experimentally and therapeutically. Naloxone is often administered in opioid overdose situations, as it is able to bind to opioid receptors and reverse opioid induced respiratory depression by blocking the actions of the opioids. There are, however, difficulties associated with this treatment, as described in Section 1.3.1 on Page 9.

Many alternatives are being investigated to develop new treatments to reverse the unwanted effects of opioid receptor agonists without producing adverse effects themselves. One such proposal is the use of peripherally selective opioid receptor antagonists to investigate, and potentially reverse or prevent, unwanted opioid effects. These are opioid receptor antagonists that have limited or no access to the CNS, so only act on peripheral mechanisms. Several opioid effects have been shown to contain a peripherally mediated component, which has led to the hypothesis that some opioid effects may be partially or completely reversed

by the use of opioid receptor antagonists that do not enter the CNS. This is advantageous as the centrally mediated opioid effects would be unaffected by treatment with these peripherally acting opioid receptor antagonists.

Prior to discussing the pharmacological characteristics of opioid receptor antagonists, a brief description of the blood brain barrier and its function is given below.

1.6.1 The blood brain barrier:

The blood brain barrier is defined as “a barrier consisting of specialised brain capillaries and astrocytes that prevents the passage of materials from the blood to the cerebro-spinal fluid (CSF) and brain” [Tortora & Grabowski, 1996]. This definition, however, simplifies the intricate design of this barrier and the importance of its correct functioning and regulation.

The blood brain barrier consists of a number of different components as shown in Figure 4. Endothelial cells are found in many regions of the body, but those present in the blood brain barrier are unique as they have few endocytic vesicles. This results in reduced pinocytotic transport of molecules through these cells. Tight junctions are also present between these endothelial cells, which, along with adhesion junctions, prevent the movement of virtually all molecules past these cells. Passive diffusion can still continue through these cells, which allows hydrophilic and small molecules to enter with relative ease. Specific transporters for essential nutrients, such as glucose and amino acids, are also present and

allow the traffic of these compounds into and out of the brain as required [Bates, 1985; Rubin & Staddon, 1999].

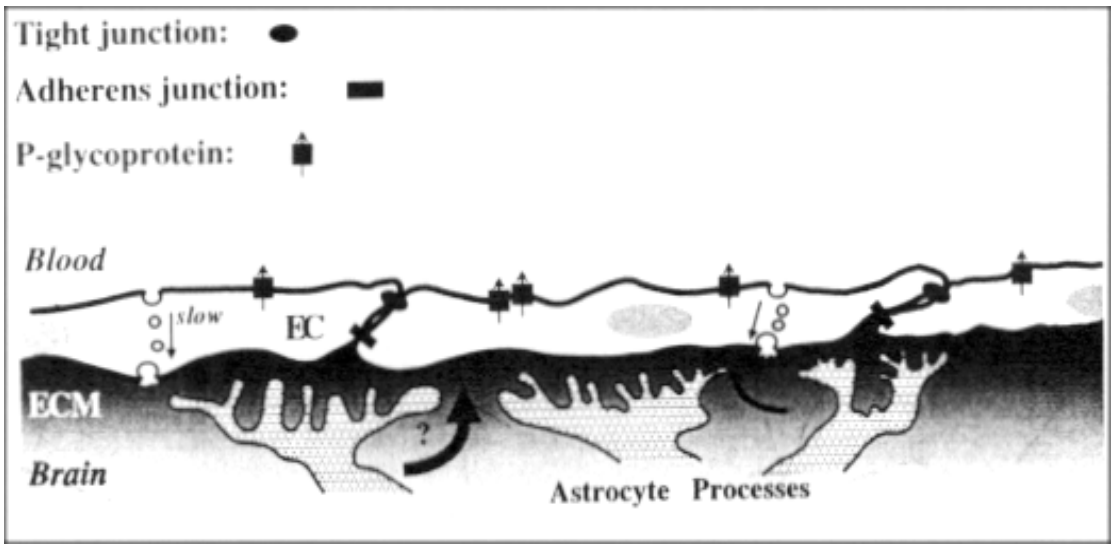

Figure 4: *Essential features of the blood-brain barrier.*

Brain capillary ECs are coupled by adherens and tight junctions, the latter limiting paracellular reflux. P-glycoprotein is expressed in the apical membrane of the endothelial cells (EC) and actively ejects certain undesired substances from the CNS. Transcytosis across brain ECs occurs slowly, minimising transcellular movement into the CNS. Astrocyte processes ensheath the ECs, although an extracellular matrix (ECM) is interposed and may release molecules that influence their phenotype. Not shown are the transporters for essential nutrients, such as glucose and certain amino acids, and for macromolecules such as transferrin. From Rubin & Staddon [1999].

Whilst it is commonly thought that the blood brain barrier prevents chemicals from entering the brain, potentially toxic compounds that gain access to the brain can also be returned to the blood stream. P-glycoprotein (PGP) (the product of the multidrug resistance gene MDR1) is the most common of these efflux transport proteins and is present in large proportions in brain capillaries [Sun *et al.*, 2003]. They function by recognising and extruding compounds including anti-cancer

agents, such as *Vinca* alkaloids, immunosuppressive drugs, such as cyclosporin A, and steroids, such as aldosterone [Schinkel *et al.*, 1996]. Other efflux transporters that have been identified include members of the multidrug resistance associated (MRP) family (MRP1, MRP4, MRP5, MRP6), the organic anion transport proteins (oatps) and the organic anion transporter (OATs). These transporters are thought to have specific substrates that enable the removal of a wide variety of compounds from the brain or CNS. The function of these proteins can have major implications on the absorption, distribution, metabolism and excretion of certain drugs [Sun *et al.*, 2003].

Astrocytes are also present in the structure of the blood brain barrier, but do not appear to play an active role in the barrier function. Their presence, however, is required for normal functioning of this defence barrier and may play an especially vital role in blood brain barrier formation and stability [Bates, 1985; Rubin & Staddon, 1999].

While the blood brain barrier can form an impermeable barrier, it is not static and can be regulated in response to disease states, such as multiple sclerosis, stroke and brain tumours, or environmental conditions, such as increased temperature [Nagy *et al.*, 1979; Sharma & Dey, 1986; Wolburg & Lippoldt, 2002]. This can allow entities as large as cells, such as macrophages and activated lymphocytes, to enter the brain. This can occur through signalling from a variety of factors, such as tyrosine phosphorylation of focal adhesion kinase or intercellular adhesion molecule 1 (ICAM-1), which allow these cells to fuse with those in the blood brain barrier and move either into or out of the CNS [Wolburg & Lippoldt,

2002]. The regulation of these modifications to the blood brain barrier is obviously complex and not fully understood at present, but a number of factors have been proposed. These include second messenger pathways, such as G-proteins and GTPases that produce regulatory proteins to modulate these signals, as well as components present in the extracellular matrix of the blood brain barrier, such as collagens, laminin and proteoglycans. These have been shown to both increase and decrease permeability, indicating that the blood brain barrier can tighten or relax in response to changing conditions [Wolburg & Lippoldt, 2002].

The blood brain barrier can also vary from one area of the brain to another, with some regions lacking the tight junctions that are present in other areas. For example, the cortex, lateral nuclei of the hypothalamus, area postrema and pineal gland are more permeable than other regions of the brain [Bates, 1985; Klaassen *et al.*, 1986]. This may be due to these areas requiring more intimate communication with blood solutes in order to perform their functions effectively. This increased accessibility in certain areas may have major implications on drug effects in the CNS [Klaassen *et al.*, 1986].

1.6.1.1 OPIOID PEPTIDES AND THE BLOOD BRAIN BARRIER:

Most opioid receptor agonists and antagonists can enter the brain and mediate central effects. King *et al.* [2001] investigated the transport of various opioids into and out of the brain. They found that β-endorphin, morphine, DAMGO and DPDPE were all dose dependently secreted into the blood after i.c.v. administration. This mechanism was also saturable, as observed with

endomorphin 1 and 2 transport [Kastin *et al.*, 2001]. Using antisense techniques, these researchers down regulated PGP mRNA production and showed that transport of these opioid receptor agonists was reduced when fewer PGP transporters were present. A significant alteration in the analgesic effects of these compounds also resulted, with antisense or mismatch treated animals requiring higher concentrations of morphine to increase tail flick latencies. Therefore, not only is PGP action important in transporting opioids out of the brain, but it also has important implications on the effects of these opioids.

Opioids also utilise influx transport mechanisms to allow them to enter the brain. Suzuki *et al.* [2002] investigated the transport mechanisms of pentazocine into the rat brain. Upon carotid injection of pentazocine, a concentration dependent and saturable increase in drug uptake was observed, indicating that a transporter mechanism was involved. The presence of other compounds that are known to enter the brain via carrier mediated systems, such as lidocaine, propranolol and mepyramine, also decreased the concentrations of pentazocine in the brain, suggesting that this compound utilises a carrier mediated system that transports other compounds. These researchers also found that other opioid analgesics, namely buprenorphine, butorphanol and eptazocine, inhibited pentazocine influx, which would suggest that this carrier system is able to recognise broad structural characteristics and is not specific to pentazocine alone. A wide range of opioids may be able to enter the brain via this carrier transporter as well as by passive diffusion through the endothelial cells of the blood brain barrier.

Despite the findings indicated in animal studies, human studies have highlighted the complicated nature of blood brain barrier transport mechanisms. One research group has investigated the role of PGP inhibition on the effects of morphine and loperamide [Sadeque *et al.*, 2000; Skarke *et al.*, 2003]. The administration of 600 to 800 mg of quinidine, which inhibits the function of PGP, to healthy humans produced significant increases in the ventilatory effects of loperamide, but did not alter the ventilatory or miotic effects produced by morphine administration. This may be due to the differences in the affinity of morphine and loperamide for the PGPs or may indicate that PGP is not an important modulator of the effects of morphine in the CNS of humans. Research is continuing to clarify these results.

The effect of opioid peptides on the function of the blood brain barrier has also been examined. Sharma *et al.* [1997] investigated the effect of naloxone and naltrexone treatment on heat stressed rats and found that 10 mg/kg i.p. doses of each opioid receptor antagonist attenuated the increased blood brain barrier permeability usually observed with heat treated animals. Brain water content was also elevated in the animals exposed to heat stress, which was reduced by naloxone and naltrexone treatment, while cerebral blood flow, which was reduced in heat stressed animals, returned to normal after opioid receptor antagonist treatment. This study suggests that opioids may be involved in hyperthermic brain injury and that blockade of opioid receptors prior to heat stress is neuroprotective and most likely due to a reduction in stress symptoms.

Opioids may also alter the function of the blood brain barrier through the hypoxic effects they can produce, particularly during opioid induced respiratory depression. Abbruscato & Davis [1999] observed that chronic hypoxia (for 48 hours) increased brain concentrations of [^{14}C]-sucrose due to increase brain permeability. This would suggest that the blood brain barrier loses integrity under hypoxic conditions, which could have major implications in opioid overdose situations.

These studies show that opioids can enter and leave the brain through transporter systems to affect the functioning of the blood brain barrier. Therefore, the blood brain barrier can regulate the effects of opioids and opioids are important in the functioning of the blood brain barrier.

