Opioids and immune function:

The role of non-classical opioid receptors and the association with pain perception

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Abstract

The discoveries of functional opioid binding receptors on immune cells suggest likely clinical relevance and exciting opportunities for future research into brain-immune communication. Reports of both classical and non-classical opioid immunomodulation can be found in the literature, however the number of opioid ligands applied to each individual experimental system is small. Therefore, a mouse splenocyte *in vitro* system was developed in which the immunomodulation by forty-one opioids was quantified, with several important characteristics observed. Firstly, 4,5-epoxymorphinan immunomodulation could be divided into five major responses, whilst there were four non-4,5-epoxymorphinan immunomodulation responses: an inverted bell-shaped curve (e.g. oxycodone and fentanyl), a concentration-dependent inhibitory response curve (e.g. buprenorphine and methadone), an inverted bell-shaped curve with induction (e.g. morphine), a concentration-dependent induction response curve (e.g. oxymorphone and nor-binaltorphimine), an induction and inhibition response (e.g. tramadol), and absence of response (e.g. hydrocodone). The magnitude and efficacy of the immunomodulation by the opioids examined demonstrated non-classical opioid rank order of effect, implying mediation via different receptors than opioid receptors previously characterised on neuronal tissue. Furthermore, both opioid receptor antagonist sensitivity and insensitivity, as well as non-stereoselectivity were observed, all agreeing with the non-classical opioid immunomodulation conclusion. Consequently, a 4,5-epoxymorphinan structure-effect relationship was developed, which differed significantly from classical μ opioid receptor hypotheses, again supporting the non-classical opioid immunomodulation conclusion. *In vivo* immunomodulation was also investigated using methadone and its enantiomers. Methadone is prescribed clinically as a racemate (50/50 mix of the two enantiomers), however only half the dose is active as only

(R)-(-)-methadone has significant μ opioid receptor activity. *In vivo* a classic opioid analgesic response was observed with the (R)-(-)-enantiomer and racemic methadone, whilst with the inactive enantiomer, (S)-(+)-methadone, produced no analgesia. However, the immunopharmacodynamic effects did not reflect this, as (S)-(+)-methadone caused significant inhibition of immune function, suggesting non-classical opioid immunomodulation. Finally, the proliferative response of peripheral blood mononuclear cells isolated from healthy subject, following exposure to morphine *in vitro*, was highly correlated with the pain tolerance of the subjects. Therefore, I have demonstrated, for the first time, an objective pain tolerance test. This discovery has potential to revolutionise pain treatment, as the immune system may become the target for future pain medications. These findings warrant further investigations into the link between the immune system and central nervous system and open the way for optimising the treatment of chronic pain.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no other material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan or photocopying.

Mark R Hutchinson

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For you, deep stillness of the silent inland
For you, deep blue of the desert sky
For you, flame red of the rocks and stones
For you, deep water from hidden springs

From the edges, seek the heartland
and when you're burnt by the journey
may the cool winds of the hovering spirit
soothe and replenish you
In the name of Christ
In the name of Christ

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Publications in support of this thesis

Hutchinson, M.R. & Somogyi, A.A. (2002). Diacetylmorphine degradation to 6-monoacetylmorphine and morphine in cell culture: implications for *in vitro* studies. Eur. J. Pharmacol., **453** 27-32.

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Abbreviations, prefixes and symbols

5-HT	Serotonin
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocytes
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte colony stimulating factor
GTP	Guanosine triphosphate
HPA axis	Hypothalamus pituitary adrenal axis
HPLC	High performance liquid chromatography
i.p.	Intra peritoneal
IC_{50}	Concentration inhibition 50% of ligand binding
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
K_d	Equilibrium dissociation constant
K_i	Inhibition constant
LC	Locus ceruleus
MAPK	Mitogen-Activated Protein Kinase
MHC	Major histocompatibility complex
mRNA	messenger Ribo Nucleic Acid
NF	Nuclear factor
NMDA	N-methyl-D-asparate
NRM	Nucleus raphe magnus
NRPG	Nucleus reticularis
ORL1	Orphan opioid receptor
PAG	Periaqueductal gray
Ra	Receptor antagonist
S.E.M.	Standard error of the mean
SD	Standard deviation
T_C	T cytotoxic cell ($CD8^+$)
TGF	Transforming growth factor
T_H	T helper cell ($CD4^+$)
TNF	Tumor necrosis factor

Opioid ligand table

Compound	Brief description
β-endorphin	Peptide μ opioid receptor ligand
β-funaltrexamine	Irreversible μ opioid receptor antagonist
3-O-Methylnaltrexone	A 4,5-epoxymorphinan which is a diacetylmorphine and morphine-6-glucuronide specific receptor antagonist
6β-naltrexol	Active metabolite of naltrexone and a 4,5-epoxymorphinan μ opioid receptor antagonist
6-Hydroxyoxymorphone	Active metabolite of oxycodone and a 4,5-epoxymorphinan μ opioid receptor agonist
6-Monoacetylmorphine	Active metabolite of diacetylmorphine and a 4,5-epoxymorphinan μ opioid receptor agonist
Alfentanil	μ opioid receptor agonist
Buprenorphine	4,5-epoxymorphinan partial μ and δ opioid receptor agonist
CGPM-9 (4-tyrosylamido-6-benzyl-1,2,3,4 tetrahydroquinoline)	Non-peptide μ opioid receptor agonist
Codeine: (-) and (+)	4,5-epoxymorphinan μ opioid receptor agonist
CTAP	μ opioid receptor antagonist
CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2)	Peptide μ opioid receptor antagonist
DADLE ([D-Ala^2,D-Leu^5]-Enkephalin)	Peptide δ opioid receptor agonist
DAME or DALA (D-Ala^2-Met^5-Enkephalinamide)	Peptide μ and δ opioid receptor agonist
DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin)	Peptide μ opioid receptor agonist
Deltorphin	Peptide δ opioid receptor agonist
Demorphin	Peptide μ opioid receptor agonist
Dextromethorphan	NMDA receptor antagonist
Dextrorphan	NMDA receptor antagonist
Diacetylmorphine (heroin)	4,5-epoxymorphinan μ opioid receptor agonist
Dihydromorphine	4,5-epoxymorphinan μ opioid receptor agonist
Diprenorphine	Opioid receptor antagonist
DPDPE ([D-$Pen^{2,5}$]-Enkephalin)	Peptide δ opioid receptor agonist
DSLET ([D-Ser^2]-Leucine Enkephalin-Thr)	Peptide δ opioid receptor agonist
DTLET (H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH)	Peptide δ opioid receptor agonist
Dynorphin A (1-17)	Peptide κ opioid receptor agonist
Endomorphin 1 & 2	Peptide μ opioid receptor agonist
Ethylketocyclazocine	κ opioid receptor agonist
Fentanyl	4-anilinopiperidine μ opioid receptor agonist
Hydromorphone	4,5-epoxymorphinan μ opioid receptor agonist
ICI 174864	δ opioid receptor antagonist
ICI 204488	κ opioid receptor agonist
Ketocyclazocine	κ opioid receptor agonist

Compound	Brief description
LAAM (levo-alpha-acetyl-methadol)	μ opioid receptor agonist
Leu-enkephalin	Peptide δ opioid receptor agonist
Levorphanol	Synthetic morphine analogue and a μ opioid receptor agonist
Met-enkephalin	Peptide δ opioid receptor agonist
Methadone (racemic, (R)-(-)) (S)-(+)-methadone is opioid inactive	3,3-diphenylpropylamines μ opioid receptor agonist
Mirfentanil	4-anilinopiperidine μ opioid receptor agonist
Morphiceptin	μ opioid receptor agonist
Morphine (-) and (+)	4,5-epoxymorphinan μ opioid receptor agonist
Morphine-3-glucuronide	4,5-epoxymorphinan metabolite of morphine
Morphine-6-glucuronide	Active metabolite of morphine and a 4,5-epoxymorphinan μ opioid receptor agonist
N-allylnormetazocine (SKF10047)	σ receptor agonist
Naloxonazine	μ opioid receptor antagonist
Naltrexone	4,5-epoxymorphinan μ opioid receptor antagonist
Naltrindole	δ opioid receptor antagonist
Nor-binaltorphimine (nor-BNI)	κ opioid receptor antagonist
Norbuprenorphine	μ opioid agonist metabolite of buprenorphine
OHM3507	Fentanyl derivative
Ohmefentanyl	μ opioid receptor agonist
Oripavine	4,5-epoxymorphinan μ opioid receptor agonist
Oxycodone	μ opioid receptor agonist
Oxymorphindole	δ opioid receptor agonist
Oxymorphone	μ opioid receptor agonist
Pethidine	4-phenylpiperidine μ opioid receptor agonist
Remifentanil	4-anilinopiperidines μ opioid receptor agonist
SUPERFIT (*cis*-(+)-3-methylfentanylisothiocyanate)	δ opioid receptor alkylating agent
Thebaine	4,5-epoxymorphinan μ opioid receptor agonist
TIPP (H-Tyr-Tic-Phe-Phe-OH)	Peptide δ opioid receptor antagonist
Tramadol	Mixed agonist
U50488	κ opioid receptor agonist
U69593	κ opioid receptor agonist

Chapter 1. Introduction

As this thesis covers the disciplines of clinical and experimental pharmacology and immunology, it is likely that the readers of this work may not be familiar with both of these disciplines. Therefore, some background information must be presented, such that a greater understanding of the discussion of the literature and data can be appreciated by a wider scientific audience.

The alluring possibility of a connection between the immune system and the central nervous system has been postulated for many years, although the specific nature of the interaction is yet to be fully comprehended. It now appears that several modes of communication are involved in the bi-directional communication between these two systems, with opioids having profound immunomodulatory potential. Due to the widespread clinical use of opioids in the front line treatment of pain, and abuse of opioids, there is a significant chance for modification of immune function. Moreover, the recent discovery of immune modulation of opioid pharmacodynamic effects highlights the importance of this relationship. A greater understanding of the interaction between the immune system and the central nervous system will have a significant influence on the clinical use and the insight into the abuse of opioids. Furthermore, it will enhance our understanding of brain-immune communication, host defence from invasive pathogens, chronic pain, nociception and the illness response as a whole.

1.1. Opiates and Opioids

The term opiate refers to compounds that are derived from the opium poppy (*papaver somniferum*), such as morphine, codeine and their synthetic analogues. Meanwhile, the term opioid refers to all compounds, both natural and synthetic, that have morphine-like actions. Opioids produce a variety of effects, the most notable being relief from pain, alterations in mood (e.g. euphoria), respiratory depression, sedation, miosis, decreased gastrointestinal motility (constipation), nausea, and vomiting (King & Miller, 1998). The first account of the use of opium for the principal indication to relieve pain and diarrhoea can be found in the writing of Theophrastus in the third century BC. However, it is almost certain that the analgesic effects of an extract of poppy seeds was known long before then, since opium was imported from Cyprus to Egypt between 1551-1436 BC. Because of the powerful euphoric effects, the non-analgesic use of opioids has certainly been known for at least this length of time.

In 1806, Friedrich Wilhelm Adam Sertürner started work on isolating the major active constituent of opium and named it morphine (after Morpheus, Ovid's god of dreams, the son of sleep) (cited by Huxtable & Schwarz (2001)). However, the chemical structure of morphine was not elucidated until early last century (Guilland & Robinson, 1923). Since this time, many structural modifications have been made to morphine, producing semi-synthetic and fully synthetic compounds with varied, but similar pharmacological actions. Morphine belongs to a class of compounds know as 4,5-epoxymorphinans, whose basic structure can be seen in Figure 1.1-1. The functional group R1 at position three has the most significant effect on ligand binding to opioid receptors and analgesic effects and R4 at position 17 produces the antagonistic affects when large bulky functional groups are present (Chen *et al.*, 1991).

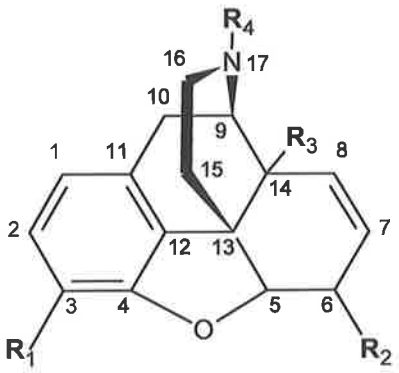


Figure 1.1-1 The basic 4,5-epoxymorphinan structure.

Once the structure of morphine had been established, attempts to synthesise opioids that were more potent, longer acting, more effective and which had fewer side effects began. This gave rise to numerous other compound classes such as morphinans, benzomorphinans, arylmorphinans, 4-phenylpiperidines, 4-anilinopiperidines and 3,3-diphenylpropylamines, all of which have differing pharmacokinetic and pharmacodynamic properties. Some examples of these non-4,5-epoxymorphinans are levorphanol (morphinan), pethidine (4-phenylpiperidine), fentanyl (4-anilinopiperidine) and methadone (3,3-diphenylpropylamine).

1.1.1. Endogenous opioids

The body is also able to synthesise its own compounds which act as opioids. The first evidence for endogenous compounds that bound to the opioid receptors was in 1975, where isolated peptides competed with morphine for binding at brain receptors (Hughes *et al.*, 1975). These peptides, which have opioid-like action, are encoded on three distinct genes. The products of these genes are known as preproopiomelanocortin, preproenkephalin and preprodynorphin. Adrenocorticotropic hormone, melanocyte-stimulating hormone and β-endorphin are derived from preproopiomelanocortin. All of these opioid peptides have the N-terminal amino acid sequence in common and this amino terminus is required for binding to

brain opioid receptors. The expression of these precursor peptides varies greatly throughout the body; preproopiomelanocortin is located centrally in the pituitary and the hypothalamus, whilst enkephalins are found centrally, peripherally and in organs like the adrenal medulla. The immune system is also capable of expressing these peptides in an active form (Cabot *et al.*, 1997a; Czlonkowski *et al.*, 1993; Miller *et al.*, 1995).

Much research has focused on the opioid peptides β-endorphin, leu-enkephalin, met-enkephalin and dynorphin, with the most intensely studied of these being β-endorphin. The distribution of the peptide opioids has been well characterised. β-endorphin can be found in neurones that run from the hypothalamus to the thalamus and brain stem, whilst enkephalins are predominately found in short interneurons distributed throughout the brain. β-endorphin is relatively stable in plasma, with a half-life of 30 minutes or more. However, enkephalins have a significantly shorter half-life of seconds in rats, and up to five minutes in humans (Turkall *et al.*, 1982). Other endogenous peptides have been discovered recently. These include endomorphin 1 and 2 (Zadina *et al.*, 1997), which have high affinity for the μ opioid receptor and nociceptin which is believed to be the endogenous ligand of the orphan opioid receptor ORL1 (Meunier, 1997; Meunier *et al.*, 1995).

1.1.2. Opioid receptors

The discovery of specific endogenous opioid agonists suggested that specific receptors for opioid compounds must be present. Evidence of the presence of these receptors came from binding studies conducted using brain and nervous tissue (Pert & Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973). Martin *et al.* (1976) proposed the existence of three types of opioid receptors which were named after the drugs used in that study, μ: morphine, κ:

ketocyclazocine and σ: N-allylnormetazocine (SKF10047). The σ receptor was later shown to be non-opioid, however Kosterlitz *et al.* (1980) later named a third opioid receptor type delta (δ for deferens) (Lord *et al.*, 1977). These opioid receptor types have been cloned, with binding and functional properties confirmed (Evans *et al.*, 1992; Satoh & Minami, 1995). The opioid receptors have recently been renamed (Dhawan *et al.*, 1996), with δ, κ and μ opioid receptors recommended to be called OP_1, OP_2 and OP_3, respectively. OP for opioid with the number a reflection of the order in which they were cloned. However, for simplicity the receptors will be referred to by their previous names throughout this thesis.

Opioid binding sites are located on both spinal cord pain transmission neurones and on the primary afferents that transmit pain messages to them. They are expressed both pre- (μ, κ and δ) and post-synaptically (μ) (Satoh & Minami, 1995) in many brain regions, some tissues in the periphery (Table 1.1-1) and in the descending inhibitory pathway (Figure 1.1-2). Although the primary effects of opioids are mediated by the central nervous system, opioid receptors exhibit a wider anatomical distribution throughout peripheral cells and tissues that give rise to hormonal, immunological and some analgesic effects (King *et al.*, 2001), whose importance and function are being intensely studied, but are currently incompletely understood (Williams *et al.*, 2001).

Table 1.1-1 Physiological effects associated with opioid receptors and their anatomical locations.

Opioid receptor	Locations	Functions
μ (OP_3)	Neocortex	Analgesia (supraspinal)
	Thalamus	Respiratory depression
	Nucleus accumbens	Addictive properties
	Hippocampus	Miosis
	Amygdala	Nausea and vomiting
	Periaqueductal gray	Reduced gastrointestinal motility
	Hypothalamus	Sedation
	Dorsal horn of the spinal cord	Euphoria
		Cardiovascular function
		Thermoregulation
κ (OP_2)	Nucleus accumbens	Analgesia (spinal)
		Miosis
		Sedation
		Dysphoria
δ (OP_1)	Olfactory bulb	Analgesia
	Neocortex	Respiratory depression
	Caudate putamen	Olfaction
	Nucleus accumbens	Nausea and vomiting
		Reduced gastrointestinal motility
		Sedation
		Euphoria
		Hallucinations

Adapted from Dhawan *et al.* (1996)

Opioid receptors belong to the family of pertussis toxin-sensitive-G-protein coupled receptors (Harrison *et al.*, 1998). G-proteins are a superfamily of receptors that have seven characteristic transmembrane domains and are coupled to guanosine diphosphate binding proteins. Opioid receptors act to inhibit adenylate cyclase, cause opening of potassium channels, inhibit voltage-gated calcium channels, and inhibit neurotransmitter release (Childers, 1988; Childers *et al.*, 1992; Minami & Satoh, 1995; Satoh & Minami, 1995; Williams *et al.*, 2001). These second-messenger events result in a reduction in the excitability of the cell due to hyperpolarisation and the inhibition of neurotransmitter release. Although

the primary effects of opioids are inhibitory, excitatory effects can occur on various neural pathways by preventing the release of inhibitory neurotransmitters (Williams *et al.*, 2001).

Opioid receptors display several "classical" characteristics that separate them from other classes of receptors. Peptide ligands require an intact NH_2-terminus, increases in sodium ion concentrations or the presence of GTP reduce opioid agonist binding but have minimal effect on antagonist affinity, the binding of a ligand to an opioid receptor is often high affinity (i.e. dissociation constant in the low nanomolar or subnanomolar concentration), binding is stereoselective, binding is blocked by "classical" opioid antagonists such as naloxone, binding to novel sites has a similar rank order of affinity or affect compared to previously characterised opioid receptors and responses are pertussis-toxin sensitive implicating receptor coupling to a G-protein (Sibinga & Goldstein, 1988). These "classical" characteristics can be used to ascertain if novel responses are mediated via an opioid receptor. However, in order for a conclusion of "classical" opioid receptor involvement, several of these characteristics must hold true.

The use (treatment of pain) and misuse (for example heroin addiction) of this class of drugs is wide-ranging. Medically, the predominant use of opioids is for the relief of moderate to severe acute, and chronic pain. The target of most clinically administered opioids is the μ receptor. However, at higher concentrations opioids cause over stimulation of opioid receptors, which can lead to changes in the expected pharmacodynamic profile of the drug, often with an increase in undesirable side effects, such as nausea and vomiting, reduced gastric motility and dysphoria (Table 1.1-1).

Opioid ligands have varying affinity for different opioid receptors and for each receptor subtype. For example, the affinity of typical opioid ligands to μ, κ, and δ opioid receptors expressed by a cell line (CHO cells) is presented in Table 1.1-2 (Satoh & Minami, 1995). The endogenous opioid peptides also have opioid receptor selectivity (Table 1.1-3), such that they produce similar effects to exogenous opioids when they bind to the same opioid receptors. Examination of the chemical structures and binding affinities of opioids for receptors allows predictions to be made for structure-effect relationships. For example, Chen *et al.* (1991) demonstrated using rat brain homogenate, that N-demethylation of 4,5-epoxymorphinans at position 17 (R_4) (Figure 1.1-1) gives rise to compounds with reduced binding affinity to the μ opioid receptor. The R_2 functional group at position 6 has little affect on binding affinity, however R_1 at position 3 is vital to this interaction, with the presence of a hydroxyl group conferring the highest affinity. It is important to note that *in vivo* metabolism can result in the conversion of a poor analgesic to a potent analgesic metabolite by revealing functional groups that allow high affinity binding to opioid receptors. An example of this is codeine metabolism to morphine. Therefore, poor *in vitro* binding does not necessarily imply poor *in vivo* analgesic activity.

Table 1.1-2 Affinity (K_i) of various opioid ligands for each classical opioid receptor type.

Ligand	μ	κ	δ
Morphine	1.4	163	>1000
Naloxone	3.9	16	95
DADLE	6.4	514	2.8
DAMGO	0.87	>1000	>1000
DPDPE	>1000	>1000	7.6
Dynorphin A (1-17)	120	5.48	45
U50488	>1000	1.4	>1000
U69593	>1000	2.3	>1000

K_i values (nM) were calculated from ligand displacement experiments using tritiated DAMGO, U69593 and DPDPE for μ, κ and δ opioid receptors, respectively (Satoh & Minami, 1995).

There have been recent reports of other receptors through which select opioids may produce pharmacodynamic responses. For example, Brown *et al.* (1997a) found two morphine-6-glucuronide binding sites in brain membranes and subsequently reported morphine-6-glucuronide and diacetylmorphine (heroin) analgesia was antagonised by 3-O-methylnaltrexone without interfering with μ, δ or κ receptor mediated responses (Brown *et al.*, 1997b). Moreover, an antisense clone of the μ opioid receptor blocked morphine, but not morphine-6-glucuronide analgesia (Brown *et al.*, 1997b). Addition of antisense oligonucleotides of different G-proteins blocked the effect of morphine-6-glucuronide but not morphine (Rossi *et al.*, 1997). This led to the conclusion that diacetylmorphine and morphine-6-glucuronide were acting not only at the μ opioid receptor, but also via a specific 3-O-methylnaltrexone-sensitive binding site (Brown *et al.*, 1997b; Rossi *et al.*, 1997). It has been suggested that alternative splicing of the μ opioid receptor produces various receptors of differing affinity including the morphine-6-glucuronide receptor (Rossi *et al.*, 1995).

Table 1.1-3 Relative binding affinities of endogenous opioids to opioid receptors.

	μ	κ	δ
β-endorphin	+++	+++	+++
Dynorphin	++	+++	+
Met-enkephalin	++		+++
Leu-enkephalin	+		+++

Adapted from Rang & Dale (1996). Binding affinity rank order + < ++ < +++

1.1.3. Opioid pharmacodynamics

The main pharmacological effects of opioids can be divided into two parts, the central nervous system (CNS) effects and the peripheral effects. Some of the classical opioid CNS effects include analgesia, euphoria, respiratory depression, antitussive action and nausea. Peripheral opioid effects include reduced motility of the gastrointestinal tract and dilatation of

cutaneous blood vessels. Many of these properties of opioids are exploited in modern medicine. Opioids are primarily used for their analgesic properties, however they are also used as antitussive and anti-diarrhoeal agents.

Pain or nociception is defined by the International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. The state of analgesia or antinociception is one in which there is reduced or complete removal of pain detection resulting from a noxious stimulus. Many theories for pain have been developed including the specificity theory, pattern theories and the gate control theory (Melzack & Wall, 1965). Much of this research focused on the dorsal horn of the spinal cord and the descending inhibitory control system. The latter was recognised when it was discovered that electrical stimulation of the periaqueductal grey (PAG) region of the brain caused significant analgesia in both rats and humans (Hosobuchi *et al.*, 1977). This system is summarised in Figure 1.1-2.

Electrical activity in small diameter primary afferent fibres of peripheral nerves is involved in the perception of noxious stimuli, or nociception. Afferent fibres are classified as A, B, or C-fibres with A-fibres further divided into subgroups α, β, δ and γ. Only the C- and Aδ-fibres carry nociceptive impulses (Markenson, 1996). They enter the spinal cord via the dorsal roots and terminate in the superficial region of the dorsal horn, generally in laminae I and II. The cells of the substantia gelatinosa in the lamina II are predominantly short inhibitory interneurons, which project to lamina I and V, and regulate transmission between the primary afferent fibres and the spinothalamic tract transmission neurons (Furst, 1999).

The sites at which opioids act to elicit analgesia are at the supraspinal, spinal and peripheral levels. Opioids act on the different opioid receptors at these sites; μ, δ and κ act at the spinal level, whilst μ acts at the supraspinal (Fields, 1993). Opioids act positively on neurones in the PAG matter and in the nucleus reticularis paragigantocellularis. These both project into the nucleus raphe magnus. 5-HT and enkephalin neurones flow to the dorsal horn to inhibit the spread of pain signals, where opioids also act directly. Noradrenergic innervation of the dorsal horn also occurs, having an inhibitory effect (Figure 1.1-2).

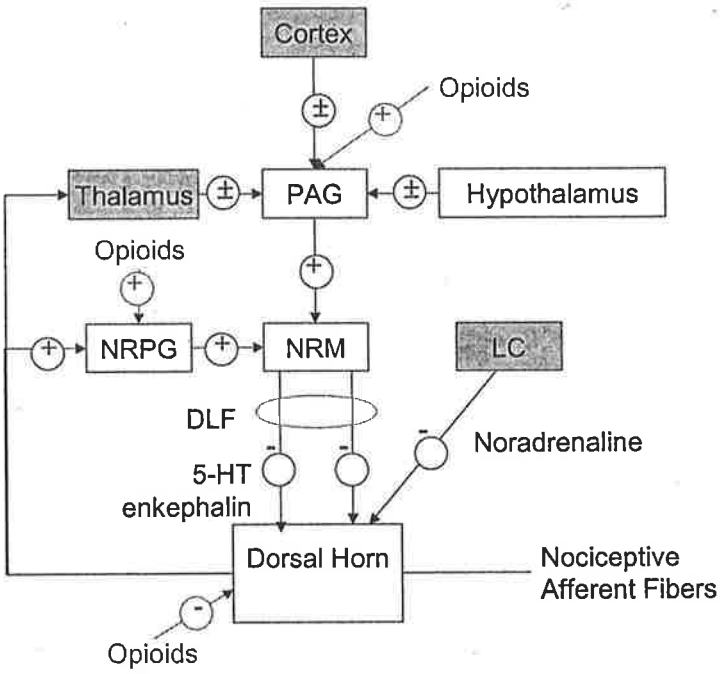


Figure 1.1-2 Descending inhibitory control system: hypothesised sites of action of opioids.

Adapted from Rang and Dale (1996). PAG - periaqueductal grey matter, NRPG - nucleus reticularis, NRM - nucleus raphe magnus and LC - the locus ceruleus.

Secondary messengers such as nitric oxide have also been implicated in morphine analgesia (Song *et al.*, 1998). Spinal nitric oxide is proposed to have a pro-nociceptive action. However, nitric oxide donors had no potentiating effect on intravenous morphine antinociception and did not produce antinociception alone. Other molecules have also been implicated in pain perception and include noradrenaline and serotonin, acetylcholine, GABA, the acidic amino acids glutamate and aspartate, substance P, neurotensin, somatostatin,

cytokines, neuropeptide Y, nerve growth factor, cholecystokinin and purines (Nozaki-Taguchi & Yamamoto, 1998).

1.1.4. Opioid tolerance

When an opioid is administered, tolerance to the drug develops and is typified by a decreased duration and intensity of action (Collett, 1998). Therefore, tolerance is defined as the state where an increase in dose is required to achieve a previously obtained pharmacological effect (Axelrod, 1968). Tolerance causes a reduction in the intensity and shortened duration of some, but not all effects of opioids, including analgesia, euphoria, sedation and respiratory depression. Tolerance to one opioid agonist often infers tolerance to other drugs of the same class. This is known as cross-tolerance (Plummer *et al.*, 1990). There are a number of different forms of tolerance, which include innate tolerance, acquired tolerance (pharmacokinetic and pharmacodynamic) and learned tolerances (behavioural and conditioned) (O'Brien, 1996). Innate tolerance refers to the genetically predetermined sensitivity to a drug that is evident upon first exposure. On the other hand, acquired tolerances are obtained following repeated administration of a drug. Pharmacokinetic tolerance involves altered metabolism and clearance (often increased) resulting in reduced concentrations of the drug. Pharmacodynamic tolerance results from a change in the organism that causes a reduced response to a given concentration of the drug, for example reduced receptor density. Learned tolerances result from compensatory mechanisms which are learned to cope with familial environments and cause a reduction in the observed drug affects.