1.6.1.2 PERIPHERAL OPIOID RECEPTOR ANTAGONISTS AND THE BLOOD BRAIN BARRIER:

The sections above illustrate that most opioid peptides have almost unrestricted access to the brain, particularly in regions of the brain not protected by a tightly maintained blood brain barrier. There are, however, compounds that have limited or no access into the brain, which restricts their central actions. These peripherally acting opioid receptor agonists and antagonists were initially developed to separate the peripheral and central effects of opioids, but their potential in therapeutic situations has now been identified, particularly in the reversal of opioid induced constipation [Yuan *et al.*, 2000].

In this project, naloxone methiodide, a peripherally acting opioid receptor antagonist and quaternary derivate of naloxone, was investigated. This

compound is readily available in Australia, unlike the quaternary derivative of naltrexone, methylnaltrexone (naltrexone methiodide), which is currently under patent protection in the USA (United States Patent No. 6,559,158, Use of methylnaltrexone and related compounds to treat chronic opioid use side effects). Due to the lack of research investigating naloxone methiodide, reference will also be made to methylnaltrexone, which is thought to act in a similar manner to naloxone methiodide.

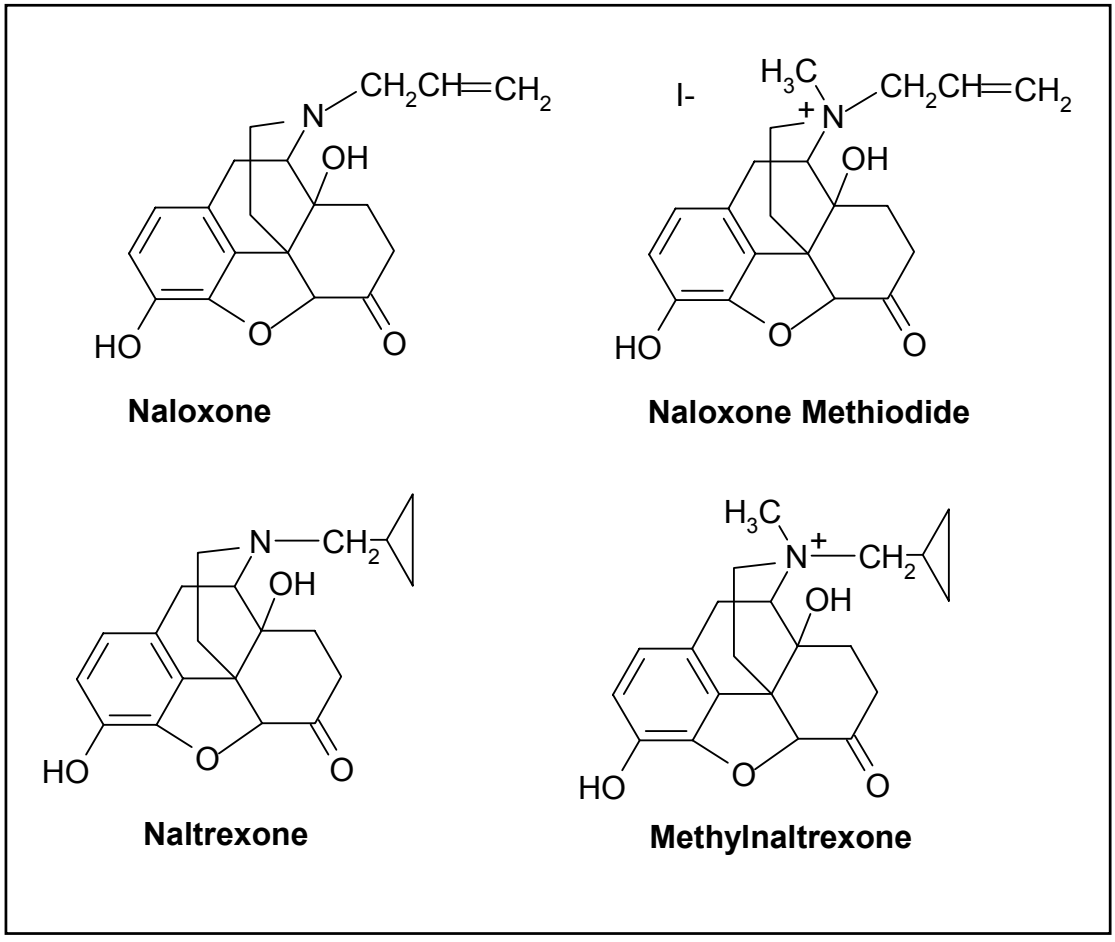

Figure 5: *The chemical structure of naloxone and its quaternary derivative, naloxone methiodide and naltrexone and its quaternary derivative, methylnaltrexone.*

The structures of naloxone and naltrexone and their quaternary derivatives are shown in Figure 5. The addition of an alkyl substituent (in this case a methyl group) onto the tertiary nitrogen atom of the ring structure of naloxone produces a quaternary amine that retains its opioid receptor antagonist activity but, due to its greater polarity and reduced lipid solubility, is thought not to cross the blood brain barrier [Brown & Goldberg, 1985; Zimmerman *et al.*, 1994]. This hypothesis, however, has not been conclusively investigated. To provide a background to this project, the properties of naloxone methiodide will be discussed initially.

1.6.2 Properties of naloxone methiodide:

1.6.2.1 STEREOCHEMISTRY OF NALOXONE METHIODIDE:

Investigation of naloxone methiodide by Iorio & Frigeni [1984] showed that on a ^{1}H- nuclear magnetic resonance (NMR) spectrum, naloxone methiodide has two methyl signals 8 Hz apart at δ 3.37 and 3.45, and has a melting point of 208-210°C. Naloxone methiodide also contains an iodide ion but its role in the activity of naloxone methiodide, if any, has not been determined. Iodine is important in the synthesis of hormones in the thyroid gland, but large doses can inhibit the release of hormones from the thyroid gland, particularly after chronic administration [Bagchi *et al.*, 1985]. The acute i.p. administration of 2000 µg of sodium iodide produced no significant changes in T_3 (triiodothyronine), T_4 (thyroxine) or TSH (thyroid stimulating hormone) concentrations and TSH receptor or Tg (thyroglobulin) mRNA levels over 24 hours. NIS (sodium/iodide symporter) mRNA decreased after 6 hours, TPO (thyroid peroxidase) mRNA decreased after 24 hours and NIS protein expression decreased after 24 hours [Eng *et al.*, 1999]. This shows that if iodide administration produces any effects,

they will occur several hours after naloxone methiodide administration. This delay in the effects of iodine have also been shown in acute and subacute toxicity studies that have also shown that observable effects only occur after much greater concentrations of iodine are administered than those used in this study [Webster *et al.*, 1957; Webster *et al.*, 1966].

Control studies conducted in the current research project using equivalent potassium iodide concentrations did not indicate any toxicity or changes in behaviour, respiration or analgesia (Unpublished results). Therefore, it is unlikely that any of the effects observed in this thesis are due to the action of the iodide present in naloxone methiodide, as these effects require a longer duration of action than that investigated in this study and much higher concentrations of iodide.

1.6.2.2 POTENCY OF NALOXONE METHIODIDE:

The N-methylation of naloxone to form naloxone methiodide produces a compound that maintains its opioid receptor antagonist activity, but has reduced potency. This was first shown in organ bath experiments investigating the effect of opioid receptor antagonists on guinea pig ileum. Valentino *et al.* [1983] found that the EC_{50} dose required to produce a contraction by ileum from morphine dependent guinea pigs was 26 times higher for naloxone methiodide than naloxone. Killian *et al.* [1981] examined the effectiveness of these two opioid receptor antagonists on reversing the effects of morphine on electrically stimulated guinea pig ileum and observed a potency ratio of 27.8:1 for naloxone methiodide compared to naloxone. In separate studies, Bianchetti *et al.* [1982,

1983] found that the K_e for antagonism of the actions of morphine on guinea pig ileum was 8 times and 28 times higher for naloxone methiodide than naloxone. Guinea pig ileum studies by Iorio & Frigeni [1984] reported that the K_e for the response to electrical stimulus was over 1500 times greater for naloxone than naloxone methiodide, and naloxone methiodide had 5 times less antagonist activity than naloxone in the hot plate test for analgesia. These studies indicate that naloxone methiodide has lower potency than naloxone, which may be due to its decreased affinity for opioid receptors.

Radioligand binding experiments in a number of species have also shown that naloxone methiodide has a lower binding affinity for opioid receptors than naloxone. Bianchetti *et al.* [1983] showed that naloxone methiodide was 20 times less potent than naloxone in displacing [^{3}H]-naltrexone from rat brain membranes. Valentino *et al.* [1983] and Manara *et al.* [1982] investigated the displacement of [^{3}H]-etorphine by naloxone and naloxone methiodide in rat brain homogenates and found that naloxone methiodide had approximately 30 times and 13 times lower affinity for binding to opioid receptors, respectively.

Similar studies using selective radioligands have been undertaken in tissues derived from a number of species. Surprisingly, these have not included the mouse. Deviche [1997] compared naloxone and naloxone methiodide binding to μ and δ opioid receptors in brain slices taken from the avian strain, *Junco hyemalis.* The displacement of [^{3}H]-DAMGO and [^{3}H]-DPDPE binding was 11.5 times and 26.9 times lower for naloxone methiodide than naloxone. Magnan *et al.* [1982] used guinea pig brain homogenates to investigate μ, δ and κ receptor binding using

the selective ligands [^{3}H]-DAMGO, [^{3}H]-DPDPE and [^{3}H]-ethylketazocine. They found that naloxone methiodide was approximately 10 times less effective in inhibiting the binding of these radioligands compared to naloxone.

Therefore, naloxone methiodide appears to be less effective in producing a response at opioid receptors than its tertiary equivalent, naloxone. *In vivo* experiments have also indicated that higher concentrations of naloxone methiodide are required to produce effects similar to naloxone. These studies will be discussed in more detail in a following section describing the functional studies that have been conducted using naloxone methiodide (See Section 1.6.3 on Page 76).

1.6.2.3 PHARMACOKINETICS AND PHARMACODYNAMICS OF PERIPHERALLY SELECTIVE OPIOID RECEPTOR ANTAGONISTS:

The pharmacokinetics of naloxone methiodide have not been described, but it is thought to behave in a similar manner to naloxone. Naloxone is rapidly metabolised by glucuronidation, N-dealkylation and 6-oxo group reduction to form conjugated N-dealkylated and 6-OH metabolites that can then conjugate with glucuronic acid [Weinstein *et al.*, 1974]. Naloxone is rapidly excreted, with approximately 30% appearing in the urine within 6 hours of i.v. administration [Fishman *et al.*, 1973]. This results in a half life of approximately 1 hour, clearance of 260 L/hr and a volume of distribution of 370 L [Chiang & Hawks, 2003; Ngai *et al.*, 1976].

Functional data has also led to the suggestion that naloxone methiodide is metabolised to an active metabolite, which could be naloxone [Ramabadran,

1982]. This could have significant implications on the site of action and effects of naloxone methiodide, and will be addressed in this research project (See Publication 3 on Page 133).