The mechanisms underpinning tolerance have yet to be fully elucidated. Functional de-coupling of the opioid receptor from the G-protein has been postulated (Williams *et al.*,

2001). Others have suggested the down regulation of endogenous opioids and receptors, or the involvement of the N-methyl-D-asparate (NMDA) receptor (Elliott *et al.*, 1994). Nitric oxide and nitric oxide synthase have also been intensely researched due to their involvement in the development of tolerance (Aley & Levine, 1997; Dambisya & Lee, 1996; Kolesnikov *et al.*, 1993; Kolesnikov *et al.*, 1992; Pasternak, 1995) , however this area is poorly understood. Recently, spinal glia have been implicated in this opioid phenomena, but further research is required.

1.1.5. Opioid dependence and withdrawal (abstinence syndrome)

Physical dependence to opioids is revealed when an opioid agonist is abruptly discontinued or when an opioid antagonist, such as naloxone, is administered and withdrawal is precipitated. Withdrawal is typically evident as anxiety, irritability, chills and hot flushes, joint pain, lacrimation, rhinorrhea, diaphoresis, nausea, vomiting, abdominal cramps and diarrhoea. These symptoms are often the opposite of those experienced when using the opioid. The mildest form of withdrawal is similar to viral "flu-like" syndromes. For opioids with short half-lives, the onset of withdrawal symptoms can occur within 6 to 12 hours and peak at 24 to 72 hours after discontinuation (King & Miller, 1998). For opioids with longer half-lives, the onset of the abstinence syndrome may be delayed for 24 hours or more after drug discontinuation and may be of milder intensity (King & Miller, 1998).

In the late 1800s it was noted that opioid dependent patients had an increased susceptibility to infection (cited by Pacifici *et al.* (1994)). Since this time a significant body of evidence has been gathered demonstrating a clear link between the opioid and immune systems. To fully appreciate these links a background of the basic function of the immune system is required.

1.2. Infection and immunity

"The cells and molecules of the immune system act together in an exquisitely dynamic and adaptable network whose complexity rivals that of the nervous system"

Adapted from Kuby (1997)

The traditionally perceived role of the immune system is host defence and protection. These protections can be from foreign origins (e.g. parasite or bacteria) or self origins (e.g. cancer). The immune system is comprised of two parts: innate and acquired immunity. Innate immunity is non-specific and often provides the first line of defence. This "protective barrier" is often sufficient to protect the host from invading pathogens; however, if the pathogens evade or penetrate through the innate immunity, a specific immune response is mounted. These two components of the immune system do not operate independently; instead, they synergise producing a more effective defence system. A brief summary of the immunology related to this thesis follows and is based on the work of Kuby *et al.*, (1997) and Roitt *et al.*, (1997).

1.2.1. Innate immunity

The first barrier which invading pathogens encounter is that of innate immunity. There are a number of types of defence barriers. Firstly, there are anatomical barriers such as the skin and mucous membranes. These barriers block entry, inhibit the growth of pathogens and expel pathogens out of the body. Secondly, there are biophysiological barriers, which include temperature, low pH and defensive enzymatic systems as well as microflora. Thirdly, there are phagocytic and endocytic barriers such as cells which phagocytose and kill whole pathogens. Finally, there are inflammatory barriers, where tissue damage or infection causes

the release of signalling compounds which induce an influx of phagocytic cells to clear away the infection.

1.2.2. Acquired immunity

Acquired immunity requires the presence of a functional immune system capable of recognising and subsequently eliminating foreign pathogens or mutated self, for example cancer. This part of the immune system exhibits several characteristic attributes: specificity (the ability to detect very specific antigens); diversity (the ability to recognise many antigens); memory (the ability to "remember" previous encounters with antigens); and recognition (the ability to recognise an antigen as foreign and to respond to its presence). The acquired immune system is capable of specifically recognising highly specific structural shapes from many types of pathogens. It is able to carry out this function whilst still distinguishing between self and non-self.

1.2.2.1. The organs of the immune system

The cells of the immune system are unique in their characteristics, as they do not reside in a single organ; rather they circulate throughout the body between circulating blood, tissues and lymphatic system, lymphoid organs and back to the blood circulation. The recirculation of immune cells is a highly controlled process, which enables the cells to efficiently survey their environment for antigens of pathogens and non-self. The characteristics of the cells can change as they move from one physiological environment or location to another, optimising the function of the cell for the conditions. Furthermore, differentiation can also occur if the cells receive an activation signal.

The organs of the immune system can be divided into primary and secondary lymphoid organs. The primary lymphoid organs are the bone marrow and thymus as they are the sites of development and maturation of lymphocytes. The secondary lymphoid organs, lymph nodes and the spleen, are not crucial for the generation of lymphocytes, but are integral in providing an environment conducive to cellular maturation and presentation of antigens collected from the periphery. The organisation of the lymphatic system rivals that of peripheral neuronal projections, such that a majority of the body is drained by a complex network of lymphatics.

1.2.2.2. The cells of the immune system

The generation of an effective immune response involves two major cell types: lymphocytes and antigen presenting cells. These two cell types are derived from the same single cell type; the pluripotent stem cell found in the bone marrow and can be differentiated into two major lines - lymphoid and myeloid cell types. The antigen presenting cells are primarily of the myeloid lineage, with lymphocytes being of the lymphoid line. Lymphocytes leave the bone marrow and circulate through the blood and lymph systems. There are two major populations of lymphocytes, B cells and T cells.

1.2.2.3. B cells

The B cells leave the bone marrow with membrane bound antibody molecules. Antibodies are glycoproteins comprising two identical heavy and two identical light polypeptide chains joined by disulphide bonds. The cleft that is formed between the heavy and light chains forms the antigen-binding site. When a B cell first encounters an antigen, which recognises its antibody-binding site, it begins to divide rapidly. From this one cell comes other B cells,

which undergo further differentiation into memory B cells and plasma cells. Memory B cells have a longer life span and express membrane bound antibodies with the same specificity as the original cell. Plasma cells express large quantities of the soluble form of the antibody, which can then bind to the original activating antigen, immobilising it and making it an easy target for phagocytosis. This type of immune response is known as a humoral response.

1.2.2.4. T cells

The T cells that leave the bone marrow then migrate to the thymus where they undergo further maturation, during which time they express a distinctive antigen binding receptor. Unlike the B cell antibody which is able to bind to and recognise antigens independently, the T cell receptor can only recognise antigens which are associated with another cell membrane protein, known as the major histocompatibility complex (MHC). When a naive T cell (one which has not encountered antigens) engages antigens associated with a MHC molecule, it differentiates into memory T cells and numerous effector T cells.

There are two major populations of T cells that leave the thymus: T helper (T_H) and T cytotoxic (T_C) cells. These two populations are easily distinguished from one another by the presence of the membrane glycoproteins $CD4^+$ (found on T_H cells) and $CD8^+$ (found on T_C cells). These glycoproteins are in close proximity to, and associated with, the T cell receptor.

There are two forms of the MHC molecule: type I and type II. All nucleated cells in the body express the MHC I molecule. This complex presents a sample of the intracellular proteins which are present at any one time in the cell. Conversely, MHC II molecules are only expressed on specialised antigen presenting cells and these present antigens produced from the phagocytosis and subsequent macromolecule degradation. The difference in expression

and function of the MHC molecules results in different T cell responses. T_H can only recognise antigens in association with MHC II present on antigen presenting cells, whilst T_C cells only recognise antigens associated with MHC I.

Once T_H cells recognise and interact with the MHC II and antigen complex, the cell is activated and secretes numerous growth hormones, collectively known as cytokines. These cytokines are vital for the activation of T_C cells, macrophages, B cells and various other cells which take part in the immune response.

T_C cells, which recognise the MHC I and antigen complex can become activated (in the presence of T_H derived cytokines) and differentiate into cytotoxic T cells (CTL). These cells do not express cytokines, instead they lyse target cells expressing the MHC I and antigen complex. Therefore, T_C cells play a critical role in recognising viral and cancerous activity in all cell types, whilst T_H cells provide the "help" required to activate the immune system through the release of cytokines. These types of immune responses are known as cell mediated responses.

1.2.2.5. Natural Killer cells

Natural Killer cells are large granulated lymphocytes which are devoid of the membrane molecules which distinguish T cells and B cells. They play an important role in recognising and destroying tumour cells. In some cases, Natural Killer cells make direct contact with tumour cells in a non-specific manner. However, this process can also be mediated via anti-tumour antibodies bound to tumour cells which are recognised by receptors expressed on Natural Killer cells.

Natural Killer cells and T_C cells are able to destroy the target cells by the release of cytotoxic molecules such as perforin, degradative enzymes and cytokines.

1.2.2.6. Antigen presenting cells

Activation of the immune system requires cytokines released by T_H cells. It is essential that the activation of T_H cells be tightly controlled as an immune response to self could result in a fatal autoimmune disease. To ensure regulation of T_H cell activation, T_H cell can only recognise antigen associated with MHC II complex, which is expressed by the antigen presenting cells. These cells, which include macrophages, B cells and dendritic cells, have two vital properties. Firstly, they express MHC II and secondly they are capable of delivering co-stimulatory signals that are necessary for T_H cell activation.

1.2.2.7. Mononuclear cells

"Mononuclear cell" is the encompassing term for phagocytes. These cells are extremely important in the first line of defence against invading pathogens, and in activation of the immune system by the release of signal hormones (cytokines) and co-stimulation required for T_H cell survival. The mononuclear cells exist in two forms; in the systemic system, monocytes circulate around the body, these cells then undergo extravasation, that is, they move from the capillary into the surrounding tissue, where they then reside and undergo further differentiation into macrophages. Some macrophages move into certain tissues and become fixed, whereas others remain free. The free macrophages move by amoeboid movement through the tissues. The fixed macrophages serve different functions in different tissues and are named to reflect their location: alveolar macrophages in the lung; microglial

cells in the brain; kupffer cells in the liver; histiocytes in the connective tissue; and mesangial cells in the kidney.

Macrophages are very important in the phagocytosis and killing of invading pathogens and presentation of the degraded products to the rest of the immune system (acting as antigen presenting cells). They are able to kill cells by phagocytosing them, then breaking down their cell membrane using reactive oxygen species or peptides which punch holes in the membrane (defensins) or by the release of signalling hormones which cause cell death.

1.2.2.7.1. Macrophage derived signalling hormones

The immune system has a very complex signalling communication system which consists of low molecular weight proteins called cytokines. The cytokines secreted from macrophages and other cell types are vital for the activation of the immune system. Interleukin 1 (IL-1) acts on T_H cells as a co-stimulatory factor to promote survival and subsequent proliferation. Initially the actions of cytokines were thought to be limited to the immune system, however, they have now been shown to have far wider effects. For example, IL-1 also acts on the thermoregulatory centre in the hypothalamus, leading to the fever response (Saigusa, 1990). Other cytokines and factors, which are secreted by macrophages, include:

Cytokine	Major Function
IL-1	Activation of inflammation
Interferon alpha (IFN-α)	Causes an antiviral state in uninfected cells
Tumour necrosis factor (TNF-α)	Kills tumour cells
IL-6	Promotes proliferation and differentiation
Complement proteins	Bind to invading pathogen's cell walls and cause lysis, and enhance phagocytosis

1.2.3. Cytokines and Chemokines

Cytokines are, in general, soluble low molecular weight polypeptide hormones (proteins or glycoproteins), which are secreted from cells to affect the growth and metabolism of the same cell (autocrine) or of another cell (paracrine) and in some cases even of a cell in another region of the body (endocrine). Cytokines bind to specific receptors on the surface of target cells that are coupled to intracellular signalling and second messenger pathways. Cytokines have been grouped into families based on homology of the amino acid sequence, chromosomal location, and in some cases functional homology. Usually the name of the family is based on the initial determination of the structure of the first member. These families include interleukin (IL), chemokine, interferon (IFN), colony stimulating factor (CSF), transforming growth factor (TGF), tumour necrosis factor (TNF) and heregulin. Many cytokines have now been identified in these families with many more still to be discovered. The actions of some of the more common cytokines are summarised in Table 1.2-1 and a schematic diagram of the network of communication between immune cells is shown in Figure 1.2-1. This table and figure will also be of use for future reference when opioid immunomodulation is discussed.

Cytokine receptors exhibit high affinity with dissociation constants ranging from 10^{-10} to 10^{-12} M. Due to this high affinity, very low concentrations of cytokines can cause a biological effect. Chemokines are a family of low molecular weight cytokines. These compounds act as chemoattractants and act by binding to cell membrane receptors and activating G-proteins. Cytokine expression and action were originally thought to be limited to the immune system. However, the cell types which are found to express cytokines and their receptors is steadily growing and will be discussed in greater detail later in this chapter.

Table 1.2-1 Summary of some common cytokines, their producers and primary functions

Cytokine	Predominant producer cells	Major functions
IL-1α & β	Monocytes and many other cells	Activation of T cells and inflammatory cells
IL-2	T cells	Activation and proliferation of T cells, pro-monocytes, NK cells
IL-3	T cells	Differentiation and propagation of early myeloid progenitor cells
IL-4	T cells	Activation and proliferation of T cells, B cells; inhibition of macrophage activation
IL-5	T cells	Proliferation of B cells; maturation of eosinophils; inhibition of macrophage activation
IL-6	Macrophages and many other cells	Activation of B cells, T cells and other cells
IL-7	Stromal cells	Maturation of T cells and B cells
IL-8	Monocytes	Chemotaxis and activation of granulocytes; chemotaxis of T cells
IL-9	T cells	Propagation of mast cells, megakaryocytes
IL-10	T cells	Propagation of mast cells; inhibition of cellular immune reactions
IL-11	Stromal cells	Maturation of lymphocytes; proliferation of myeloid progenitor cells
IL-12	Monocytes	Activation of T cells, Natural Killer cells
IL-13	T cells	Activation and proliferation of B cells, inhibition of cellular immune reactions
IL-14	T cells	Activation of B cells
IL-15	Epithelial cells, T cells	Activation and proliferation of T cells, Natural Killer cells
IL-16	T cells	Chemotaxis of T cells
IFN-α	Monocytes	Induction of antiviral activity; inhibition of tumour cell growth; activation of cells
IFN-β	Fibroblasts	
IFN-γ	T cells	Induction of antiviral activity; activation of macrophages; immunoregulation
TNF-α	Monocyte and many other cells	Activation of many cells; induction of apoptosis cachexia; shock
TNF-β	T lymphocytes	

T_H cells are also a major source of cytokines and therefore play a central role in the cytokine network. Two subpopulations of T_H cells exist that produce different cytokines and hence, have differing biological activity, T_H1 and T_H2. The roles of the T_H1 and T_H2 cells also differ substantially with T_H1 cells responsible for activation of T_C cells and delayed-type hypersensitivity, whilst T_H2 cells are primarily involved in B cell helper functions.

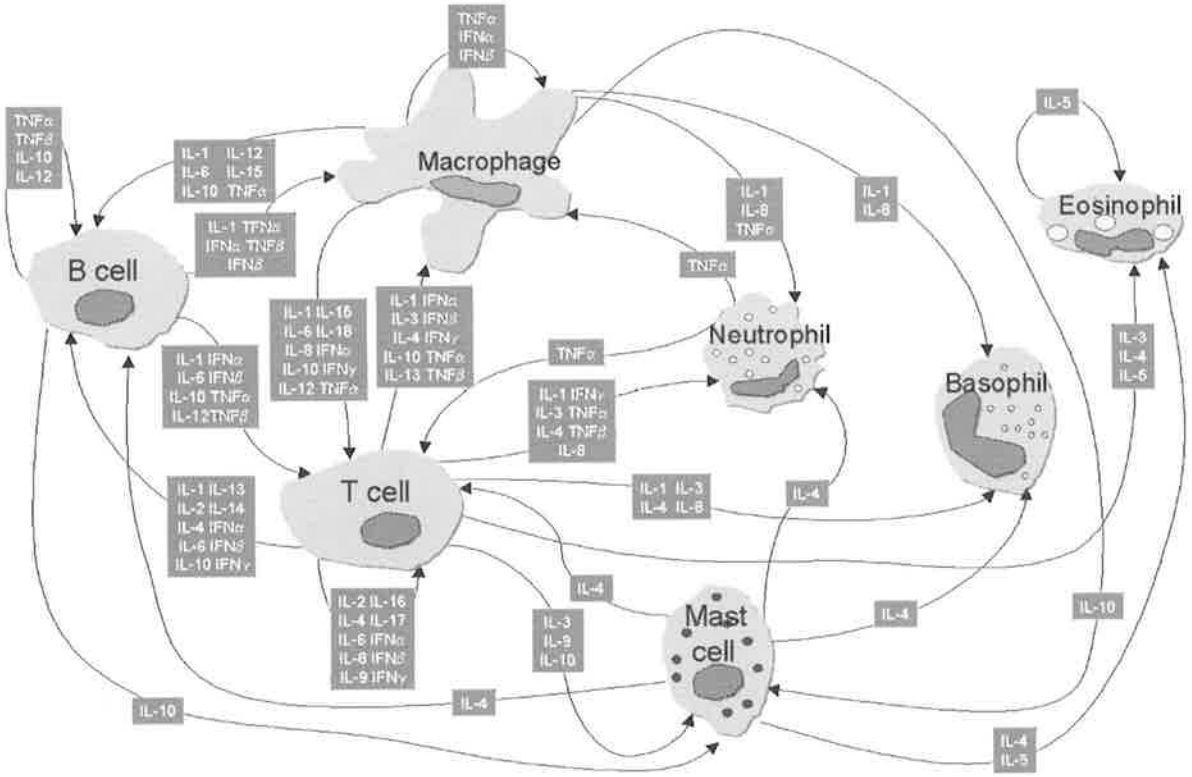


Figure 1.2-1 Schematic diagram of the network of cytokine communication between immune cells, demonstrating the complexity of the signalling pathways.

Summarised from Kuby (1997) and Roitt (1997).

Now with a greater appreciation and understanding of both the immune and opioid systems, the link between these two systems can be discussed.

1.3. Immune opioid receptors

In 1979, Wybran *et al.* (1979) demonstrated a clear link between the immune and opioid systems. They showed that *in vitro* morphine was able to inhibit T cell rosetting (the binding of sheep red blood cells to the surface of T cells which can be observed under a light microscope) in a naloxone reversible manner at low concentrations (10 nM). Subsequently, numerous research groups have used a plethora of both *in vitro* and *in vivo* infection and immunity models to investigate opioid-induced alterations in immune function. For opioids to exert a "classical" neuronal effect on immunocompetent cells, receptors that bind opioid ligands must be expressed by the target cells (Madden *et al.*, 1998). Discovering and understanding the cell membrane receptor(s) and associated secondary messenger system(s) will allow greater insight into the intended action of opioids on the immune system.

As previously described, μ, κ and δ opioid receptors are seven transmembrane G-proteins. These are subsequently negatively associated with adenylate cyclase (Childers, 1993). These classical opioid receptors display saturable stereospecific binding which can be blocked by selective opioid receptor subtype antagonists (Sibinga & Goldstein, 1988). Opioid agonist binding is also altered by sodium ions, whilst antagonist (naloxone) binding is unaffected. Guanine nucleotides inhibit binding of agonists, reducing agents decrease the binding affinity, and dissociation constants are in the low nanomolar range. A review of the available immune opioid receptor expression follows. For further information on immune opioid receptors refer to quality reviews published in this area (Bidlack, 2000; Carr & Blalock, 1989, 1990; Carr *et al.*, 1991b; Hedner & Cassuto, 1987; Madden *et al.*, 1998; McCarthy *et al.*, 2001b; Sharp, 1996, 2001; Sharp *et al.*, 1998b; Sibinga & Goldstein, 1988).

1.3.1. μ opioid receptor

1.1.1.1. μ opioid receptor ligand receptor binding

The first evidence of the binding of opioid ligands to receptors expressed by human immunocompetent cells that resembled the μ opioid receptor was reported by Lopker *et al.* (1980), who found stereoselective binding of dihydromorphine to freshly isolated human peripheral blood monocytes (K_d = 10 nM) and granulocytes (K_d = 8 nM). These data were supported by Mehrishi *et al.* (1983) and Falke *et al.* (1985). Mehrishi *et al.* (1983) demonstrated tritiated naloxone binding to peripheral blood mononuclear cells, which was displaced by morphine under low sodium concentration conditions, thereby agreeing with neuronal opioid receptor data, suggesting the expression of a classical opioid receptor. Falke *et al.* (1985) found freshly isolated human polymorphonuclear leukocytes bound naloxone, diprenorphine and ethylketocyclazocine. Moreover, naloxone binding was stereoselective, again asserting the possible presence of classical opioid receptors on immune cells. Interestingly, there was no specific binding of the μ and δ opioid ligand DADLE to the isolated cells, giving the first indication that these receptors expressed by the immune system were not identical to their neuronal counterparts, as they displayed uncharacteristic ligand selectivity (Falke *et al.*, 1985).

Specific human immune cell populations were investigated for their opioid receptor expression, partially to increase the sensitivity of the binding assays, due to the high background noise, but also to characterise opioid ligand binding to individual cell types. Madden *et al.* (1987) found purified T cells from human peripheral blood had a specific binding site for naloxone. The mean K_d was about 50 nM for three subjects, whilst one subject had a lower K_d = 35 nM. Interestingly, the B_{max} varied greatly amongst all subjects

with almost a 5-fold range of values. The bound naloxone was partially displaced by various opiate agonists, including morphine (56%), β-endorphin (61%), met- (40%) and leu-enkephalin (40%), DADLE (78%) and DADL-enkephalinamide (66%), but was not displaced by levorphanol or dextrorphan, thereby displaying ligand selectivity (Madden *et al.*, 1987). Interestingly, Kay *et al.* (1990) found that naloxone binding to phytohemagglutinin-stimulated cells was antagonised by both the classical opioid ligand β-endorphin and the cytokine IL-2. The antagonist activity of IL-2 provided tantalising evidence for bi-directional communication between the opioid and immune systems.

In subsequent studies Madden *et al.* (1996) found no measurable morphine binding sites on peripheral T cells freshly isolated from human peripheral blood, despite their previous findings (Madden *et al.*, 1987). However, 24 hour stimulation of these cells with phytohemagglutinin produced a measurable, saturable morphine binding site. Further incubation for up to 72 hours produced a higher affinity-binding site, which could be further enhanced by stimulation with IL-2, in contrast to the findings for δ opioid receptors discussed later (1.1.1.10, Sharp *et al.* (1997)). Morphine was displaced by naloxone, β-endorphin and morphiceptin, but not by DAMGO, dextrorphan or levorphanol (Madden *et al.*, 1993; Madden *et al.*, 1996), once again demonstrating opioid ligand selectivity. The binding of morphine to the high affinity inducible site was optimal at NaCl concentrations of 150 mM, which was significantly different to neuronal μ opioid receptors where optimal binding occurs at less than 50 mM NaCl (Madden *et al.*, 1996). Interestingly, ligand binding was inhibited by 2-mercaptoethanol and glutathione (sulphydryl reagents) unlike central μ opioid receptors, but dithiothreitol (disulphide splitting reagent) and trypsin also inhibited ligand binding confirming the protein nature of the binding site (Madden *et al.*, 1993; Madden *et al.*, 1996).

These controversial findings confirmed the hypothesis that immunocompetent cells express an opioid-like, although non-classical opioid receptor.

In contrast to the above reports, Palm *et al.* (1996) were unable to detect tritiated morphine, DAMGO, DPDPE or U69593 binding to membranes of phytohemagglutinin- and IL-1-stimulated human peripheral blood mononuclear cells. Likewise, Mendelsohn *et al.* (1985) were unable to detect specific tritiated naloxone binding to human mononuclear cells (B and T lymphocytes and monocytes). The reasons for these discrepancies may lie in experimental differences, as the background noise associated with opioid ligand binding to immunocompetent tissue was very high (Madden *et al.*, 1993).

From the available human data, there is evidence for a non-classical opioid receptor whose expression by isolated peripheral blood mononuclear cells is altered by mitogenic stimulation and has uncharacteristic opioid ligand selectivity. However, due to likely experimental difficulties the site is hard to detect using ligand binding assays, possibly leading to the conflicting reports in the literature. The availability of animal immune tissues has allowed greater investigation of opioid receptor expression in these ligand binding assays.

Using a rat model, Ovadia *et al.* (1989) demonstrated that splenocytes but not thymocytes possessed specific and displaceable binding sites for tritiated naloxone, which was increased by mitogen stimulation in splenocytes only, with B cells shown not to bind naloxone. These data indicated that the naloxone binding receptor expression is cell- and compartment-specific since Ovadia *et al.* (1989) found expression on splenocytes but not thymocytes. They found that labelled naloxone binding was displaced by morphine and unlabelled naloxone but not with leu-enkephalin or β-endorphin. This opioid ligand selectivity also was observed in human tissue (mentioned previously and discussed in detail later in the chapter, 1.1.1.5.1).

Binding was also profoundly inhibited by the presence of NaCl (120 mM) and GTPγs. GTPγs inhibition of binding is consistent with the receptor being coupled to a G-protein, whilst inhibition of naloxone binding by high NaCl is consistent with neuronal opioid receptors, but differs from the findings of Madden *et al.* (1993) discussed previously.

Unlike Ovadia *et al.* (1989), Roy *et al.* (1991) demonstrated low affinity morphine binding (K_d = 50 nM) by stimulated (IL-1 and phytohemagglutinin) murine thymocytes and even lower affinity binding (K_d = 93 nM) by unstimulated cells (Roy *et al.*, 1992). As the cell proliferation increased so did morphine binding. However, morphine binding was non-stereoselective, indicating expression of a non-classical opioid binding site. IL-1 alone was not able to increase the binding of morphine to cell membrane preparations which is contrary to the previous findings of Wiedermann *et al.* (1989) and Ahmed *et al.* (1985) (discussed in greater detail later in this chapter, 1.1.1.17). IL-1 stimulation alone was able to increase tritiated morphine binding, although only to about half of the combination of phytohemagglutinin and IL-1, or phytohemagglutinin stimulation alone. Treatment of the cells with phorbol myristate acetate, which activates protein kinase C, mimicked IL-1 and phytohemagglutinin-induced increases in morphine binding. These data suggest that IL-1 may enhance morphine binding via a second messenger system. However, direct phosphorylation of the necessary proteins by phorbol myristate acetate causing direct enhancement of morphine binding site expression cannot be ruled out (Roy *et al.*, 1991). Morphine binding was also sensitive to cations. Most notably binding was inhibited by Mg^{2+}, Ca^{2+}, Mn^{2+} and Cl^-, which is unlike the pattern of sensitivity seen for brain opioid receptors (Roy *et al.*, 1992). These differences again implicate a non-classical opioid receptor. Roy *et al.* (1992) also found an intriguing rank order of displacement by various opioid ligands of tritiated morphine from activated thymocyte membranes (primarily associated with $CD4^+$ cell population) (IC_{50}): (+)-morphine (active) (30 nM) > (-)-morphine (inactive) (50 nM) >

ethylketocyclazocine (100 nM) = etorphine (100 nM) = diprenorphine (100 nM) = dihydromorphinone (100 nM) = nalbuphine (100 nM) > naloxone (1 μM) > dynorphin (1-13) (5 μM) > β-endorphin = DPDPE = DAMGO (10 μM) > U50488 (> 1 mM) (Roy *et al.*, 1992). Hence, Roy *et al.* (1992) found non-stereoselective displacement by morphine and also found that alkaloid ligands displaced tritiated morphine more than opioid peptide ligands. The receptor characterised by Roy *et al.* (1992) is unlike the "classical" neuronal opioid receptors expressed centrally which suggests that opioid ligand binding to this site may result in substantially different responses to those anticipated based on neuronal opioid receptor binding data.

In an attempt to circumvent experimental problems associated with detecting ligand binding, different techniques have been employed to characterise opioid binding sites on immune cells. Carr *et al.* (1988a) raised an antibody that recognised opiate receptors on cells of the immune system, which blocked specific binding of tritiated dihydromorphine, β-endorphin, met-enkephalin and naloxone to receptors on murine splenocytes. Using fluorescein isothiocyanate (FITC)-labelled naltrexone (addition of FITC at the 6 position) Lang *et al.* (1995) found low levels of specific binding to rat lymphocytes, monocytes and neutrophils. DAMGO, DADLE and unlabelled naltrexone blocked FITC-labelled naltrexone binding, thereby demonstrating that low levels of each drug bound to each of the cell populations that were analysed (lymphocytes, monocytes and neutrophils). Patrini *et al.* (1996) established that mouse splenic T and B cells and macrophages have specific naloxone binding sites. Interestingly, following subcutaneous injection, *in vivo* morphine (20 $mg.kg^{-1}$) or methadone (12.5 $mg.kg^{-1}$) caused decreased splenocyte naloxone binding 20 minutes, 1 and 3 days after injection, in a naltrexone-sensitive manner (administered *in vivo*). The effect of morphine and methadone was similar, although each cell population responded differently. This study did

not determine whether the reduced naloxone binding caused by *in vivo* exposure to morphine and methadone was due to the direct action of the administered opioid at the immunocompetent cell level or via central pathways. Nor did it ascertain the physiological significance of the reduced naloxone binding, although this is undoubtedly important.