While the pharmacokinetics of naloxone methiodide have not been well investigated, studies have been undertaken using methylnaltrexone and, despite the differences between these two compounds, these studies will be discussed below. In a pharmacokinetic and metabolism study by Misra *et al.* [1987], rats were injected with 4 mg/kg i.v. [^{3}H]-methylnaltrexone, and blood and tissue samples taken at several time points for radioactivity counting. The pharmacokinetic parameters calculated were 0.45 hours for $t_{1/2\alpha}$ and 2.94 hours for $t_{1/2\beta}$, an elimination rate constant of 0.86 hours^{-1}, a volume of distribution of 0.82 L/kg and a total body clearance of 5.25 ml/min/kg. This study also reported that small concentrations of methylnaltrexone were present in the brain as shown by the brain to plasma concentration ratio of methylnaltrexone increasing from 0.031 to 0.228 over 6 hours. The decay of methylnaltrexone out of the brain was also slow with a reported $t_{1/2\beta}$ of 7.61 hr, which suggested that once it reaches the brain it diffuses out very slowly.

This slow diffusion was also shown when [^{3}H]-naloxone or [^{3}H]-naloxone methiodide were injected into the nucleus raphe pontis and the radioactivity in brain sections determined for up to 1 hour after administration [Schroeder *et al.*, 1991]. Naloxone was removed from the hindbrain more rapidly that naloxone methiodide, with 20% of the measurable naloxone methiodide still present in the brain after 60 minutes. Naloxone methiodide concentrations in the liver were

also higher than that of naloxone, but there were no differences in blood concentrations between these two opioid receptor antagonists. Therefore, naloxone methiodide appears to diffuse out of the brain more slowly than naloxone, which is most likely due to its lower lipophilicity compared to naloxone.

The study by Misra *et al.* [1987] also showed that methylnaltrexone undergoes N-dealkylation and 6-keto group reduction to form the metabolites naltrexone, 7,8-dihydro-14-hydroxynormorphine and 7,8-dihydro-14-hydroxynormorphinone in the plasma ratios of 6:32:63. Naltrexone was present in the brain in concentrations of 5.3 and 4.3 ng/gram of tissue at 0.25 and 0.5 hours after administration. Therefore, methylnaltrexone appears to enter the brain in small concentrations, which could bind to opioid receptors, and is metabolised to naltrexone that can readily enter the CNS to produce opioid receptor antagonist effects. This would indicate that methylnaltrexone is not a peripherally selective opioid receptor antagonist, but the limitations of this study will be described below.

Kim *et al.* [1989] also conducted studies to determine the concentrations of methylnaltrexone in the blood and several regions of the brain using high performance liquid chromatography (HPLC) with coulometric electrochemical detection. Rats were administered 10 mg/kg or 30 mg/kg i.p. methylnaltrexone and blood and brain samples taken up to 2 hours after injection. After the administration of 10 mg/kg, the concentration of methylnaltrexone in serum peaked at 2.9 µg/ml after 15 minutes and reduced to 0.2 µg/ml after 2 hours. Methylnaltrexone was detected in the hippocampus, hypothalamus, pons-medulla

and midbrain, but the concentrations were 5 to 7% of that found after the administration of 10 mg/kg naltrexone [Kim *et al.*, 1988]. This suggests that methylnaltrexone can enter the brain, but only in very low concentrations which, given its lowered affinity for opioid receptors, may not be high enough to produce functional changes.

Whilst the studies by Kim *et al.* [1988] and Misra *et al.* [1987] do suggest that methylnaltrexone can enter the brain, one major deficiency in their experimental design is that no consideration was given to the blood present in the brain samples analysed. The methylnaltrexone present in the blood in this brain tissue would be detected by HPLC and radioactivity counting, and may have resulted in methylnaltrexone being measured when it was not in the brain tissue itself. Therefore, despite methylnaltrexone being measured in the brain in these experiments, it may still be a peripherally selective opioid receptor antagonist. Evidence is still lacking to confirm whether naloxone methiodide or methylnaltrexone can enter the CNS. One of the experiments conducted in this research project has addressed this issue (See Publication 3 on Page 133).

Kotake *et al.* [1989] investigated the metabolism of methylnaltrexone in mice, rats, dogs and humans to identify any species differences and determine if naltrexone was a major metabolite. 10 mg/kg s.c. N-[^{14}C-methyl]methylnaltrexone was administered to mice, rats and dogs and expired air samples collected for 120 minutes after dosing. The amount of $^{14}CO_2$ collected was used as an indicator of the N-demethylation of the methyl-group of N-

methylnaltrexone. Three dogs were also administered 2 mg i.v. N-[^{14}C-methyl]methylnaltrexone and urine and faeces collected over 120 hours to determine the rate of elimination of radioactivity. The amount of exhaled labelled $^{14}CO_2$ was species dependent with the percentage of dose excreted in 2 hours being 1.17% for rats, 0.48% for mice and 0.14% for dogs. In the dogs examined, 51% of the methylnaltrexone dose was recovered over 120 hours in the urine with a total of 83% of the dose administered being excreted in the urine and faeces.

In the human studies, 2 mg N-[^{14}C-methyl]methylnaltrexone was injected i.v. with breath, blood and urine samples collected to determine the elimination of radioactivity following drug administration [Kotake *et al.*, 1989]. In the five patients investigated, only one had detectable $^{14}CO_2$ concentrations in their expired breath, which was only present for 1 hour after methylnaltrexone administration. This suggests that N-demethylation in humans is extremely slow and not a major route of metabolism. The urine samples indicated that 40 to 60% of the drug administered was present in the urine after 24 hours, confirming that renal excretion plays an important role in the removal of methylnaltrexone. Blood sampling indicated a biphasic disappearance of radioactivity with a rapid distribution phase lasting 6 to 9 minutes followed by a slower elimination phase ranging from 238 to 1320 minutes. The volume of distribution was calculated to be between 120 to 1961 L and plasma clearance of the radioactivity from 100 to 416 ml/min. In three of the patients, renal clearance was estimated to be 80% of the plasma clearance, which was consistent with the large percentage of radioactivity excreted in the urine. From these results it would appear that N-demethylation of methylnaltrexone to naltrexone is not a significant factor in the

functional effects observed with this drug, particularly as this metabolism is not rapid. It cannot, however, be excluded, especially if larger doses of quaternary opioid receptor antagonists are administered.

The most comprehensive evaluation of the profile of quaternary opioid receptor antagonists in humans was conducted by Yuan *et al*. [1996, 1997] who investigated both the oral (19.2 mg/kg) and i.v. (0.45 mg/kg) administration of methylnaltrexone to opioid naïve subjects. Upon oral administration, variable results were found with a C_{max} (maximum concentration) of 165±251 ng/ml, t_{max} of 115.7±101.8 minutes, area under the curve (AUC) calculated to be 418.8±388.4 ng/ml·hr, β-half life equal to 204.1±82.4 minutes and the fraction excreted unchanged over 0 to 3 hours and 3 to 6 hours being 0.3±0.39% and 0.34±0.37%, respectively [Yuan *et al.*, 1997]. After i.v. administration, C_{max} was 3299.4±1027.1 ng/ml, AUC was 676.8±122.6 ng/ml·hr, volume of distribution was 115.1±53.1 L/kg, β-half life was 131.2±48.7 minutes, clearance was 52.5±12.8 L/hr and fraction excreted unchanged over 3 hours was 41.9±11.8% [Yuan *et al.*, 1996]. Further investigation with 0.1 mg/kg and 0.3 mg/kg i.v. doses also found that the C_{max} (110±55 ng/ml for 0.1 mg/kg and 287±101 ng/ml for 0.3 mg/kg) were reached by 16.7 and 20 minutes after administration, while the fractions excreted unchanged over 0 to 6 hours were 51.7% and 47.3% [Osinski *et al.*, 2002]. These studies indicated that there is high individual variability in the pharmacokinetics of methylnaltrexone, but this compound can be administered both i.v. or orally.

This same research group has also investigated the pharmacokinetic profile of methylnaltrexone in anaesthetised rabbits [Murphy *et al.*, 2001]. 0.66 mg/kg

methylnaltrexone was administered via an epidural catheter, with blood and CSF samples collected for 180 minutes. Small concentrations of methylnaltrexone were identified in the CSF (up to 50 ng/ml, 15 minutes after administration) while higher concentrations were detected in the serum samples. This study, therefore, suggests that large concentrations of methylnaltrexone do not cross the dural membrane after epidural administration, so high proportions of this quaternary opioid receptor antagonists would not be present in the CNS.

These experiments have also highlighted that the naloxone methiodide administered must be extremely pure and contain no naloxone contamination. If naloxone is present, it may produce opioid receptor antagonist effects and lead to incorrect conclusions regarding the effects of naloxone methiodide. This is particularly important if high concentrations of naloxone methiodide are administered as it may be the naloxone impurities, not naloxone methiodide, that are responsible for the reported effects [Bianchi *et al.*, 1982; Milne *et al.*, 1990]. Milne *et al.* [1990] conducted NMR experiments to determine the spectra for both naloxone and naloxone methiodide. They found that there was less than 0.5% naloxone contamination in the naloxone methiodide used in their experiments.

In this research project, analysis of the naloxone methiodide (purchased from Sigma-Aldrich, Australia) using HPLC with electrochemical detection found that there is less than 0.005% naloxone contamination (Unpublished data). Therefore, it is unlikely that any naloxone contamination present in the naloxone methiodide administered in these experiments would be responsible for the opioid receptor antagonist effects reported. Functional studies that indicate naloxone

methiodide can reverse peripherally, but not centrally, mediated opioid effects may also suggest that contaminating naloxone is not present in the naloxone methiodide administered at pharmacaologically effective concentrations. These will be discussed in further detail in the next section.

1.6.3 Functional studies investigating peripherally selective opioid receptor antagonists:

Despite the limited information available regarding the properties of naloxone methiodide, it has been used to determine if peripheral mechanisms are involved in many opioid effects. This current research project is particularly concerned with opioid induced respiratory depression, analgesia and withdrawal, but an understanding of the research that has been conducted regarding other opioid effects, and its limitations, provides important background information for this study.

The quaternary derivative of naloxone has been given a variety of different names, such as methylnaloxone and methylnaloxonium, but for continuity naloxone methiodide will be used throughout. Other compounds such as naloxone methobromide have also been investigated but, as the substitution of an iodide group with a bromide group does not greatly affect the actions of these antagonists, they have been referred to as naloxone methiodide in this thesis [Debarbieux *et al.*, 1998; Romanovsky *et al.*, 1994].

1.6.3.1 CONSIDERATIONS WHEN EVALUATING FUNCTIONAL STUDIES:

It is important to note that several limitations exist in the current literature that has

used naloxone methiodide to identify peripheral mechanisms or determine its effectiveness in antagonising opioid effects. These are discussed below and will be re-iterated where relevant in the functional studies described. Some of the deficiencies in previous literature have been addressed in this research project, as shown in the research aims (See Section 1.7 on Page 114).