Other studies expanded the range of μ opioid receptor ligands shown to bind to splenocytes. Wu *et al.* (1999) found tritiated ohmefentanyl (a high affinity μ opioid receptor agonist), bound to a single naloxone-sensitive site on murine splenocytes, although the stimulation status of the cells is unclear. Radulescu *et al.* (1991) demonstrated tritiated BIT (2-(p-ethoxy-benzyl)-1-[N,N-diethylamino]-ethyl-5-isothiocyanatobenzimidazole) binding to splenic lymphocytes and gave a rank order of potency for competing ligands as: BIT > etonitazine > naltrindole > (-)-naloxone > U69593 > CYCLOFOXY (17-cyclopropylmethyl-3,14-dihydroxyl-4,5-α-epoxy-6-β-fluoromorphinan) > oxymorphindole = (+)-naloxone. These data curiously showed that the δ opioid receptor antagonist naltrindole was more effective than naloxone at displacing BIT, which is a μ opioid receptor ligand. Furthermore, the κ opioid ligand U69593 was effective at displacing BIT from the splenocyte binding site, but BIT selective opioid receptors in the brain were insensitive to U69593, suggesting a unique opioid binding site on splenocytes (Radulescu *et al.*, 1991). In contrast to the effectiveness of U69593, Roy *et al.* (1992) found U50488 was the least effective at displacing morphine from thymocyte membranes, indicating a possible difference between splenocyte and thymocyte opioid binding receptors. Roy *et al.* extended their search for μ ligand binding receptors to tumour cell lines. Using the murine macrophage cell line Bac 1.2F5, Roy *et al.* (1996) found a low affinity morphine binding site. The site displayed non-classical opioid characteristics, as both stereoisomers of morphine had similar IC_{50} values, ((+)-morphine (active) 50 nM, (-)-morphine (inactive) 70 nM) which agreed with their previous findings using activated

thymocytes (Roy *et al.*, 1992). Moreover, the relative potency of various opioid compounds at displacing labelled morphine was: DADLE > β-endorphin = naloxone > diprenorphine > etorphine. κ opioid ligands were the least potent in competing for morphine binding. In contrast to Roy *et al.* (1996), Shapira *et al.* (1997) found no displacement of the non-selective opioid diprenorphine by DADLE using the mouse thymoma cell lines R1.1 and R1.EGO, thereby demonstrating opioid peptide insensitivity.

The data reported by Patrini *et al.* (1996) for morphine binding are in partial agreement with Ovadia *et al.* (1989) who demonstrated that concanavalin A-activated splenocytes but not thymocytes possess specific and displaceable binding sites for tritiated naloxone. However, unlike Patrini *et al.* (1996) who showed B cells bound fluorescently-labelled naloxone, Ovadia *et al.* (1989) saw no decrease in tritiated naloxone binding in B cell depleted splenocytes. This tritiated naloxone binding was displaced by morphine and naloxone but not by opioid peptides, which is consistent with data for different opioid ligands (Falke *et al.*, 1985; Ovadia *et al.*, 1989; Shapira *et al.*, 1997). Opioid binding sites were similar in freshly isolated human peripheral blood lymphocytes (Falke *et al.*, 1985; Lopker *et al.*, 1980; Mehrishi & Mills, 1983) and displayed classical opioid characteristics. However, when individual immune cell populations were examined, differences between morphine and naloxone binding appeared (Madden *et al.* (1987) compared to Madden *et al.* (1996), and Ovadia *et al.* (1989) compared to Roy *et al.* (1991)). Naloxone binding displayed more classical opioid characteristics than the morphine binding data. Therefore, it is possible that naloxone and morphine were binding to two distinct receptors with some cross reactivity, as evident by displacement data. Alternatively, they may have been binding to a single receptor, antigenically similar to neuronal opioid receptors whose agonist and antagonist specificity is different to its neuronal counterparts, as discussed previously. Due to these and other

unanswered questions, the search for the expression of μ opioid receptor mRNA and protein by immunocompetent cells began.

1.1.1.2. μ opioid receptor mRNA expression

Mu opioid receptor mRNA has been successfully isolated from a variety of human and animal immune tissues resulting in much conflicting data (summarised in Table 1.3-1). Using the same primers Chuang *et al.* (1995b), Chang *et al.* (1998a) and Suzuki *et al.* (2000) found expression of μ opioid receptor mRNA in numerous immune cell types. Interestingly, Suzuki *et al.* (2000) found that after incubation with 10 μM morphine, μ opioid receptor mRNA levels increased 350% at 12 hours in a naloxone-sensitive fashion, but returned to control levels after this time. These data partially agree with opioid binding data in 1.1.1.1, although the *in vitro* effect of morphine exposure to increase μ opioid receptor mRNA expression does not concur with the *in vivo* data of Patrini *et al.* (1996) who demonstrated reduced morphine and methadone binding just 20 minutes after the injection. However, the results potentially highlight the differences between direct and indirect opioid effects on immune cells.

In contrast to the positive identification of μ opioid receptor previously mentioned, Gaveriaux *et al.* (1995) found no μ opioid receptor mRNA in a wide range of cell types, even after stimulation with phytohemagglutinin and concanavalin A. Interestingly, using the same primers as Gaveriaux *et al.* (1995), Makarenkova *et al.* (2001) also found no μ opioid receptor mRNA in dendritic cells derived from human peripheral blood mononuclear cells or untreated immature cells, but detected μ opioid receptor mRNA in murine bone marrow-derived cultured dendritic cells, suggesting species and tissue source are important determinants of μ opioid receptor mRNA expression. The mRNA expression was significantly increased

(300%) in TNF-α activated mature cells compared to untreated immature cells, implicating the μ opioid receptor in activation or control of the response elicited by TNF-α (Makarenkova *et al.*, 2001).

Table 1.3-1 Summary of μ opioid receptor mRNA expression in immune tissues

Cell type	Species	Expression	Study
B cell line (25-9-17S)	Mouse	✗	Gaveriaux *et al.* (1995)
B cell line (CH27)	Mouse	✗	Gaveriaux *et al.* (1995)
B cell line (EBV)	Human	✗	Gaveriaux *et al.* (1995)
B cell line (Raji)	Human	✓	Chuang *et al.* (1995b)
Dendritic cells	Human	✗	Makarenkova *et al.* (2001)
Dendritic cells	Murine	✓	Makarenkova *et al.* (2001)
Lymph node cells	Mouse	✗	Gaveriaux *et al.* (1995)
Lymph node cells	Porcine	✗	Pampusch *et al.* (1998a)
Macrophage cell line (J774)	Mouse	✗	Gaveriaux *et al.* (1995)
Macrophage cell line (P388d_1)	Mouse	✗	Gaveriaux *et al.* (1995)
Macrophages – Alveolar	Porcine	✗	Pampusch *et al.* (1998a)
Microglial cells – Foetal	Human	✓	Chang *et al.* (1998a)
Monocyte cell line (U937)	Human	✗	Gaveriaux *et al.* (1995)
Monocytes/macrophages	Human	✓	Chuang *et al.* (1995b)
Neutrophils	Porcine	✗	Pampusch *et al.* (1998a)
Peripheral blood mononuclear cells	Human	✗	Gaveriaux *et al.* (1995)
Peripheral blood mononuclear cells	Monkey	✓	Chuang *et al.* (1995b)
Peripheral blood mononuclear cells	Monkey	✓	Suzuki *et al.* (2000)
Peripheral blood mononuclear cells	Porcine	✗	Pampusch *et al.* (1998a)
Peritoneal macrophages	Rat	✓	Sedqi *et al.* (1995)
Peyer's patch cells	Porcine	✓	Pampusch *et al.* (1998a)
Peyer's patch cells – Ileal	Porcine	✗	Pampusch *et al.* (1998a)
Peyer's patch cells – Jejunal	Porcine	✗	Pampusch *et al.* (1998a)
Polymorphonuclear cells	Human	✓	Chuang *et al.* (1995b)
Polymorphonuclear cells	Monkey	✓	Chuang *et al.* (1995b)
Splenocytes	Mouse	✗	Miller *et al.* (1996)
Splenocytes	Mouse	✗	Gaveriaux *et al.* (1995)
Splenocytes	Porcine	✗	Pampusch *et al.* (1998a)
T and B cell hybrid (CEM×174)	Human	✓	Chuang *et al.* (1995b)
T and B cell hybrid (CEM×174)	Human	✓	Suzuki *et al.* (2000)

Cell type	Species	Expression	Study
T cell line - immature (CEM)	Human	✖	Gaveriaux *et al.* (1995)
T cell line - immature (HSB2)	Human	✖	Gaveriaux *et al.* (1995)
T cell line - immature (MOLT-4)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (11.10)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (CTLL)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (EL-4)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (Jurkat)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (X63-IL-4)	Mouse	✖	Gaveriaux *et al.* (1995)
T cells ($CD4^+$)	Human	✓	Chuang *et al.* (1995b)
Thymocytes	Mouse	✖	Sedqi *et al.* (1996)
Thymocytes	Porcine	✓	Pampusch *et al.* (1998a)
Thymocytes	Porcine	✖	Pampusch *et al.* (1998a)

✓ indicates a positive expression result, whilst ✖ indicates a negative expression result was obtained.

Although the data are conflicting, they support the hypothesis raised previously of a non-classical opioid receptor when discussing the μ opioid receptor ligand binding data (1.1.1.1). To further characterise the receptor that binds opioids expressed by immunocompetent cells, the translation of mRNA into peptides and expression of membrane bound receptors has been assessed.

1.1.1.3. μ opioid receptor protein/western blot analysis

Translation of the μ opioid receptor mRNA to proteins in immunocompetent cells was detected by Western Blot analysis using antibodies raised against the neuronal μ opioid receptor (Suzuki *et al.*, 2000). A single band of approximately 50 kDa was detected in protein isolated from concanavalin A-stimulated monkey peripheral blood mononuclear cells and from cultured CEM×174 cells (T cell and B cell hybrid). As previously mentioned for mRNA, Suzuki *et al.* (2000) demonstrated increased μ opioid receptor protein levels in CEM×174 cells which correlated with the increased mRNA levels following 24 hour incubation with 10 μM morphine. This response was antagonised by pre-treatment with

naloxone. Morphine incubation with monkey peripheral blood mononuclear cells also increased levels of μ opioid receptor protein after 24 hours (10 nM and 10 μM morphine) of a 36 hour (10 μM morphine) incubation. These data conflict with previously presented mRNA expression data but agree with ligand binding evidence. In contrast, Makarenkova *et al.* (2001) found no μ opioid receptor protein using dendritic cells derived from human peripheral blood mononuclear cells, untreated immature cells, and murine bone marrow-derived cultured dendritic cells.

Using a different method, Radulescu *et al.* (1991) found tritiated BIT irreversibly labelled a surface protein with an apparent molecular weight of 58 kDa from enriched B and T cell populations. Binding was blocked completely by unlabelled BIT and etonitazine, whilst binding was partially blocked by U69593 (72%), DAMGO (47%), CYCLOFOXY (a fluorinated analogue of naltrexone) (30%), (-)-naloxone (30%) and was unaffected by (+)-naloxone. Interestingly, the δ opioid receptor agonist oxymorphindole augmented BIT binding by almost 100%, although this ligand competed for the BIT binding site very poorly (1.1.1.1, Radulescu *et al.* (1991)). A similar augmentation of BIT binding by oxymorphindole was obtained for brain derived μ opioid receptors. The δ opioid receptor antagonist naltrindole reduced BIT binding, whilst δ ligands DPDPE, *cis*-(+)-3-methylfentanylisothiocyanate (SUPERFIT) and leu-enkephalin and the κ ligand dynorphin A had no effect. The significance of the interaction between ligands is not clear, however, as with the mRNA data, these protein data lack unequivocal proof of the expression of a classical μ opioid receptor by immunocompetent cells.

1.1.1.4. μ opioid receptor knockouts (*in vivo* influence)

In an attempt to identify the physiological significance of the effect of opioids on the immune system, knockout models were employed. Gaveriaux-Ruff *et al.* (1998) showed that immune function in mice lacking the μ opioid receptor gene and hence the μ opioid receptor was not affected by chronic daily morphine injections. Wildtype and knockout mice had identical basal immune parameters indicating that the loss of the μ opioid receptor had no effect on the development of a functional immune system. In wildtype animals, morphine administration induced atrophy of both the spleen and thymus. There was no change in the relative proportions of $CD4^+$ and $CD8^+$ T cells, although there was an overall reduction in the number of immune cells, with the percentage of double-positive $CD4^+CD8^+$ thymocytes diminished. This may be due to apoptosis of double positive cells after chronic morphine treatment. Natural Killer cell activity was also significantly depressed in wildtype animals. None of these effects were present in μ opioid receptor knockout mice, indicating the importance of the μ opioid receptor in morphine-induced immunomodulation. However, these data do not indicate at which level morphine was acting: centrally or directly via opioid receptors expressed on immunocompetent cells.

Using μ opioid receptor knockout mice implanted with a morphine pellet for 48 hours, Roy *et al.* (1998a) investigated a number of immune functions. In contrast to Gaveriaux-Ruff *et al.* (1998), Roy *et al.* (1998a) demonstrated that chronic morphine treatment resulted in reduced thymic and splenic weight in both sets of mice, with naltrexone only partially reversing this effect in both animal groups. A reduction in IL-2 secretion by T cells and proliferation of splenic and thymic cells from both sets of mice were also observed, however the baseline proliferation of either thymic or splenic T cells was less in the knockout mice compared to the

wildtype (Roy *et al.*, 1998a). Morphine induced a naltrexone-sensitive decrease in phagocytic activity of peritoneal macrophages from wildtype animals implanted with morphine pellets, whilst μ opioid receptor knockout mice only had a non-significant decrease in phagocytic activity from wildtype. Macrophages from μ receptor knockout animals implanted with morphine pellets, displayed a reduction in lipopolysaccharide-induced IL-1 and IL-6 secretion which was similar to that observed in wildtype animals. Interestingly, TNF-α secretion by μ opioid receptor knockout animals was increased (10%), whilst morphine caused a decrease (45%) in TNF-α production in wildtype animals. As previously mentioned, binding studies conducted on knockout mice demonstrated that only unlabelled morphine was able to displace labelled morphine from the binding site, whilst DAMGO, DPDPE and U50488 had no effect (Roy *et al.*, 1998a). Based on these results it appears that the effects of morphine on phagocytosis and TNF-α secretion are mediated through the μ opioid receptor, since these effects were not seen in knockout mice (Roy *et al.*, 1998a), although these results disagree with Gaveriaux-Ruff *et al.* (1998). However, the reduction in thymic and splenic weight, inhibition of proliferation, IL-2 synthesis, and IL-1 and IL-6 secretion appear to be mediated via a naloxone-insensitive morphine receptor. It is possible that some of these effects are mediated via other opioid receptor subtypes such as δ or κ.

In further studies, Roy *et al.* (2001b) used μ opioid receptor knockout and wildtype animals that received either a 48 hour exposure to placebo and morphine pellets or intracerebroventricular injections of μ, κ or δ antagonists. Morphine treatment resulted in a 5-fold increase in plasma corticosterone concentrations following pellet implantation in the wildtype animals. However, in the μ opioid receptor knockout mice there was no increase in plasma corticosterone following morphine administration, although the concentrations in these mice were higher at baseline compared to wildtype. The μ opioid receptor antagonist

CTOP, was able to inhibit the increase in plasma corticosterone following morphine pellet implantation, whilst the δ (naltrindole) and κ (nor-binaltorphimine) opioid receptor antagonists did not antagonise this response. Therefore, the increase in corticosterone concentration following morphine treatment is likely to be mediated by the μ opioid receptor since the response was not seen in μ opioid receptor knockout animals and only the μ opioid receptor antagonists caused inhibition of this response in the wildtype group (Roy *et al.*, 2001b). Thymic and splenic weights were reduced following morphine pellet implantation, which was only partially reduced (~28%) by naltrexone in wildtype animals as had been observed in their previous study (Roy *et al.*, 1998a). However, contrary to their previous findings only a 28% reduction in thymic weight was observed in the μ opioid receptor knockout animals and this was not to the same extent as seen in wildtype animals (85%) and furthermore, this reduction was not reversed by naltrexone. Therefore, the 28% reduction in thymic weight observed in μ opioid receptor knockout animals was not mediated by the μ opioid receptor and was independent of corticosterone, and resulted from action via a naloxone-insensitive morphine binding site (Roy *et al.*, 2001b). Injection of dexamethasone alone induced a 40% reduction in thymic weight in both animal types. Moreover, addition of morphine caused the response to increase to 95% in wildtype animals and 68% in μ opioid receptor knockout animals. Therefore, an intact opioid receptor is still required to obtain the maximal reduction in thymic weight. The location of the μ opioid receptor expression was not established as the effect may be mediated via neuronal sympathetic innervation of the thymus, which is inhibitory when activated (Vizi *et al.*, 1995). These data confirm the results of previous studies showing a naloxone-insensitive morphine-binding site on immune cells. However, they contradict the findings of a similar knockout animal study by Gaveriaux-Ruff *et al.* (1998) who showed the immune systems of μ opioid receptor knockout animals were not affected by morphine. Nonetheless, these data do reiterate the existence of

physiologically relevant and functionally active non-classical opioid receptors that modulate the function of the immune system.

1.1.1.5. μ opioid receptor characteristics

As has been previously described, opioid exposure, mitogen or cytokine stimulation of immune cells induces upregulation of the expression of opioid binding sites. Kraus *et al.* (2001) found that stimulation with IL-4 induces μ opioid receptor mRNA in human peripheral blood cells, Raji, U-937 and HMEC-1 cell lines, and in dendritic cells. In unstimulated immune cells, the μ opioid receptor gene appears to be silent or expressed at less than 5 copies per cell which was the lower limit of detection of the assay (Kraus *et al.*, 2001). It is possible that this dormant characteristic of the μ opioid receptor has led to some of the negative results of μ opioid receptor mRNA expression reported previously. Interestingly, μ opioid receptor transcription was up-regulated by IL-4 in cultures of rat neurons (Kraus *et al.*, 2001). This result provides more evidence of immune to brain communication. When peripheral blood monocytes were differentiated to dendritic cells using IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), the resultant cells did not express μ opioid receptor mRNA despite incubation with IL-4 for 9 days. Further incubation with IL-4 alone was able to induce μ opioid receptor mRNA expression, suggesting that GM-CSF may inhibit IL-4-induced μ opioid receptor mRNA expression. The IL-4 effect was associated directly to cis-active μ opioid receptor promoter elements and function in a manner suggesting they alone can mediate IL-4 induction of the μ opioid receptor gene (Kraus *et al.*, 2001). Furthermore, these response elements were shown to bind STAT6 transcriptional factors. Interestingly, a single nucleotide polymorphism has been found within the IL-4 response element (Hoehe *et al.*, 2000). Kraus *et al.* (2001) demonstrated that transfection of the mutant

allele reduced the trans-activating potential by 50%. This genetic polymorphism may affect the phenotype of subjects carrying this polymorphism and may explain some interindividual variations previously observed in ligand binding to human immune cells by Madden *et al.* (1987).

1.1.1.5.1. Novel μ_3 opioid receptor

An opioid receptor that binds opioid alkaloids but not opioid peptides is expressed on cells of human and invertebrate immune systems (Stefano *et al.*, 1993). It has been designated the μ_3 opioid receptor based on its affinity for morphine. The research that led to the discovery of this receptor and its subsequent characterisation will be discussed.

Stefano *et al.* (1993) demonstrated the presence of morphine-like and codeine-like substances in various tissues of the invertebrate mollusc *Mytilus edulis* and the expression of opioid ligand binding sites on *Mytilus* immunocytes and human peripheral blood monocytes. Scatchard analysis revealed a single binding site with a K_d = 42 nM and a B_{max} = 2.0 pM.g of protein^{-1} for monocytes and a K_d = 56.7 nM and B_{max} = 2.3 pM.g of protein^{-1} for *Mytilus* immunocytes using tritiated dihydromorphine. Opioid peptides were ineffective at displacing dihydromorphine, in comparison to morphine and etorphine (Stefano *et al.*, 1993).

The μ_3 opioid receptor has subsequently been characterised on feline (Dobrenis *et al.*, 1995) and human astrocytes (Makman *et al.*, 1996), human peripheral blood granulocytes (Makman *et al.*, 1995a), macrophage cell lines (Makman *et al.*, 1998; Makman *et al.*, 1995b) and neuronal cells (Cruciani *et al.*, 1994). All these data demonstrate characteristic selectivity of the opioid binding to the μ_3 opioid receptor. For example, tritiated morphine binding to

human astrocytes was displaced by morphine, etorphine, naloxone, diprenorphine, and morphine-6-glucuronide, but not by morphine-3-glucuronide, fentanyl, benzomorphans, or enkephalins, dynorphin, β-endorphin or other opioid peptides (Makman *et al.*, 1996). Therefore, these receptors are distinguishable from those naloxone-insensitive sites described by Roy *et al.* (1998a; 2001b; 1992; 1991). Using a different receptor source (macrophage cell line), μ_3 opioid receptors were also shown to be naloxone-sensitive (Makman *et al.*, 1998) contrary to reports in a recently published review (Sharp *et al.*, 1998b). Similarly, using granulocyte membranes it was found that μ_3 opioid receptors had no detectable affinity for endogenous opioid peptides (DADLE, DAMGO, DPDPE, DAMA, dynorphin A (1-17) deltorphin I, met-enkephalin, and β-endorphin) or for cyclazocine, naltrindole and codeine (Makman *et al.*, 1995a).

Morphine binding to μ_3 opioid receptors on granulocyte and undifferentiated macrophage cell line HL-60 membranes was stereoselectively increased by levorphanol (dextrorphan was without effect) (Makman *et al.*, 1995a; Makman *et al.*, 1998) in a similar fashion to oxymorphindole augmenting the binding affinity of BIT (Radulescu *et al.*, 1991). The levorphanol-induced increase in morphine binding also potentiated the inhibition of granulocyte activation induced by TNF-α in a naloxone-sensitive manner (Makman *et al.*, 1995a). Interestingly, stimulation of HL-60 cells, resulting in differentiation of these cells into a macrophage phenotype, caused the complete loss of the levorphanol effect on binding with no change in morphine affinity or receptor concentration (Makman *et al.*, 1998). However, differentiation of these cells into a granulocyte phenotype had no effect on levorphanol-induced increases in binding affinity. Other macrophage cell types, for example J774.2, RAW264.7 and those previously mentioned such as peripheral blood mononuclear

cells and microglia, differ from granulocytes since receptor binding is not affected by levorphanol.

Further characterisation revealed that the μ_3 opioid receptor appeared to be much more sensitive to inactivation by reduced glutathione than classical μ, δ and κ opioid receptors (Makman *et al.*, 1996; Makman *et al.*, 1998). The μ_3 receptor appears to be linked to heterotrimeric G-protein(s), based on GppNHp effects on agonist binding (Makman *et al.*, 1998). However, contrary to findings for classical opioid receptors, binding of morphine to various cell line μ_3 receptors is not influenced by pertussis toxin or cholera toxin.

Based on the relative binding affinities, differences in glutathione-, pertussis toxin- and cholera toxin-sensitivity, it is apparent that the receptor which has been designated the μ_3 opioid receptor is not closely related to other classical opioid receptors (Makman *et al.*, 1998). It is also apparent that μ_3 opioid receptors expressed by granulocytes and undifferentiated macrophages differ significantly from those expressed by differentiated macrophages and astrocytes due to their different levorphanol sensitivity. This may be due to differences in receptor subtype, the presence of additional components, or receptor coupling in either cell type (Makman *et al.*, 1998). Despite these dissimilarities, the physiological significance of the receptor has been highlighted by Fricchione *et al.* (1997) who found peripheral blood mononuclear cells from patients with histiocytic medullary reticulosis did not express the μ_3 opioid receptor.

Previously reported classical opioid effects may actually be attributable to the μ_3 opioid receptor. For example, Makman *et al.* (1994) suggested that the findings of Ovadia *et al.* (1989) (1.3.1) were in part due to the μ_3 receptor rather than conventional μ opioid receptor

subtypes. In addition, Lopker *et al.* (1980) probably demonstrated dihydromorphine binding to μ_3 receptors on peripheral blood mononuclear cells, as Cabot *et al.* (1997b) alluded to in their discussion of the lung opioid binding sites. The binding sites described by Madden *et al.* (1993; 1996) have a number of similarities to the μ_3 receptor reported by Makman *et al.* (1995a), but differ from them in β-endorphin affinity and optimal salt ion concentration for ligand binding (Madden *et al.*, 1998).

In summary, the majority of these data support the hypothesis that there are μ opioid ligand binding sites on immunocompetent cells. However, their characteristics are not exactly the same as for neuronal μ opioid receptors. These receptors appear to be functional and opioid ligands can act either directly or in combination with centrally mediated processes. Therefore, further characterisation of these types of receptors is required.

1.3.2. κ opioid receptor

1.1.1.6. κ opioid receptor ligand binding

A κ opioid receptor-like binding site on immune cells was first demonstrated by Fiorica *et al.* (1988) who showed tritiated bremazocine was efficiently displaced by U50488 (IC_{50} = 0.57 μM) from the T cell line EL-4. However, levorphanol and dextrorphan showed little stereospecificity in their displacement of bremazocine (IC_{50} = 3 μM and 2 μM, respectively) and the affinity of bremazocine for the receptor expressed by EL-4 cells was significantly lower than for neuronal κ opioid receptors, thereby indicating non-classical κ opioid characteristics of this binding site. Moreover, codeine had a higher affinity for the receptor than morphine (IC_{50} = 60 μM) and was similar to naloxone (IC_{50} = 69 μM). Therefore, the binding site characterised by Fiorica *et al.* (1988) had an uncharacteristic κ opioid receptor binding profile. A U69593 binding site on splenocytes and the macrophage cell line $P388d_1$ characterised by Carr *et al.* (1989) also displayed non-classical κ opioid characteristics. The μ (naltrexone and DAMGO) and δ (met-enkephalinamide) opioid receptor ligands were without effect, whilst U50488 stereoselectively displaced U69593 with the highest affinity (IC_{50} = 8 nM). Although, dynorphin (1-13) was unexpectedly ineffective except at very high concentrations (Carr *et al.*, 1991b; Carr *et al.*, 1989). Therefore, dynorphin (1-13) also displayed ligand selectivity and hence, non-classical κ opioid characteristics.

In contrast, classical κ opioid receptor binding has also been reported. Using the mouse thymoma cell line R1.1, tritiated naloxone binding was efficiently displaced by κ selective compounds U50488 and U69593, whilst μ (DAMGO) and δ (ICI 174864 and DSLET) ligands were less effective (Bidlack *et al.*, 1992). Moreover, stereoselectivity was observed for the displacement of tritiated naloxone by isomers of pentazocine. These results were

supported by subsequent studies by Shapira *et al.* (1997), Bidlack *et al.* (1992) and Lawrence *et al.* (1992). Further research demonstrated that the binding site was coupled to a G-protein and had similarities with the classical κ_{1b} opioid receptor (Bidlack *et al.*, 1995; Bidlack *et al.*, 1992; Lawrence & Bidlack, 1992). Interestingly, differences in G-proteins were observed between various related R1.1 cell lines (Bidlack *et al.*, 1995; Lawrence *et al.*, 1995c). Further characterisation of the κ opioid receptor expressed on R1.1 cells revealed a sulphydryl group at or near the binding site (Joseph & Bidlack, 1994) and a pertussis toxin-sensitive inhibitory guanine nucleotide binding protein mediates the link between the R1.1 κ opioid receptor and adenylyl cyclase (Lawrence & Bidlack, 1993). All these data supported the classical κ opioid nature of the receptor.

Further advances were made in the research investigating the distribution of κ opioid receptors using κ agonists labelled with an immunofluorescent tag (Bidlack *et al.*, 1996; Lawrence *et al.*, 1995a; Lawrence *et al.*, 1995b). Using this method κ opioid binding sites were resolved on mouse thymocytes and splenocytes (Bidlack *et al.*, 1996; Lawrence *et al.*, 1995a), with 58% of thymocytes labelled with the κ opioid receptor immunofluorescence tag. Sixty four percent of the $CD4^+$ cells were also positive for κ opioid receptors, whilst 60% of $CD8^+$ cells were positive. Similar percentages of both immature and mature T cells bound the κ opioid immunofluorescent label (Ignatowski & Bidlack, 1998b). Bidlack *et al.* (2001) demonstrated that *in vitro* mitogen stimulation with concanavalin A and phytohemagglutinin caused an increase in κ opioid receptor expression, as determined by agonist binding.