- The difference in potency between naloxone and naloxone methiodide has often been overlooked when naloxone methiodide is administered peripherally (For more details refer to Section 1.6.2.1 on Page 66). These differences have not been well defined in rodent models and may also depend on the opioid action being investigated, but it is well accepted that higher doses of naloxone methiodide are required to produce the same effects as naloxone. Inadequate naloxone methiodide dosing has often resulted in the presentation of inconclusive data, as it is not known if the lack of effect observed is due to the low dose of opioid receptor antagonist administered or if naloxone methiodide does not antagonise the effect being examined. This is particularly pertinent, as naloxone methiodide has frequently been used as a tool to distinguish between centrally and peripherally mediated opioid effects, and may have led to incorrect conclusions regarding the site of action of several opioid effects. This inadequate dosing may have also hampered the potential use of quaternary opioid receptor antagonists to treat peripherally mediated adverse effects due to their apparent lack of efficacy.

- It is difficult to reach conclusions from previous studies when it is not known if naloxone methiodide is a peripherally selective opioid receptor antagonist. Whilst naloxone methiodide is thought not to readily enter the brain, it has been detected in brain samples, suggesting it can gain entry into the CNS (See Section 1.6.2.3 on Page 69). Despite this finding, naloxone methiodide has continued to be used to elucidate peripherally and centrally mediated opioid effects. Definite conclusions from this previous research, however, cannot be made without confirmation that naloxone methiodide does not act at central opioid binding sites.

- Many previous researchers have used low doses of opioid receptor agonists to simulate endogenous opioid concentrations or those used therapeutically when determining the effect of naloxone methiodide on opioid induced effects. Whilst these experiments still provide information, the results obtained are more relevant to the understanding of the physiology of respiration and the role of endogenous opioids rather than the mechanisms relating to opioid overdose. Larger opioid receptor agonist doses may induce other mechanisms of action that would not be activated or observed when lower doses are administered. As this project is primarily concerned with the reversal of opioid effects in overdose situations, it is vital that the effects of naloxone methiodide are investigated after the administration of doses of opioid receptor agonists that induce significant respiratory alterations.

- The effect of naloxone methiodide treatment after the administration of a variety of opioid receptor agonists is also not well characterised. Whilst morphine and opioid receptor agonists specific to opioid receptor types are most commonly administered in these experiments, the effect of other less specific opioid receptor agonists should also be examined. Of particular relevance in this study are opioid receptor agonists that are frequently used by humans and have the potential to produce respiratory depression. These include methadone and heroin.

- There is a deficiency in the literature regarding the effects of peripherally acting opioid receptor antagonists after chronic opioid treatment. As indicated in previous sections (See Sections 1.4.1.2 and 1.4.2.2 on Pages 14 and 26), it is unclear how tolerance develops to the respiratory effects of opioids, and if it is ever complete. It is, therefore, of importance to investigate if the effects of naloxone methiodide treatment differ after the chronic administration of opioids compared to acute opioid treatment.

While the following sections illustrate that naloxone methiodide has often been used in opioid related research, the considerations listed above are vital in the evaluation of this past research. These factors compromise much of the data that have been produced and highlight the need for additional well designed investigations. These issues have been addressed in this research project and will be further discussed in Section 1.7 on Page 114.

1.6.3.1.1 Food & water intake:

Naloxone suppresses drinking in rats deprived of water or administered hypertonic saline, isoproterenol (a β-adrenergic receptor agonist) or angiotensin II (a vasoconstrictor) [Brown & Holtzman, 1981]. This is thought to occur through disruption of the endogenous opioid system that modulates water consumption, but it is not known if this is a centrally or peripherally mediated action. Brown & Holtzman [1981] attempted to determine the location of this activity in rats by s.c. or i.c.v. injection of naloxone, naltrexone or their quaternary derivatives. The tertiary opioid receptor antagonists produced a decrease in water intake at doses ranging from 1 to 10 mg/kg s.c. while the quaternary derivatives had no effect. Naloxone methiodide and methylnaltrexone, however, were effective after i.c.v. administration, indicating that they can alter this action. Cooper & Turkish [1983] also tested if taste conditions affected these results, but s.c. naloxone methiodide still produced no changes in water, saccharin or saline intake. Katovitch *et al.* [1990] investigated the combined effects of naloxone methiodide (1.28 mg/kg s.c.), isoproterenol and angiotensin II, but again observed no significant changes with this antagonist treatment.

Whilst it would appear from these studies that central mechanisms control water intake, the doses of naloxone methiodide and methylnaltrexone administered (up to 10 mg/kg s.c.) did not compensate for the differences in potency between the quaternary opioid receptor antagonists and their tertiary counterparts (See Section 1.6.2.1 on Page 66). This is highlighted by a study investigating food intake in food deprived pigeons [Deviche *et al.*, 1984]. Fluid consumption was decreased in both pigeons and rats and food intake in pigeons was also reduced

after naloxone treatment. Equimolar concentrations of naloxone methiodide did not produce any changes in food consumption, but concentrations twice that of naloxone significantly decreased food intake. Therefore, if higher doses of naloxone methiodide were used in the water intake studies, an effect similar to that of naloxone may have been observed.

1.6.3.1.2 Drug self-administration:

The involvement of peripheral opioid mechanisms in drug self-administration has been investigated using naloxone methiodide. Franck *et al.* [1998] reported that naloxone methiodide (3 mg/kg i.p.) had no effect on ethanol intake, while the same doses of naltrexone and naltrindole (a δ opioid receptor antagonist) significantly decreased the amount of ethanol consumed. Experiments investigating heroin self-administration have shown that naloxone methiodide can effectively increase heroin self-administration when injected i.c.v. or directly into the nucleus accumbens or ventral tegmental area [Vaccarino *et al.*, 1985a,b]. When injected i.p., naloxone methiodide was only slightly effective at the highest dose tested (30 mg/kg i.p.) while approximately 10 times lower doses of naloxone and naltrexone produced significant increases in heroin administration [Koob *et al.*, 1984]. Therefore, it would appear that peripheral mechanisms are involved in drug self-administration, but this should be confirmed using higher doses of naloxone methiodide.

1.6.3.1.3 Gastrointestinal effects:

The peripheral mechanisms involved in gastrointestinal motility have received considerable attention. Early studies showed that naloxone methiodide could

reverse opioid induced constipation in mice, rats and dogs but, as expected, required larger doses than naloxone [Bianchetti *et al.*, 1982; Bianchetti *et al.*, 1983; Bianchi *et al.*, 1982; Bianchi *et al.*, 1983; Manara *et al.*, 1982; Russell *et al.*, 1982].

Naloxone methiodide has been utilised by Pol *et al.* [1995] to demonstrate that both the same dose of naloxone and naloxone methiodide increased gastrointestinal transit times in mice with croton oil induced diarrhoea, but naloxone was three times as effective as naloxone methiodide. The authors suggested that this was due to inhibition of the actions of endogenous opioid peptides in the croton oil treated animals. These studies were then extended to show that morphine, n-methylmorphine (the quaternary derivative of morphine), fentanyl, PL017 (a peripheral μ opioid receptor agonist), U-50488H (a κ opioid receptor agonist) and ICI-204448 (a peripheral κ opioid receptor agonist) could increase gastrointestinal transit times in normal and croton oil treated mice [Pol *et al.*, 1996a,b]. Naloxone methiodide was effective in antagonising the effects of these opioid receptor agonists in the croton oil treated mice, but was only partially effective in the control animals without diarrhoea, indicating that gastrointestinal transit involves both peripheral and central mechanisms.

These researchers have also shown that the administration of a variety of opioid receptor agonists can reduce the inflammation and diarrhoea induced by acute and chronic croton oil treatment, and that both naloxone and naloxone methiodide can prevent these opioid receptor agonist effects [Valle *et al.*, 2000; Valle *et al.*, 2001]. Similar results were reported after the administration of

fedotozine, a peripherally acting opioid receptor agonist, to rats with surgically induced digestive ileus [Riviere *et al.*, 1993]. Naloxone methiodide can also significantly increase gastric acid secretion in 14 day old rats, but not in 20 day old rats, indicating that the role of opioids in gastrointestinal functioning changes during development [Rao *et al.*, 1995].

Schulz *et al.* [1979] showed that morphine decreased intestinal motility in rats administered charcoal meals, and that this morphine effect could be reversed by naloxone or naloxone methiodide treatment. Higher doses of naloxone methiodide compared to naloxone were required to produce antagonism of i.v. or i.c.v. morphine effects and only ever reached 50% inhibition of peristalsis, while naloxone produced complete antagonism. Naloxone methiodide also completely antagonised the constipatory effects of loperamide, a peripherally acting opioid receptor agonist, again confirming a peripheral component in the gastrointestinal effects of opioids.

Broccardo *et al.* [1998] investigated the effect of a peripherally active δ opioid receptor agonist, SNC80, and found that while pre-treatment with 5 mg/kg i.p. naloxone prevented the inhibition of gastrointestinal transit produced by this drug, 5 mg/kg i.p. naloxone methiodide did not. While the authors concluded that SNC80 is largely acting centrally, this may be incorrect as the dose of naloxone methiodide may not have been large enough to produce observable effects, particularly at δ opioid receptor sites.

Naloxone methiodide has been used to investigate the gastrointestinal protection produced by opioid receptor agonist administration. Scoto & Parenti [1996]

reported that i.c.v. or i.p. administered thiorpan (an inhibitor of 'enkephalinase' endopeptidase, which increases enkephalin concentrations) and DALA (([D-Ala^2-Met^5]enkephalinamide) a synthetic enkephalin analogue) could protect against lesions induced by cold-restraint stress in rats. These actions could be reversed by i.p. injections of naloxone, and naloxone methiodide. Yates *et al.* [2001] also observed that naloxone and naloxone methiodide could maintain normal secretory and permeability parameters in intermittently stressed animals, therefore maintaining normal mucosal physiology, but did not have a protective effect in continuously stressed animals. The use of naloxone methiodide in these experiments has identified that peripheral opioid receptors may contribute to the gastrointestinal protection observed after opioid treatment.

In a study by Sengupta *et al*. [1999], the visceromotor response to colorectal distension was measured after the i.v. administration of morphine and EMD 61753 (a peripherally selective opioid receptor agonist) with or without the pre-treatment with 0.03, 0.3 or 2 mg/kg i.v. naloxone methiodide. Both morphine and EMD 61753 attenuated the visceromotor response to the noxious stimulus and naloxone methiodide antagonised these effects, but lower doses were required to antagonise the morphine effects than the EMD 61753 effects. This shows that naloxone methiodide is an effective antagonist against noxious visceral stimulus in the inflamed rat colon.

Clinical trials have recently been conducted, and are continuing, to investigate the use of methylnaltrexone and a recently developed peripherally acting opioid receptor antagonist, alvimopan (ALV 8-2698), to treat post-operative ileus and

reduce constipation in opioid maintenance patients [Foss *et al.*, 1997; Schmidt, 2001; Yuan *et al.*, 1996; Yuan *et al.*, 1997; Yuan *et al.*, 2000]. The i.v. or orally administered methylnaltrexone was shown to effectively reverse morphine induced increases in oral-cecal transit time in normal subjects [Yuan *et al.*, 1996; Yuan *et al.*, 1997]. In chronic methadone users, i.v. methylnaltrexone significantly decreased oral-cecal transit time compared with no changes after placebo treatments [Yuan *et al.*, 2000]. Alvimopan has also been shown to decrease colonic transit time and oral-cecal transit time in healthy subjects administered loperamide or morphine. Opioid bowel dysfunctions in opioid dependent patients have also been reversed by alvimopan. In opioid naïve patients who underwent surgery and received opioids for acute pain, alvimopan treatment shortened the time to achieve normal bowel function and reduced the duration of hospital stays without compromising analgesia [Schmidt, 2001]. These studies have highlighted the effectiveness of peripherally acting opioid receptor antagonists on the reversal of adverse gastrointestinal effects, suggesting that they may also be clinically effective for a variety of other peripherally mediated opioid effects.