Subsequent studies have confirmed previous research and shown that κ opioid receptors are widely expressed on various immunocompetent cells and this expression decreases with maturity (Ignatowski & Bidlack, 1998a, 1999). For example, mouse thymocytes displayed

substantial levels of fluorescently tagged κ opioid label binding ($CD4^+$ 91% positive and $CD8^+$ 86% positive), whilst splenocyte labelling was lower ($CD4^+$ 23% positive and $CD8^+$ 14% positive) (Ignatowski & Bidlack, 1999). Splenocyte-derived B cells, Natural Killer cells and macrophages also displayed various levels of κ opioid receptor labelling (Ignatowski & Bidlack, 1999). Interestingly, substantial numbers of resident peritoneal macrophages were positively labelled for the κ opioid receptor (54%). However, when thioglycollate was used to enrich the macrophage population, the κ opioid labelling disappeared. Activation of non-thioglycollate elicited macrophages with lipopolysaccharide *in vivo* did not alter the κ opioid receptor expression. A possible explanation for the lack of receptor expression caused by thioglycolate exposure is the influx of immature monocytes lacking κ opioid receptors into the peritoneal cavity (Ignatowski & Bidlack, 1999). Kirst *et al.* (2002) searched for κ opioid receptor expression using a monoclonal antibody and demonstrated κ opioid receptor expression on murine splenic dendritic cells. Moreover, specific saturable binding of tritiated U69593 was detected using membrane preparations of these cells in a partially naloxone-sensitive manner (3 mM displaced 30nM tritiated U69593 to ~ 40% of control values).

These data suggest that a receptor similar to the κ opioid receptor is expressed on a large proportion of phagocytic cells and T cells although the expression appears to be dependent on the maturation status of the cell. It is important to highlight that despite the binding data being similar to the neuronal κ opioid receptor, it is not identical to the receptor expressed by the cells of the CNS, suggesting there may be a non-classical κ opioid receptor. To further investigate this, κ opioid receptor mRNA expression has also been studied.

1.1.1.7. κ opioid receptor mRNA expression

The expression of κ opioid receptor mRNA has been demonstrated in numerous immune tissues. A summary of the available literature is presented in Table 1.3-2. The expression of the κ opioid receptor transcript has been found to be constitutive in CEM×174 cells and can be up-regulated by treatment with morphine, in a naloxone and nor-binaltorphimine-sensitive fashion (Suzuki *et al.*, 2001a; Suzuki *et al.*, 2001b). The κ opioid receptor mRNA expressed by R1.1 cells was cloned by Belkowski *et al.* (1995c) who found it had 99.8% sequence homology to the neuronal κ opioid receptor but identified a 30 base pair insertion. Meanwhile, Chuang *et al.* (1995a) found 99% homology in the nucleotide sequence isolated from human peripheral blood mononuclear cells, human $CD4^+$ cells, monkey peripheral blood mononuclear cells and T cell lines CEM×174 and Jurkat T-4 with corresponding placenta κ opioid receptor cDNA. This translated to 100% homology in amino acid sequence. Further analysis of the R1.1 cell line and primary peritoneal macrophages revealed an additional intron-exon splice variant (Alicea *et al.*, 1998). Therefore, it is possible that splice variants of the κ opioid receptor are expressed by cells of the immune system, although the influence of this insert on receptor function and ligand selectivity is unknown (McCarthy *et al.*, 2001b). Moreover, it is probable that the characteristics of responses obtained from opioid exposure to immune cells are different to those previously experienced with neuronal cells and hence neuronal κ opioid receptors. This splice variant may explain some of the discrepancies observed between neuronal and immune responses to opioid exposure.

Table 1.3-2 Summary of κ opioid receptor mRNA expression in immune tissues

Cell type	Species	Expression	Study
Alveolar macrophages	Porcine	✓	Pampusch *et al.* (1998a)
Alveolar macrophages - cultured	Porcine	✓/✗	Pampusch *et al.* (1998a)
Astrocytes	Rat	✓	Tryoen-Toth *et al.* (1998)
B cell line (25-9-17S)	Mouse	✗	Gaveriaux *et al.* (1995)
B cell line (CH27)	Mouse	✗	Gaveriaux *et al.* (1995)
B cell line (EBV)	Human	✓	Gaveriaux *et al.* (1995)
$CD4^+$ cells	Monkey	✓	Chuang *et al.* (1995a)
Keratinocytes	Mouse	✓	Kirst *et al.* (2002)
Lymph node cells	Mouse	✗	Gaveriaux *et al.* (1995)
Lymph nodes - inguinal	Porcine	✓	Pampusch *et al.* (1998a)
Lymph nodes - mesenteric	Porcine	✓	Pampusch *et al.* (1998a)
Macrophage - Peritoneal	Mouse	✓	Alicea *et al.* (1998)
Macrophage cell line (Bac 1.2F5)	Mouse	✗	Roy *et al.* (1996)
Macrophage cell line (J774)	Mouse	✗	Gaveriaux *et al.* (1995)
Macrophage cell line ($P338d_1$)	Mouse	✗	Gaveriaux *et al.* (1995)
Microglia	Human	✓	Chao *et al.* (1996a)
Microglia	Rat	✓	Tryoen-Toth *et al.* (1998)
Monocyte cell line (U937)	Human	✗	Gaveriaux *et al.* (1995)
Neutrophils	Porcine	✓	Pampusch *et al.* (1998a)
Peripheral blood cells – stimulated	Human	✗	Wick *et al.* (1996)
Peripheral blood mononuclear cells	Human	✓	Gaveriaux *et al.* (1995)
Peripheral blood mononuclear cells	Human	✓	Chuang *et al.* (1995a)
Peripheral blood mononuclear cells	Porcine	✓	Pampusch *et al.* (1998a)
Peripheral blood mononuclear cells	Porcine	✓	Pampusch *et al.* (1998a)
Peyer's patches – ileum	Porcine	✓	Pampusch *et al.* (1998a)
Peyer's patches – jejunum	Porcine	✓	Pampusch *et al.* (1998a)
Splenocytes	Mouse	✗	Gaveriaux *et al.* (1995)
Splenocytes	Porcine	✓	Pampusch *et al.* (1998a)
Splenocytes - unstimulated	Porcine	✓	Pampusch *et al.* (1998a)
Splenocytes – activated	Mouse	✗	Miller *et al.* (1996)
Splenocytes - freshly isolated	Porcine	✓	Pampusch *et al.* (1998a)
Splenocytes - stimulated	Porcine	✓	Pampusch *et al.* (1998a)
T and B cell hybrid (CEM×174)	Human	✓	Suzuki *et al.* (2001b)
T and B cell hybrid (CEM×174)	Human	✓	Suzuki *et al.* (2001a)

Cell type	Species	Expression	Study
T and B cell hybrid (CEM×174)	Human	✓	Chuang *et al.* (1995a)
T cell line (11.10)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (CEM)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (CTLL)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (EL-4)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (HSB2)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (Jurkat)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (Jurkat)	Human	✓	Chuang *et al.* (1995a)
T cell line (MOLT-4)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (MOLT-4)	Human	✖	Wick *et al.* (1996)
T cell line (R1.1)	Mouse	✓	Belkowski *et al.* (1995c)
T cell line (R1.1)	Mouse	✓	Belkowski *et al.* (1995b)
T cell line (X63-IL-4)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell lines (CEM-3)	Human	✓	Wick *et al.* (1996)
Thymocytes	Porcine	✓	Pampusch *et al.* (1998a)
Thymocytes	Porcine	✓	Pampusch *et al.* (1998a)
Thymocytes – activated	Mouse	✖	Sedqi *et al.* (1996)
Thymocytes - stimulated	Porcine	✓/✖	Pampusch *et al.* (1998a)

✓ indicates a positive expression result, whilst ✖ indicates a negative expression result was obtained.

Interindividual variability in κ opioid receptor mRNA expression in humans has also been reported. Gaveriaux *et al.* (1995) found κ opioid mRNA in one out of three lymphocyte preparations and two out of three monocyte preparations. Stimulation of a κ opioid receptor negative lymphocyte preparation with phytohemagglutinin did not alter the κ opioid mRNA status of the preparation. The influence this has on changes in opioid pharmacodynamic effects on immunocompetent cells in these subjects is unknown.

As alluded to previously, κ opioid receptor expression by immunocompetent cells appears to be linked with maturation. Tryoen-Toth *et al.* (1998) found the expression of κ opioid receptor mRNA in immature astrocytes is much higher than in their mature counterparts, whilst there is no such difference between expression levels in oligodendrocytes of differing maturity. Moreover, the κ opioid receptor mRNA expression is under the control of cAMP,

with higher cAMP levels producing a strong reduction in κ opioid receptor levels in astrocyte and oligodendrocyte cultures (Tryoen-Toth *et al.*, 1998).

Despite some reports of the absence of κ opioid receptor mRNA, the majority of data support the ligand binding data, which point to the expression of a κ opioid-like receptor by immunocompetent cells. However, the characteristics of this receptor are not identical to neuronal κ opioid receptors and therefore the receptor expressed on immune cells will be called a κ opioid-like receptor.

1.1.1.8. κ opioid receptor knockout study

Utilising a κ opioid receptor knockout mouse model, Gaveriaux-Ruff *et al.* (2003) showed a small reduction in thymus cellularity and $CD4^+$ cell ratio. This coincided with a slight increase in immature $CD4^+CD8^+$ lymphocytes. There was a 36% increase in immune cell number in κ opioid receptor knockout mice, but no change in immune cell distribution amongst the various cell populations analysed. The performance of κ opioid receptor knockout cells in proliferation and activity tests was unaffected as were basal immunoglobulin levels compared to wildtype animals. However, following immunisation using a T cell dependent antigen (keyhole limpet hemocyanin), knockout animals produced significantly higher levels of antigen-specific total immunoglobulin, IgM, IgG1 and IgG2a antibodies compared to wildtype animals (Gaveriaux-Ruff *et al.*, 2003). These data suggest that endogenous compounds which act via the κ opioid receptors, expressed on immunocompetent cells or centrally, may exert an inhibitory tone on antibody production. Furthermore, these data also implicate the κ opioid system as an important suppressor for the humoral response. However, the locations of the functional receptors that altered this response are not known.

1.3.3. δ opioid receptor

1.1.1.9. δ opioid receptor ligand binding

Early studies suggested that the δ opioid receptor expressed on immunocompetent cells was slightly different to previously characterised neuronal opioid receptors. Ausiello *et al.* (1984a; 1984b) found that the Jurkat T cell line binding of leu-enkephalin was only reversed by very high concentrations of naloxone and morphine. Moreover, ligand selectivity was seen with DADLE, a δ opioid receptor ligand, which did not bind specifically to human peripheral blood mononuclear and non-adherent cells (Mendelsohn *et al.*, 1985) or human polymorphonuclear leucocytes (Falke *et al.*, 1985). The first possible δ opioid receptor was identified by Carr *et al.* (1988c) using purified T and B cells from mouse splenocytes and human peripheral blood lymphocytes and monocyte enriched leukocytes. Using a specific δ opioid receptor ligand, SUPERFIT, they demonstrated binding to a 70000 kDa protein under reducing conditions and 58000 kDa under non-reducing conditions. The binding of SUPERFIT could not be blocked by DAMGO but was blocked by DADLE and DPDPE, and partially by naloxone. SUPERFIT was also found to bind to the macrophage cell line $P388d_1$ in a DADLE-reversible manner, whilst DAMGO and naloxone were less effective (Carr *et al.*, 1989). When compared with previously mentioned data for κ opioid receptor binding (1.1.1.6 above), it appears that there are at least two opioid binding sites from different classes on the same immune cell.

Subsequent studies identified δ opioid receptor ligand binding sites on human granulocytes (Stefano *et al.*, 1992), NALM 6 (pre-B acute lymphoblastic leukaemia cell line) and Jurkat cell line (Heagy *et al.*, 1999), mouse splenocytes (Barsegov, 1999; Johnson *et al.*, 1982) and dendritic cells (Makarenkova *et al.*, 2001). In the majority of these studies a single binding

site was identified, however Stefano *et al.* (1992) demonstrated the presence of two high affinity-binding sites with differing antagonist sensitivities.

Once again, the majority of these data indicate the expression of a δ-like opioid receptor on various immune cells, although there are several differences between the binding sites characterised here and those reported for neuronal opioid receptors.

1.1.1.10. δ opioid receptor mRNA expression

The murine δ opioid receptor mRNA has been extracted from numerous immune tissues (summarised in Table 1.3-3) and cloned from stimulated thymocyte cultures (Sedqi *et al.*, 1996). The deduced amino acid sequence was similar to that in the brain. From the early studies it became apparent that the degree of stimulation of the cells dictated the expression of δ opioid receptor mRNA (Table 1.3-3). A number of studies suggested that increased proliferation increased expression, although extended lengths of culturing actually decreased δ opioid receptor mRNA levels. This should be kept in mind when designing *in vitro* pharmacodynamic experiments using isolated cell cultures (Sharp *et al.*, 1997).