1.6.3.1.4 Thermoregulation:

Opioid systems are known to play an integral role in thermoregulation, and exogenous opioid administration can produce hyperthermia, hypothermia or biphasic temperature responses. These are dependent on the species used, dose of opioid receptor agonist administered and experimental conditions employed [Adler *et al.*, 1988]. Peripherally selective opioid receptor antagonists have been used to determine if these changes contain a peripheral component. Naloxone and its quaternary derivative increased tail skin temperature and

decreased rectal temperatures after i.c.v. injection into female morphine dependent rats [Katovich *et al.*, 1986]. 1 mg/kg s.c. naloxone produced these same effects, while doses up to 9 mg/kg s.c. naloxone methiodide produced only slight changes in temperature.

Handler *et al.* [1995] investigated the interaction between neurotensin and opioid receptor agonists on body temperature and observed that 100 mg/kg s.c. naloxone methiodide had no effect on neurotensin induced hyperthermia, but did block the actions of U50 (a κ selective opioid receptor agonist) alone or in combination with neurotensin. The conflicting results produced by these two studies highlight the difficulties present in evaluating the peripheral effects of opioid actions through studies utilising naloxone methiodide. Only further research will clarify if there is a peripheral component to the thermoregulatory changes induced by opioids.

1.6.3.1.5 Withdrawal:

The investigation of the mechanisms of withdrawal and their site of action has produced varied results. Early studies by Ramabadran [1982] and co-workers [Ramabadran *et al.*, 1982] reported that in acutely opioid dependent mice (administered 100 mg/kg s.c. morphine 4 hours before antagonist treatment) naloxone administration at doses higher than 3 mg/kg s.c. produced significant withdrawal signs, while doses of naloxone methiodide up to 30 mg/kg s.c did not produce any observable effects. In their thermoregulation studies, Katovich *et al.* [1986] also noted that while 1 mg/kg s.c. naloxone produced withdrawal signs, such as writhing and teeth chatter, the administration of up to 9 mg/kg s.c. naloxone methiodide did not produce any of these symptoms.

A similar result was also found when 5 mg/kg s.c. naloxone methiodide administration only produced diarrhoea, while the same dose of naloxone induced wet dog shakes, teeth chattering, ptosis, rearing, digging and escape behaviours in morphine dependent rats [Hamlin *et al.*, 2001]. This study also showed that while naloxone methiodide did not precipitate withdrawal, it did increase Fos-immunoreactive neurons in the brain, which occurs during opioid withdrawal.

Milanes *et al.* [2001] administered 1 mg/kg s.c. naloxone and 5 mg/kg s.c. naloxone methiodide to opioid dependent rats and while naloxone produced significant withdrawal, only teeth chattering was noted in 5 out of 8 of the naloxone methiodide treated animals and jumping was reported in 1 animal. They also observed that both naloxone and naloxone methiodide treated rats displayed enhanced cardiac noradrenaline and dopamine turnover, an indicator of withdrawal in cardiac tissue, which suggests this effect is mediated by mechanisms outside the CNS.

Rohde *et al.* [1997] observed that treatment of opioid tolerant rats with 10 mg/kg s.c. naloxone methiodide produced diarrhoea in 50% of animals, but no other withdrawal signs were observed. The administration of intrathecal (i.t.) naloxone methiodide (10 µg) produced body movements only in the hind part of the body, indicating antagonism only at the spinal level. This was in contrast to 1 mg/kg s.c. and 10 µg i.t. naloxone administration, which produced significant withdrawal.

Other studies have shown that only slight withdrawal is produced when high doses of naloxone methiodide (500 ng) are injected i.c.v. into morphine dependent rats, while no withdrawal symptoms are noted in rats administered 10 mg/kg s.c. naloxone methiodide, or when this opioid receptor antagonist is administered to opioid naïve mice [Hand *et al.*, 1988]. Akaoka & Aston-Jones [1991] also observed that in morphine dependent animals, neurons in the locus coeruleus, the brain region thought to be responsible for withdrawal symptoms, were not activated with either naloxone or naloxone methiodide administration. The authors comment that this may have been due to the neurons being completely tolerant to the circulating morphine and hence unable to respond to the opioid receptor antagonists administered.

In an extension of rodent models, a study using morphine dependent rhesus monkeys reported the precipitation of withdrawal in naloxone methiodide treated animals, but 100 times higher doses of naloxone methiodide than naloxone were required [Valentino *et al.*, 1983]. This would indicate that at high doses, naloxone methiodide could enter the CNS to precipitate withdrawal effects. The severity of this withdrawal was not reported, nor is it known if the withdrawal symptoms produced by these high doses of naloxone methiodide were comparable to those produced by naloxone. This study does, however, suggest that naloxone methiodide or its metabolites can gain access to the brain to produce centrally mediated withdrawal in primates.

These studies, therefore, indicate that the administration of adequate doses of naloxone methiodide does not produce significant withdrawal, and if any

withdrawal signs are observed, they appear to be of peripheral origin. They suggest that peripheral mechanisms do not play a predominant role in opioid withdrawal in rodents or monkeys, as very high doses of naloxone methiodide were required to produce any significant changes in behaviour. They also highlight that the mechanisms involved in producing withdrawal in opioid naïve and dependent rodents are complex and require further investigation to fully elucidate.

1.6.3.1.6 Locomotor activity and behavioural alterations:

Several studies have been conducted exploring the effect of naloxone methiodide on a variety of behavioural changes produced by opioids and other drugs. Brown *et al.* [1983] observed that morphine induced catalepsy could be reversed by peripherally administered naloxone and higher doses of naloxone methiodide. The injection of naloxone methiodide i.c.v. or into various brain regions could also reverse heroin induced hyperactivity, alfentanil induced catatonia, rigidity and increases in electromyographic activity [Amalric & Koob, 1985; Amalric *et al.*, 1986; Blasco *et al.*, 1986]. The central administration of naloxone methiodide, however, does not provide any information regarding the central or peripheral mechanisms involved in these effects. The reasoning behind the use of peripherally acting opioid receptor antagonists in these experiments, therefore, is unclear.

Naloxone methiodide administered i.c.v. attenuated gross changes in movement produced by amphetamine, while 5 mg/kg s.c. naloxone methiodide produced no changes [Jones & Holtzman, 1992]. This result can either be

interpreted to confirm a central role for opioid receptors in amphetamine induced changes in locomotor activity, or may be a result of the administration of sub-effective dose of naloxone methiodide. The suggestion that the dose of naloxone methiodide was inadequate is most likely as 5 mg/kg s.c. naloxone was required to produce significant changes in a similar study [Hooks *et al.*, 1992].

Increases in immobility time produced by interferon-α administration in the mouse forced swimming test was also reduced by 1 mg/kg s.c. naloxone, but not the same dose of naloxone methiodide, while 1 nmol of naloxone methiodide injected intracisternally was effective [Makino *et al.*, 2000]. Conclusions regarding the peripheral or central site of these opioid actions cannot, however, be drawn without larger peripherally administered doses of naloxone methiodide being tested.

Injections of naloxone have been shown to produce aversions to sensory stimuli in opioid dependent and non-dependent rats and i.c.v. administered naloxone methiodide also produced dose dependent place aversion behaviour in these animals [Hand *et al.*, 1988]. Withdrawal symptoms, such as wet dog shakes and diarrhoea, were only observed in opioid dependent rats after 500 ng i.c.v. naloxone methiodide administration. 10 mg/kg s.c. naloxone methiodide, however, produced no aversion behaviour in either dependent or naïve rats, while 4 µg/kg naloxone was effective. This would suggest that aversive behaviour is mediated by central mechanisms. Cross-sensitivity to naltrexone is observed with naloxone, but not naloxone methiodide, indicating that this enhanced sensitivity is mediated via the CNS [Schindler *et al.*, 1993].

Peripherally administered naloxone methiodide has, however, been shown to reverse the effects of [leu]-enkephalin in place preference tasks and active avoidance tasks [Martinez *et al.*, 1988]. Naloxone methiodide has also been shown to affect attention and signal processing during the early stages of learning in rabbits, while naloxone is more effective in later effects [Hernandez *et al.*, 1997]. The authors, therefore, suggest that learning and memory contain a peripherally mediated opioid component, particularly during the early stages of development.

1.6.3.1.7 Immune and hormonal responses:

The influence of endogenous and exogenous opioids on the immune system has recently been of interest, and naloxone methiodide has been utilised to determine if these effects contain a peripheral component. Opioids are also implicated in changes in hormonal production and protein expression, but the site of action of these effects is largely unknown.

Naloxone methiodide was used by De Simoni *et al.* [1993] to determine if the effects of interleukin-1 (IL-1) on interleukin-6 (IL-6) induction were mediated in the periphery. Naloxone treatment (10 mg/kg i.p.) prior to IL-1 administration attenuated the expected increase in IL-6 concentrations, but the same dose of naloxone methiodide did not alter IL-6 release. Opioids, therefore, can play a role in this response and while it appears that this IL-1 activity takes place in the CNS, the administration of larger doses of naloxone methiodide is required to confirm this site of action.

Morphine has been shown to suppress the activity of natural killer cells (NK cells). To determine if this mechanism is centrally or peripherally mediated, chronically morphine treated mice were exposed to continuous infusions of naloxone or naloxone methiodide, using osmotic mini-pumps [Freier & Fuchs, 1994]. NK cell activity was then determined after 16 hours. Morphine abolished almost all NK cell activity and 1 and 5 mg/kg naloxone treatment completely reversed this effect, while naloxone methiodide was only slightly effective at these doses. Peripherally located opioid receptors do not appear play a predominant role in morphine induced suppression of NK cells, but this should be confirmed with higher doses of naloxone methiodide.

Pain associated with arthritis can be reduced using opioids and Binder & Walker [1998] used naloxone methiodide to determine if the analgesia produced by the κ opioid receptor agonist, asimadoline, contained a peripheral component. They administered naloxone methiodide for 21 days at doses of 6 mg/kg/day or 48 mg/kg/day to rats with induced adjuvant arthritis and found that this treatment reversed the anti-arthritic effects of asimadoline. Therefore, it appears that asimadoline acts via peripherally located opioid receptors, and may be a viable treatment for this type of long-term pain without the adverse effects associated with the current μ opioid receptor agonist treatments.

Gestreau *et al.* [2000] investigated the effects of equimolar concentrations of naloxone (2 mg/kg s.c.) and naloxone methiodide (2.58 mg/kg s.c.) on c-Fos protein expression in various regions of the rat brain. c-Fos itself is not a direct

marker of hormonal or immune responses, but is a marker of neuronal activity, which may occur with these types of responses. Naloxone increased c-Fos expression in the nucleus tractus solitarius, area postrema, rostral ventrolateral medulla, Kölliker-Fuse nucleus, supramammillary nucleus and the amygdala, whilst naloxone methiodide did not produce differences when compared to control tissue. These results can either be interpreted to suggest that peripheral mechanisms are not involved in regulating c-Fos expression in brain regions, or suggest that naloxone methiodide at this dose cannot enter the brain.

Naloxone can increase luteinizing hormone (LH) secretion and significantly potentiate NMDA induced LH release in male and female rats. Naloxone methiodide has been used in several studies to determine if this effect is peripherally mediated. Early studies indicated that 5 mg/kg s.c. naloxone and naloxone methiodide increased LH concentrations in intact and gonadectomised rats, and a similar result was also observed with nalmefene methiodide in intact rats [Panerai *et al.*, 1983; Simpkins *et al.*, 1991]. An extension of these studies found that in female hypogonadal mice, 3 mg/kg i.v. naloxone methiodide exhibited the same effects as 3 mg/kg i.v. naloxone administration. On their own, these two opioid receptor antagonists produced no significant alteration on LH secretion, but produced significant increases in LH secretion with the administration of NMDA. Therefore, this hormonal response to NMDA involves opioid modulation in peripheral tissues, which these authors suggest may be in an area such as the median eminence or the pituitary gland [Miller *et al.*, 1995].

1.6.3.1.8 Antinociception:

Naloxone methiodide has been frequently used to research the peripheral mechanisms involved in opioid induced antinociception. A variety of results have been obtained from these studies, which are dependent on several factors including the doses of opioid receptor agonists and antagonists administered and the pain tests used.

Early studies using the hot plate test by Bianchi *et al.* [1982] reported that doses of naloxone methiodide higher than 16 mg/kg s.c. could lower morphine induced antinociception, but Ramabadran *et al.* [1982] found that s.c. administration of up to 30 mg/kg had no effect. Zimmerman *et al.* [1994] reported that the ED_{50} for precipitation of diarrhoea in morphine dependent mice was 0.05 mg/kg for naloxone and 0.31 mg/kg for naloxone methiodide, while the AD_{50} for antagonism of morphine analgesia was 0.08 mg/kg for naloxone and 1.5 mg/kg for naloxone methiodide.

Inflammatory pain induced by intraplantar injection of carrageenan was blocked by intraplantar or s.c. administration of naloxone methiodide when injected 30 minutes, but not 4 hours, after carrageenan treatment [Rios & Jacob, 1982, 1983]. This hyperalgesic action may be a result of antagonism of the mediators of inflammation, such as prostaglandins. These researchers also showed that naloxone methiodide did not reverse morphine induced analgesia with doses up to 30 mg/kg s.c. or 30 µg injected intraplantarly. A similar study by Perrot *et al.* [2001] showed that naloxone methiodide administration resulted in lower pain thresholds after morphine treatment but only in the animals previously injected

with carrageenan. This suggests that endogenous opioids produce a peripheral analgesic effect with continuous pain, which can be blocked with naloxone methiodide administration.

Abbot [1988] reported that naloxone methiodide (10 mg/kg s.c.) could partially antagonise the analgesic effect produced by high doses of ethylketocyclazocine (a potent μ and κ opioid receptor agonist) after inducing pain by injecting formalin into the hind paw of rats. Naloxone methiodide alone was not effective in reducing latencies in the tail immersion test, nor after the administration of morphine in either the formalin or tail immersion test. From these results, the authors suggested that peripheral opioid mechanisms operate to produce analgesia with high doses of ethylketocyclazocine, but this does not occur with morphine or in the tail immersion test. It was concluded from this lack of effect in the tail immersion test, that naloxone methiodide is not demethylated to naloxone, so naloxone is not responsible for the analgesia observed. Higher doses of naloxone methiodide, however, should be administered to confirm this conclusion.

Further studies using the formalin test have shown that naloxone methiodide can attenuate the effects produced by DAMGO, U-50, and DPDPE as well as the reversal of the attenuation of formalin induced flinching and increased paw blood flow produced by the opioid receptor agonist remifentanil [Hong & Abbott, 1995; Taylor *et al.*, 1997; Taylor *et al.*, 2000]. This pain test has also been used to show that analgesia can be produced by the injection of morphine into the rostral agranular insular cortex, an area of the brain involved in producing morphine

induced antinociception, and this effect can be reversed with the administration of naloxone methiodide into this brain region [Burkey *et al.*, 1996].

Capsaicin has been frequently used to study pain mechanisms, and Barrett *et al.* [2003] used this compound to investigate the sex differences in hyperalgesia and μ opioid induced anti-hyperalgesia in Fischer 344 rats. Capsaicin was injected into the tail of male and female rats and their responses to the tail flick test determined. Capsaicin reduced the tail flick latency times in all animals, with females experiencing more pain, and hence lower latency times, than males. Morphine reduced the pain produced by the capsaicin injections when injected systemically, locally into the tail or centrally, with the locally injected morphine being 10 times more potent than the morphine administered systemically. Male and female capsaicin treated rats were then locally administered 1 mg of morphine and treated with 10 μg of naloxone methiodide i.c.v.. While morphine increased the tail flick latencies in both sexes, but to a slightly greater effect in females, naloxone methiodide produced no alterations. The authors concluded that as centrally administered naloxone methiodide could not attenuate the morphine induced effects, these effects are peripherally mediated, but these findings could also result from other factors, such as the administration of an inadequate dose of naloxone methiodide.

The peripheral and central components of stress-induced analgesia after 90 seconds of continuous foot shock were examined by Chance & Nelson [1986]. Both 5 mg/kg i.p. naloxone and naloxone methiodide reduced the antinociceptive responses produced. Morphine induced analgesia was also tested, and the

morphine induced effects were antagonised by 5 mg/kg i.p. naloxone. Systemic administration of 5 mg/kg naloxone methiodide did not reduce the increased tail-flick latencies, but did attenuate the morphine induced antinociception when injected i.c.v. (50 µg). The authors suggest that endogenous endorphins are released into the periphery upon continuous stress, which can be antagonised by naloxone and naloxone methiodide, while morphine induced antinociception involves other central opioid mechanisms that are not affected by the peripherally acting naloxone methiodide.

The effect of opioid receptor agonist and naloxone methiodide treatment has also been investigated in rats with peripheral mononeuropathy induced by constriction of the sciatic nerve in the hind paw [Kayser *et al.*, 1995]. Naloxone methiodide was effective in reversing morphine induced antinociception when injected into the nerve affected paw, suggesting that peripheral sites of action are involved in this type of pain. Studies in these rats were extended to show that the analgesic effects of morphine were enhanced by treatment with (+)-HA966 (a glycine/NMDA receptor antagonist) but naloxone methiodide could still reverse this analgesia. This indicates that NMDA mediated actions are involved in this analgesia, and contain a peripherally mediated component [Martinez *et al.*, 2002].

The study conducted by Milne *et al.* [1990] has become the most frequently cited publication to confirm that naloxone methiodide is a peripherally selective opioid receptor antagonist. In this study, the effect of morphine administration and subsequent naloxone or naloxone methiodide administration was examined in rats using the tail flick assay. In normal rats, 6 mg/kg i.p. morphine produced the

expected increase in tail flick latency, which was antagonised by naloxone (2 mg/kg i.p.) but not naloxone methiodide (5 mg/kg i.p.). In a group of spinalised rats, however, morphine still produced a significant increase in tail flick latency, but both naloxone methiodide and naloxone could antagonise this morphine induced antinociception. The results from this article, therefore, do not confirm that naloxone methiodide is a peripherally selective opioid receptor antagonist and other workers evaluating these results have drawn incorrect conclusions from this research.

As mentioned in other studies, it is not known if the lack of effect of naloxone methiodide in normal animals is due to limited access of this opioid receptor antagonist into the brain, or results from the administration of an inadequate dose. The experimental design used by Milne *et al.* [1990] did not address this point. In addition, as stated by the authors, there is no evidence to explain why naloxone methiodide would be more effective in spinalised rats. The authors did suggest that this may be due to the halothane anaesthetic administered during surgery, but this is unlikely as the surgery was conducted at least one day prior to naloxone methiodide administration. Another suggestion was that vasogenic oedema induced by CNS injury could break down the blood brain barrier, but again there is little evidence to indicate this is a major factor. The authors' final suggestion was that spinal transection could allow the entry of naloxone methiodide into the cerebrospinal fluid through diffusion, but further evidence is required to confirm if this is responsible for the effects observed.

Spinal transection has been shown to reduce the concentration of systemically administered morphine in the brain and spinal cord compared to intact rats

[Advokat & Gulati, 1991]. This is confirmed by Milne *et al.* [1990], as after morphine administration the maximum tail flick latencies in the spinalised animals were lower than in the intact animals. How or why this could alter the effects of subsequent naloxone methiodide administration is not known. A possible hypothesis is that the lower concentrations of morphine in the CNS provide less analgesia, so the doses of naloxone methiodide administered would be adequate to reverse this level of analgesia, but not the analgesia present in the intact, normal animals. This remains to be investigated, and again highlights that this article cannot be used to confirm that naloxone methiodide cannot enter the brain to induce centrally mediated effects.

The effect of naloxone methiodide treatment on analgesia produced by peripherally acting opioid receptor agonists has also been conducted. Methylmorphine injected peripherally and centrally could inhibit the licking induced by peripheral pain stress, but naloxone methiodide (10 mg/kg i.p.) could only reverse this antinociception following peripheral methylmorphine administration [Oluyomi *et al.*, 1992]. This suggests that there is both a central and peripheral component to opioid induced analgesia, and these doses of naloxone methiodide can only target the opioid actions that occur in the periphery.

Using the abdominal writhing test, 10 mg/kg s.c. naloxone methiodide effectively blocked the analgesic effect of loperamide (an opioid receptor agonist with limited access into the brain) indicating a peripherally mediated effect [Reichert *et al.*, 2001]. Naloxone and naloxone methiodide could also reverse the anti-

hyperalgesic effects of loperamide and morphine in the formalin test, with naloxone methiodide requiring 30 to 100 fold higher doses than naloxone to produce the same effect [Shannon & Lutz, 2002]. Naloxone methiodide, however, was not effective in reversing the effects of intracisternally administered loperamide, but could still reverse the effects of morphine when administered in this manner. Therefore, a peripheral component to the effects of morphine and loperamide exists, but naloxone methiodide cannot reverse the antinociception produced by centrally administered loperamide.

Wu *et al.* [1997] used the tail flick test to examine the analgesic effect of M-6-G, which has limited brain uptake and is, therefore, thought to produce analgesia through predominantly peripheral actions. 10 μmol/kg s.c. naloxone effectively reversed the analgesia produced after peripheral administration of M-6-G (5 mg/kg s.c.), but naloxone methiodide at the same dose produced no effect. However, higher doses of naloxone methiodide must be administered to confirm this result. This is illustrated in a study by Spampinato *et al.* [2003] who antagonised the analgesia produced by s.c. administration of Tyr-L-β-Pro-Trp-Phe-NH_2; Endo1-β-Pro with 30 mg/kg i.p. naloxone methiodide to show that a peripheral component is involved in the antinociceptive action of this novel endomorphin-1 analogue.

A study investigating the analgesic effects of EMD 61753 (asimadoline), a peripherally acting κ opioid receptor antagonist, found that naloxone methiodide could reverse the antinociception produced by this agonist, but not the hyperalgesia that was later observed [Machelska *et al.*, 1999]. The authors

suggested that the asimadoline antinociception contains κ opioid receptor mediated effects in the periphery, but the hyperalgesia was due to a non-opioid receptor mechanism. Similar antinociception was also observed with two peripherally selective κ opioid peptides, FE 2000665 and FE 2000666 [Binder *et al.*, 2001]. Naloxone methiodide administered s.c. could completely attenuate the effects of these compounds after intraplantar or s.c. administration, but not when FE 2000665 was administered i.t. or when naloxone methiodide was administered i.t. and FE 2000665 systemically. Therefore, naloxone methiodide could reverse the peripherally induced analgesia, but was unable to cross the blood brain barrier either into the brain, to reverse the actions of i.t. administered opioid receptor agonists, or out of the brain, to reverse systemically administered opioid receptor agonists. This study also showed that s.c. naloxone methiodide could reverse the inflammatory effects of these two opioid receptor agonists or U-69,593, indicating that this κ opioid effect is peripherally mediated.

A potent μ receptor agonist peptide, [Dmt^1]DALDA ([2',6'-dimethyltyrosine]Tyr-D-Arg-Phe-Lys-NH_2), was examined for its potential to act as an analgesic without many of the centrally mediated side effects of morphine [Riba *et al.*, 2002]. Morphine was effective in producing analgesia in the tail flick assay and its effect was reversed by both naloxone methiodide and naloxone, but only naloxone could reverse the analgesia produced by s.c. [Dmt^1]DALDA. When both [Dmt^1]DALDA and naloxone methiodide were injected either i.c.v. or i.t., naloxone methiodide was even more effective than naloxone in reversing the analgesia induced by this opioid receptor agonist. This study again shows that naloxone

methiodide can only act in the periphery, unless it is administered directly into specific brain regions.

Resiniferatoxin (a vanilloid receptor-1 agonist) administration into the bladder produces visceral pain similar to that of abdominal pain and was used by Craft *et al.* [1995] to examine the antinociceptive effects of a variety of opioid receptor agonists. Systemic administration of morphine, U50-488 (a κ opioid receptor agonist), BW373U86 (a δ opioid receptor agonist), DAMGO (a μ opioid receptor agonist) and CI-977 (a κ opioid receptor agonist) reduced abdominal licking, and while i.p. administration of naloxone methiodide was not as effective as naloxone it still reversed these effects. Upon i.c.v. administration, naloxone methiodide blocked the antinociceptive effects of morphine but not BW373U86 or CI-977, indicating these agonists do not act centrally when administered systemically. Naloxone methiodide was also effective in reversing the analgesia produced when these opioid receptor agonists were administered into the bladder. This study, therefore, shows that while the effects of opioid receptor agonists may differ, the administration of the correct doses of naloxone methiodide can prevent their peripherally mediated antinociceptive effects.

Varied results have been obtained in the studies investigating the effect of naloxone methiodide treatment on antinociception, but there is growing evidence to show that adequate doses of naloxone methiodide can prevent the analgesic actions of a variety of opioid receptor agonists. This suggests that opioid induced antinociception contains a significant peripheral component that can be targeted by peripherally selective opioid receptor antagonists.

1.6.3.1.9 Cardiovascular effects:

With the discovery of opioid receptors in cardiac tissue, many studies have aimed to determine if the opioid effects observed contain a central or peripheral component, or a combination of both [Barron, 2000]. In anaesthetised rats given a dose of i.v. morphine to produce a reduction in heart rate of 10%, both i.v. naloxone and naloxone methiodide could reverse this effect, but a 15 times higher dose of naloxone methiodide was required [Kiang *et al.*, 1983]. These results were also replicated in rats administered i.v. etorphine, confirming a peripheral component to the cardiovascular changes produced by opioid receptor agonists [Dashwood *et al.*, 1983].

In conscious dogs, i.v. morphine produced a biphasic cardiovascular response with significant increases in heart rate and mean arterial blood pressure, which is then followed by decreases in these parameters after 20 minutes [Given *et al.*, 1986]. Pre-treatment with 1 mg/kg i.v. naloxone and naloxone methiodide prevented the initial increases in blood pressure, but not the changes in heart rate, which may be due to non-opioid mechanisms controlling this response. Naloxone prevented the subsequent decreases in heart rate and mean arterial pressure, but no effect was observed with naloxone methiodide when administered either before or after morphine treatment. This suggests peripheral mediation of the initial increases in mean arterial pressure, but central mediation of the following depression of cardiovascular parameters. Higher doses of naloxone methiodide, however, may have shown that peripheral components are also involved in this delayed action.

A study investigating the cardiorespiratory effects of endomorphin 1 & 2, DAMGO and morphine in conscious rats found that pre-treatment with 1 mg/kg i.v. naloxone and naloxone methiodide could attenuate the decreases in mean arterial blood pressure and heart rate produced by these opioid receptor agonists [Czapla *et al.*, 2000]. A study by Negri *et al.* [1998] also found that [Lys^7]dermorphin decreased arterial blood pressure in conscious, restrained rats, which was reversed by 0.1 mg/kg s.c. naloxone and partially reversed by 3 mg/kg s.c. naloxone methiodide. This suggests that peripherally mediated mechanisms contribute significantly to the cardiovascular effects of these opioid receptor agonists.

Ellenberger *et al.* [2003] investigated the identity and location of opioid receptors involved in anaesthesia associated hypertension. They injected anaesthetised rats with 1 mg/kg naloxone or naloxone methiodide i.v. before 2% isoflurane exposure for 20 minutes. The isoflurane loading on the anaesthetised animals caused an initial reduction in mean arterial blood pressure, which increased slightly after 1.5 minutes and was followed by a gradual decrease that continued for 20 minutes. Pre-treatment with naloxone or naloxone methiodide attenuated the initial rapid decrease in blood pressure and then gradually decreased over the 20 minutes, as seen in the control animals. Whilst the change in blood pressure was not completely reduced, this study does indicate that opioid receptors are involved in isoflurane induced hypertension, and they appear to be located in the periphery.

Naloxone methiodide has also been used to determine if ischaemic pre-conditioning of the heart involves peripheral or central mechanisms. High doses of naloxone methiodide (10 mg/kg i.v.) and naloxone (3 mg/kg i.v.) reduced the effect of ischemic pre-conditioning, indicating that this cardioprotective effect is mediated by a peripheral opioid mechanism [Schultz *et al.*, 1997]. A similar cardiac mechanism was observed by Chien *et al.* [1999], who investigated tolerance to myocardial ischaemia by ischaemic pre-conditioning in rabbits.

Weber *et al.* [2001] examined the effect of myocardial stunning on conscious dogs and found that while naloxone could improve ischaemic and post-ischaemic systolic impairment and reduce the severity of myocardial stunning, the same dose of naloxone methiodide (63 µg/kg) produced no effects. Maslov *et al.* [2003], however, illustrated that the abolition of cardiac electrical instability produced by DPDPE in rats with experimental cardiosclerosis could be prevented by 5 mg/kg naloxone methiodide administration. The antifibrillatory effect of DPDPE, therefore, appears to be mediated by peripherally located δ opioid receptors.

A study in a pregnant sheep model has also illustrated the peripheral component to opioid related cardiovascular effects. Noradrenaline and sodium nitroprusside were administered i.v. to produce a hypertensive or hypotensive stimulus, respectively. DALDA (H-Tyr-D-Arg-Phe-Lys-NH_2), a µ opioid peptide, suppressed the baroreflex sensitivity to the hypertensive stimulus of noradrenaline, but had no effect on the hypotensive actions of sodium

nitroprusside. Prior treatment with naloxone methiodide abolished the action of DALDA, indicating that this effect is mediated through a peripheral opioid system [Kett *et al.*, 1998]. A study in foetal sheep has also shown that 0.5 mg/kg i.v. DALDA produces a gradual increase in foetal heart rate, which is attenuated by both naloxone and naloxone methiodide. If naloxone methiodide is unable to cross the foetal blood brain barrier, this study again indicates that there is a peripheral component to the cardiovascular effects of opioids [Holsey *et al.*, 1999].

During haemorrhage in rats and humans, an early sympathoexcitatory response occurs to maintain blood pressure, which is followed by an inhibitory effect to significantly lower blood pressure. Naloxone has been shown to restore blood pressure after severe haemorrhage, but it is not known if this effect is centrally or peripherally mediated. Ang *et al.* [1999] observed that i.t. and intracisternal administration of naloxone methiodide could prevent the fall in blood pressure that occurs with haemorrhage and increase heart rate compared to control animals. When administered i.v., however, naloxone methiodide did not produce any changes compared to control animals. Therefore, this would suggest that the effect of naloxone after severe haemorrhage is due to a central mechanism. Doses of naloxone methiodide higher than 100 nmol i.v., however, may have produced effects similar to that of naloxone.

Variable results have been obtained from studies involving the use of naloxone methiodide to investigate opioid induced cardiovascular changes, but generally confirm that peripheral opioid mechanisms are involved in these effects.

1.6.3.1.10 Respiration:

As shown above, peripherally acting opioid receptor antagonists, such as naloxone methiodide, have been used to elucidate peripheral opioid mechanisms. While the data are still inconclusive for many actions, they do suggest that peripheral mechanisms may be involved in a number of opioid effects. These previous findings have led to the investigation of peripheral mechanisms in opioid induced respiratory depression in this research project. Prior to the initiation of this project, very little research had been conducted on the effects of peripherally acting opioid receptor antagonists on respiration, but more research has been published over the duration of the project. The currently available literature is summarised below.

Whilst not directly related to opioid induced respiratory depression, the antitussive effect of opioids has been investigated to determine if peripheral mechanisms are involved. Karlsson *et al.* [1990] used nebulised citric acid to induce cough and bronchoconstriction in guinea pigs, and reported that codeine, morphine and pethidine could inhibit these effects when administered intramuscularly (i.m.) or nebulised. Irrespective of the route of administration, the effects of these opioid receptor agonists were reversed by i.m. naloxone. The effect of the peripheral opioid receptor antagonist, levallorphan methyl iodide, was also tested on guinea pigs treated with nebulised codeine and completely reversed the codeine antitussive effects, indicating that peripheral mechanisms control this opioid effect.

A similar study by Callaway *et al.* [1991] found that the frequency of coughing could be reduced by inhaled or i.p. injected morphine, DALDA, dextromethorphan or codeine, and that the inhaled morphine effects could be reversed by the i.p. administration of either naloxone or naloxone methiodide. Recently, it has been shown that while non-opioid compounds such as pinacidil (an ATP sensitive K^+ channel opener) and moguisteine ((R,S)-2-(2-methoxyphenoxy)-methyl-3-ethoxycarbonyl-acetyl-1,3 thiazoladine), a non-opioid antitussive drug) can reduce capsaicin induced cough in guinea pigs, naloxone methiodide does not antagonise the effect of these compounds. It could, however, reverse the antitussive effect of dihydrocodeine in the same model [Morita *et al.*, 2002]. These studies, therefore, suggest that opioid induced antitussive effects can be reversed by peripherally acting opioid receptor antagonists, indicating a peripheral opioid mechanism. This provides strong evidence for peripherally acting opioid receptor antagonists, such as naloxone methiodide, being able to antagonise the respiratory effects of opioids by targeting peripheral mechanisms.

The effect of naloxone methiodide on respiration without exogenous opioid receptor agonist administration has been investigated in several studies. Schlenker & Inamdar [1995] examined the effect of endogenous opioids on respiration in conscious golden Syrian hamsters, which express 400 times greater concentrations of enkephalin like peptides in their adrenal glands than rats. Under normal air breathing conditions, 1 mg/kg s.c. naloxone and naloxone methiodide produced significant increases in VO_2, with naloxone methiodide having a greater effect than naloxone despite the same doses being administered. Breathing frequency, tidal volume and minute volume were

unaltered by the naloxone, naloxone methiodide or saline treatments. Under hypoxic conditions, breathing frequency remained unaltered, but tidal volume and minute volume increased with both antagonist treatments. Only significant increases in tidal volume and minute volume were noted in the naloxone treated hamsters under hypercapnic conditions. This study, therefore, illustrates that respiration is modulated by endogenous opioids and that both naloxone and naloxone methiodide can antagonise these effects. If naloxone methiodide is unable to enter the CNS, this study also indicates a peripheral component to these opioid receptor antagonist effects.

Lee *et al.* [2000, 2001] also investigated respiratory modulation by endogenous opioids in obese Zucker rats. Obese rats have increased levels of endogenous opioids compared to lean rats and are, therefore, more sensitive to opioid receptor antagonist actions [Schlenker & Farkas, 1995]. 5 mg/kg s.c. naloxone and naloxone methiodide produced no changes in the lean rat groups, and no changes were observed in the 6 or 16 week old obese rats treated with naloxone methiodide [Lee *et al.*, 2000]. Naloxone treatment in 6 week old obese rats produced increases in tidal volume and minute volume, which also occurred under hypercapnic breathing conditions. During exercise testing under normal breathing conditions naloxone methiodide again had no effect on either the lean or obese rats tested. Naloxone produced no significant changes on the lean rats, but in the 6 week old obese animals significantly increased peak aerobic activity.

In their following study examining sustained hypoxia, naloxone methiodide again produced no changes in respiratory parameters. In young obese rats, naloxone

initially increased tidal volume and minute volume, and over time maintained elevated tidal volume while minute volume returned to normal and respiratory rate decreased [Lee *et al.*, 2001]. Therefore, it appears that naloxone can produce subtle changes in respiration in young obese animals, while naloxone methiodide does not modulate the respiratory effects of endogenous opioids. This may be either due to these effects being centrally mediated, or due to the dose of naloxone methiodide administered being too low to produce observable effects. Given that the effects of naloxone were minimal, it would be of interest to investigate the effects of higher doses of both naloxone and naloxone methiodide, as more pronounced respiratory effects may be observed.

Limited studies have been conducted examining the effect of naloxone methiodide on the changes in respiration produced by exogenously administered opioid receptor agonists. In foetal sheep, i.c.v. administration of naloxone methiodide reversed the morphine induced (2.5 mg/hr i.v.) changes in respiration, indicating that if naloxone methiodide is not peripherally selective and can gain access to the CNS it would be able to antagonise opioid induced changes in respiration [Szeto *et al.*, 1991].

In a study investigating etorphine induced cardiovascular changes, i.v. administration of 69 mmol/kg naloxone or 593 mmol/kg naloxone methiodide alone did not produce any changes in respiratory rate, but after etorphine administration naloxone reversed the 40% respiratory depression that developed [Dashwood *et al.*, 1983]. Despite the administration of higher doses, naloxone methiodide had no effect on the respiratory depression produced by etorphine,

suggesting that peripheral mechanisms are not involved in this opioid action. Negri *et al.* [1998] also showed that 3 mg/kg s.c. naloxone methiodide did not reverse the respiratory changes in rats administered [Lys^7]dermorphin or morphine under normal air or hypoxic conditions while 0.1 mg/kg s.c. naloxone blocked these effects. This lack of effect was also noted in rats treated with 1 mg/kg i.v. naloxone methiodide after the administration of DAMGO, endomorphin 1 & 2 and morphine, despite naloxone methiodide completely attenuating the cardiovascular effects of these opioid receptor agonists [Czapla *et al.*, 2000].

To date, only one clinical study has investigated the effect of a peripherally acting opioid receptor antagonist on respiratory parameters [Amin *et al.*, 1994]. Healthy males were administered 0.125 mg/kg i.v. morphine and 40 minutes later given 5 µg/kg naloxone, 0.3 mg/kg methylnaltrexone or saline. The volunteers were subjected to hypoxic challenges prior to morphine administration, 40 minutes after morphine administration, and 40 minutes after antagonist or saline administration. The respiratory responses were measured during these hypoxic challenges. If at any time during the hypoxic treatments SpO_2 decreased to lower than 70%, O_2 was immediately given. All results were discussed in relation to the slope of the regression analysis determined using the minute ventilation and O_2 desaturation results collected, or from calculations to determine the Ve_{80} or predicted ventilation at SpO_2 = 80%.

Morphine produced a significant decrease of approximately 50% in the slope of the regression analysis. The Ve_{80} values were also decreased to approximately 60% of pre-morphine treatment values. 40 minutes after naloxone infusion, these

slopes returned to 85 to 90% of control slopes, which were not significantly different from pre-treatment values. Saline and methylnaltrexone infusions at this time were approximately 60% of control values, a significant depression compared to pre-treatment values. At 80 minutes after antagonist infusion, the methylnaltrexone slope was no longer different to pre-treatment values but Ve_{80} was still significantly lowered, while the values after saline treatment were still significantly depressed. Therefore, the naloxone treatment provided adequate reversal of the morphine induced decreases in respiration while methylnaltrexone was not as effective.

The results of this study do not suggest a significant peripheral component to the respiratory effects of opioids, but several limitations complicate the analysis of these findings. Measurements were only taken 40 minutes after antagonist treatment, so any of the effects that occurred before this time would not be included in the analysis. A different, and more complete, profile of effects may have been observed if measurements were taken more frequently. Measurements were only taken during the hypoxic treatments, so it is not known if the morphine administered actually depressed respiration under normal breathing conditions and, therefore, if any significant antagonism of effects by naloxone or methylnaltrexone would be expected. It would also have been of interest to determine the effects of the morphine, naloxone and methylnaltrexone effects under normal breathing conditions, and not with the added stress of hypoxia, as this would provide a better indication of the effects of these opioid receptor agonists and antagonists. The actual changes in respiratory parameters with these treatments were not presented in this study, as only the results from

regression analyses were reported. This makes it difficult to draw any conclusions from this study without further information, as certain aspects may not have been considered with the regression analyses conducted.

Another question that arises is why naloxone and methylnaltrexone were chosen in this study. It is not known if the dose of methylnaltrexone chosen would be expected to produce effects similar to naloxone, or if they would follow the same profiles. The authors do comment that higher doses of methylnaltrexone may have resulted in a greater effect being observed, but this was not investigated.

Therefore, despite this clinical study and the animal studies discussed previously suggesting that peripheral mechanisms do not contribute greatly to opioid induced respiratory depression, there is still doubt, as these studies have not addressed all of the relevant issues. It is not known if higher doses of peripherally acting opioid receptor antagonists would produce greater effects, or if more pronounced antagonism would have been noted with greater respiratory depression. This PhD project was designed to address the major deficits in the previous literature, and extend the current findings relating to the effects of peripherally acting opioid receptor antagonists on opioid induced respiratory depression.

1.7 RESEARCH AIMS:

As shown in the previous section, a great deal of research has been conducted using naloxone methiodide as a tool to identify peripheral opioid mechanisms or to investigate its potential to prevent or treat adverse opioid effects. There are deficiencies, however, in much of this literature as listed below (For more details See Section 1.6.3.1 on Page 76)).

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

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

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

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

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

The findings from much of these existing data may have led to incorrect conclusions regarding the effectiveness of naloxone methiodide, or the involvement of peripheral mechanisms in opioid effects. This research project was, therefore, designed to investigate naloxone methiodide with respect to these

limitations and with specific interest in opioid induced respiratory depression, analgesia and withdrawal behaviour.

The main aim of this project was to determine the effectiveness of naloxone methiodide, compared to naloxone, in reversing opioid induced respiratory depression. This has been addressed in four publications with the specific aims described below.

Publication 1 - Published in European Journal of Pharmacology (2002):

The aim of this publication was to determine if naloxone methiodide could reverse opioid induced respiratory depression and analgesia without inducing withdrawal in mice treated acutely and chronically with high doses of morphine.

The first limitation in the previous literature (See above) was addressed as higher doses of naloxone methiodide compared to naloxone were administered to compensate for the suspected differences in potencies between these two opioid receptor antagonists. The second limitation was also considered as withdrawal symptoms were measured throughout the treatment regime to determine if naloxone methiodide could induce this predominantly centrally mediated effect. High doses of morphine were administered acutely or chronically, which addressed limitations 3 and 5.

Publication 2 – Published in Brain Research (2003):

The aim of this study was to compare the ability of naloxone and naloxone methiodide to displace binding to μ, δ and κ opioid receptors derived from mouse

brain homogenates, which addressed the first limitation of the previous research that has been conducted. A comparison of the affinity of naloxone and naloxone methiodide for μ, δ and κ opioid receptors has not been previously conducted in mouse brain homogenates. This is vital for the interpretation of results obtained using murine models, as conducted in these experiments.

Publication 3 – Under Review in Drug and Alcohol Dependence (2004):

This manuscript addressed many of the limitations of previous research using naloxone methiodide. The first aim was to determine if the effects of naloxone methiodide previously observed were due to actions on centrally located μ opioid receptors (Limitation 2). This was conducted by measuring the binding of [^{3}H]-DAMGO to μ opioid receptor binding sites in the brain *ex vivo*, after the *in vivo* peripheral administration of naloxone and naloxone methiodide.

The second aim involved comparing the respiratory effects of morphine, methadone and heroin to determine the doses of each opioid receptor agonist that produced similar decreases in respiratory rate. These equipotent respiratory depressive doses of morphine, methadone and heroin were then used to compare the effect naloxone and naloxone methiodide treatment on respiratory rate, withdrawal and analgesia. This addressed limitations 1, 3 and 4.

The final aim of this study was to use whole body barometric plethysmography to extend our measurements of respiratory parameters to include tidal volume and minute volume. Mice were administered methadone followed by naloxone and naloxone methiodide treatment to examine the complete respiratory changes that

occur within this model of opioid induced respiratory depression. Limitations 1, 3 and 4 were again considered in this experimental plan.

Publication 4 – Published in the European Journal of Pharmacology (2004):

This final publication addressed two main aims. The first was to validate the use of radiotelemetry to measure cardiovascular and respiratory changes continuously in rats treated chronically with methadone. The second was to examine the effect of naloxone and naloxone methiodide treatment on respiratory rate, heart rate, spontaneous locomotor activity and mean arterial blood pressure using this rat model.

This study again addressed the limitations observed in the previously published literature as the dose of methadone administered produced significant respiratory depression and adequate doses of naloxone and naloxone methiodide were injected to produce observable effects (Limitations 1, 3 & 4). This study also examined the cardiovascular and respiratory effects of naloxone methiodide after chronic opioid receptor agonist treatment (Limitation 5).

These publications will be described in further detail in the following section.