Table 1.3-3 Summary of δ opioid receptor mRNA expression in immune tissues

Cell type	Species	Expression	Study
B cell line (25-9-17S)	Mouse	✖	Gaveriaux *et al.* (1995)
B cell line (CH27)	Mouse	✓	Gaveriaux *et al.* (1995)
B cell line (EBV)	Human	✓	Gaveriaux *et al.* (1995)
B cell line (Raji)	Human	✓	Wick *et al.* (1996)
Dendritic cells	Mouse	✓	Makarenkova *et al.* (2001)
Lymph node cells	Mouse	✖	Gaveriaux *et al.* (1995)
Lymph node cells – inguinal	Porcine	✓/✖	Pampusch *et al.* (1998a)
Lymph nodes cells - mesenteric	Porcine	✓/✖	Pampusch *et al.* (1998a)
Macrophage cell line (Bac 1.2F5)	Mouse	✓	Roy *et al.* (1996)

Cell type	Species	Expression	Study
Macrophage cell line (J774)	Mouse	✖	Gaveriaux *et al.* (1995)
Macrophage cell line (KG-1a)	Human	✓	Wick *et al.* (1996)
Macrophage cell line (P338d_1)	Mouse	✖	Gaveriaux *et al.* (1995)
Macrophages – alveolar	Porcine	✓	Pampusch *et al.* (1998a)
Monocyte cell line (U937)	Human	✓	Gaveriaux *et al.* (1995)
Neutrophils	Porcine	✖	Pampusch *et al.* (1998a)
Peripheral blood lymphocytes	Human	✓	Wick *et al.* (1996)
Peripheral blood mononuclear cells	Porcine	✓/✖	Pampusch *et al.* (1998a)
Peripheral blood mononuclear cells	Human	✖	Gaveriaux *et al.* (1995)
Peyer's patch – jejunum	Porcine	✖	Pampusch *et al.* (1998a)
Peyer's patches – ileum	Porcine	✓	Pampusch *et al.* (1998a)
Splenocytes	Porcine	✓	Pampusch *et al.* (1998a)
Splenocytes	Mouse	✓	Gaveriaux *et al.* (1995)
Splenocytes	Mouse	✓	Sharp *et al.* (1997)
Splenocytes	Mouse	✓	Li *et al.* (1999)
Splenocytes	Mouse	✓	Shahabi *et al.* (2000)
Splenocytes – ($CD4^+$) stimulated	Mouse	✓	Miller (1996)
Splenocytes – ($CD8^+$) stimulated	Mouse	✓	Miller (1996)
Splenocytes - unstimulated	Mouse	✖	Miller (1996)
T cell line (11.10)	Mouse	✓	Gaveriaux *et al.* (1995)
T cell line (CEM)	Human	✓	Gaveriaux *et al.* (1995)
T cell line (CEM-3)	Human	✓	Wick *et al.* (1996)
T cell line (CTLL)	Mouse	✖	Gaveriaux *et al.* (1995)
T cell line (EL-4)	Mouse	✓	Gaveriaux *et al.* (1995)
T cell line (HSB2)	Human	✓	Gaveriaux *et al.* (1995)
T cell line (HSB-2)	Human	✓	Wick *et al.* (1996)
T cell line (Jurkat)	Human	✖	Gaveriaux *et al.* (1995)
T cell line (MOLT-4)	Human	✓	Gaveriaux *et al.* (1995)
T cell line (MOLT-4)	Human	✓	Wick *et al.* (1996)
T cell line (X63-IL-4)	Mouse	✖	Gaveriaux *et al.* (1995)
T cells – cultured	Mouse	✓	Nguyen *et al.* (2002)
T cells – freshly isolated	Mouse	✖	Nguyen *et al.* (2002)
Thymocytes	Porcine	✓	Pampusch *et al.* (1998a)
Thymocytes – stimulated	Mouse	✓	Sedqi *et al.* (1996)

✓ indicates a positive expression result, whilst ✖ indicates a negative expression result was obtained.

Li *et al.* (1999) demonstrated that δ opioid receptor mRNA could be detected in freshly obtained splenocytes from CD1 mice. However, using quantitative competitive RT-PCR they showed that δ opioid receptor mRNA expression in splenic T cells increased after stimulation with α-CD3 antibodies for 24 and 48 hours compared to the basal level in freshly isolated T cells from less than 1 copy.cell^{-1} to 22 and 42 copies.cell^{-1}, respectively. Further studies with actinomycin D suggested that α-CD3 antibodies affected δ opioid receptor mRNA expression at the transcriptional level. Following stimulation with phorbol myristate acetate and ionomycin, the expression of δ opioid receptor mRNA in splenocytes and T cells was significantly reduced compared with unstimulated cultured cells (Li *et al.*, 1999). These data suggest that signalling through the T cell receptor complex by α-CD3 antibodies regulates δ opioid receptor expression differently than phorbol myristate acetate and ionomycin. These data were supported by Sharp *et al.* (2000) and further explained by Nguyen *et al.* (2002). Nguyen *et al.* (2002) were unable to detect δ opioid receptor mRNA expression in freshly isolated T cells or in $CD4^+$ or $CD8^+$ T cells stimulated with α-CD3 antibodies alone. However, δ opioid receptor mRNA expression was induced by co-stimulation with α-CD3 and α-CD28 antibodies. This response was completely blocked by the inhibition of phosphatidylinositol 3-kinase with wortmannin. This kinase is linked to co-stimulation, suggesting that δ opioid receptor expression is strictly dependent on co-stimulation. However, co-stimulatory receptors other than CD28 are able to provide the signalling required for δ opioid receptor expression since the mRNA for this receptor was induced by concanavalin A-stimulation in splenocytes from CD28 deficient mice. Perhaps some of the discrepancies in the literature can be explained by this need for co-stimulation. If the concentration of mitogen or antibody is insufficient or the cell culture lacks other cell types, which are able to supply the co-stimulation, then no up-regulation of the expression of δ opioid receptor mRNA can occur.

1.1.1.11. δ opioid receptor protein expression

The increased level of δ opioid receptor mRNA expression caused by stimulation was shown to follow through to elevated levels of receptor protein (Miller, 1998). *In vivo* staphylococcal enterotoxin B exposure resulted in an increased proportion and increased receptor number per cell on splenic T cells from 15 to 24 hours. There was a decrease after this time (Shahabi *et al.*, 2000). Moreover, whilst the number of splenic T cells did not increase, there was a significant increase in the proportion of splenocytes that were labelled for δ opioid receptors (from 4% to 21%) and an increase in the fraction of splenic T cells which tested positive for δ opioid receptor expression (from 8% to 51%). The decline in δ opioid receptor positive cells after 24 hours was not due to the redistribution of these positive cells. Rather, δ opioid receptor expression was transiently increased in response to a signal. Others also demonstrated dendritic cell δ opioid receptor expression increased following TNF-α stimulation (Makarenkova *et al.*, 2001).

1.1.1.12. δ opioid receptor knockout

To date only one study has investigated the influence that the δ opioid receptor knockout genotype has on immune function (Gaveriaux-Ruff *et al.*, 2003). In this study, basal immunoglobulin levels were unchanged, although there was a non-significant trend to lower IgM titres in δ opioid receptor knockout animals compared to wildtype animals (0.12 $mg.ml^{-1}$ vs 0.17 $mg.ml^{-1}$, $n = 12$, $P = 0.076$). The response to immunisation with keyhole limpet hemocyanin showed only minor trends to lower immunoglobulin titres in knockout animals, suggesting that the δ opioid receptor only plays a minor role in the humoral response.

1.1.1.13. δ opioid receptor function

When a combination of deltorphin, α-CD3 and α-CD28 antibodies was applied to a Jurkat cell line transfected with a δ opioid receptor, Hedin *et al.* (1997) demonstrated that IL-2 secretion was doubled when compared to α-CD3 and α-CD28 antibody stimulation alone. This deltorphin effect was linked to the AP-1- and NF-AT/AP-1 binding sites of the IL-2 promoter. Deltorphin and DADLE were also shown to increase intracellular calcium levels in a naltrindole-reversible manner which peaked at 10 μM (Sharp *et al.*, 1996) and this response involved the activation of phospholipase C and inhibition of adenylate cyclase. Therefore, this δ opioid receptor is positively coupled to pathways leading to calcium mobilisation and negatively coupled to adenylate cyclase. Concanavalin A-induced calcium mobilisation was subsequently shown to be enhanced by β-endorphin (1 nM), also in a naltrindole-sensitive manner. DADLE was less effective but still increased intracellular calcium, whilst DAMGO and CTAP had no effect on the response. Other studies found that DADLE and deltorphin caused naltrindole and pertussis toxin-sensitive, phospholipase C and protein tyrosine kinase-dependent and Ras-independent phosphorylation of mitogen-activated protein kinases (MAPKs) ERK-1 and ERK-2, whilst nociceptin was without effect (Shahabi *et al.*, 1999). Therefore, the δ opioid receptors expressed by activated T cells are capable of attenuating T cell activation that depends on ERK phosphorylation (Shahabi *et al.*, 2000).

1.3.4. σ receptor

The σ receptor is expressed by a wide range of immunocompetent cells (Carr *et al.*, 1991a; DeHaven-Hudkins *et al.*, 1996; Ganapathy *et al.*, 1999; Garza *et al.*, 1993; Su *et al.*, 1988b; Whitlock *et al.*, 1996; Wolfe *et al.*, 1988), with very similar rank order of inhibitory potency compared to central σ receptors (Wolfe *et al.*, 1988). Moreover, Ganapathy *et al.* (1999)

concluded that Jurkat cells express the type 1 σ receptor and an alternative splice variant, which was not functional in binding assays. Interestingly, a proposed endogenous ligand for the σ receptors, progesterone, competitively bound to this "σ receptor" (Ganapathy *et al.*, 1999), as has been reviewed previously for σ receptors of other tissues (Su, 1991; Su *et al.*, 1988a; Su *et al.*, 1990). It is apparent that a σ receptor is expressed by a wide range of immunocompetent cells, however its physiological function is unknown.

1.3.5. β-endorphin receptor

β-endorphin exhibits a wide range of physiological activities with some of these being blocked by opioid receptor antagonists, naloxone and naltrexone, although other effects were not antagonised. Hazum *et al.* (1979) concluded that this was due to the action of β-endorphin via non-opioid receptors. Tritiated β-endorphin bound to cultured human lymphocytes with a K_d = 3 nM and a second low affinity site that could not be characterised due to non-specific binding (Hazum *et al.*, 1979). It has also been shown that β-endorphin is rapidly internalised following its binding to the cell surface (Falke & Fischer, 1986). Various opioid agonists and antagonists were unable to displace β-endorphin, including naloxone and morphine, confirming the non-classical nature of this binding site (Hazum *et al.*, 1979). α-endorphin was also unable to bind to the β-endorphin receptor, suggesting the COOH terminal region is important for binding, unlike neuronal opioid receptors. Leu- and met-enkephalin were shown to be the only opioids able to partially displace β-endorphin. In addition, the high affinity β-endorphin binding sites could be destroyed by digestion with trypsin but not phospholipase A2. Schweigerer *et al.* (1985) found non-opioid β-endorphin binding to a thymoma cell line (EL-4) with binding dependent on the COOH terminus of β-endorphin rather than the NH_2 terminus. Shahabi *et al.* (1990a) also found similar binding of degraded β-endorphin peptide to cultured murine splenocytes as others found for U937 cells

(Shahabi *et al.*, 1990b; Sharp *et al.*, 1991). Therefore, degradative products of β-endorphin may still act on immune cells, suggesting these metabolised products play a role in the maintenance of immune homeostasis. Others found naloxone-insensitive β-endorphin binding to murine peritoneal macrophages (Woods *et al.*, 1997) and Langerhans cells (Hosoi *et al.*, 1999).

Shahabi *et al.* (1992) raised an antibody to the complementary peptide sequence of the COOH terminus of β-endorphin to recognise the naloxone-insensitive β-endorphin receptor. The antibody successfully inhibited the binding of β-endorphin to receptors on U937 cells but did not affect the binding to brain membrane preparations (Shahabi *et al.*, 1992). Carr *et al.* (1988d) also produced an antibody that bound to opioid receptors on mouse splenocytes and blocked the binding of not only β-endorphin, but also dihydromorphine, met-enkephalin and naloxone. These data suggest that the specific β-endorphin receptor is antigenically similar to opioid receptors expressed on immunocompetent cells or that β-endorphin is still able to bind to naloxone-sensitive opioid receptors. Evidence for the latter was reported by Carr *et al.* (1988b) who found two naloxone-sensitive binding chains of the β-endorphin splenic opiate receptor using murine splenocytes.

Exposure of splenocytes to high concentrations of concanavalin A caused increased specific binding of β-endorphin after 24 and 48 hour incubations independent of its mitogenic effects (Sharp *et al.*, 1991). Jia *et al.* (1992) reported that concanavalin A stimulation of splenocytes induced the expression of a high affinity (K_d = 1 nM) receptor, in addition to a constitutive low affinity (K_d = 103 nM) β-endorphin receptor as Hazum *et al.* (1979) had observed. Importantly, the addition of exogenous IL-2 lowered the concanavalin A-induced change of both low and high affinity receptor number. Therefore, it was suggested (Jia *et al.*, 1992) that

the degree of expression of the receptors does not simply depend on the mitogenic stimulation of the cells, conclusions which agree with Sharp *et al.* (1991).

Using A20 cells which are a B cell lymphoma cell line, Shaker *et al.* (1994) showed two β-endorphin binding sites; a high affinity site (K_d = 0.09 nM) and a low affinity site (K_d = 22 nM). These affinities were significantly higher than those previously observed in splenocytes (Hazum *et al.*, 1979; Jia *et al.*, 1992). Furthermore, in contrast to observations in splenocytes (Jia *et al.*, 1992), Shaker *et al.* (1994) demonstrated that concanavalin A caused an increase in the low affinity and loss of the high affinity site in A20 cells. Other researchers have demonstrated β-endorphin binding to human complement proteins, which was dependent on the COOH terminal region and could not be displaced by other related opioids such as morphine, dynorphin (1-13) or leu-enkephalin (Schweigerer *et al.*, 1983). Therefore, it is apparent that specific β-endorphin, non-classical opioid receptors are expressed on stimulated or cultured immune cells.

1.3.6. ORL1 or the Nociceptin/orphanin FQ receptor

Halford *et al.* (1995) discovered the mRNA for the ORL1 receptor in murine lymphocytes while searching for expression of δ opioid receptor mRNA. The mRNA transcripts were detected in $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ populations and an alternative splice product was also observed. Concanavalin A and lipopolysaccharide stimulation increased the expression of ORL1 receptor mRNA, whilst, the ORL1 antisense oligonucleotide caused inhibition of lipopolysaccharide-induced proliferation and IgG and IgM production.

Increases in ORL1 mRNA expression due to stimulation by phytohemagglutinin were observed by Wick *et al.* (1995), where at least a 10-fold increase in ORL1 mRNA expression in peripheral blood leukocytes was observed. Expression was also detected in mRNA isolated

from HSB-2, CEM-3, MOLT-4 and Raji cell lines. Once again, alternative splicing of the transcript was seen, although the variation occurred in the non-coding region (Wick *et al.*, 1995).

ORL1 receptor expression has also been observed in human foetal microglia and astrocytes (Chao *et al.*, 1998b), circulating lymphocytes, monocytes, T, B and monocytic cell lines (Peluso *et al.*, 2001; Peluso *et al.*, 1998). Peluso *et al.* (2001) have also presented data that indicates ORL1 receptors expressed by immunocompetent cells are linked to G-proteins. A splice variant, lacking 15 nucleotides at the junction between exon 1 and exon 2, showed a distribution similar to the already known ORL1 receptor transcript (Peluso *et al.*, 1998), however the functional effect of this is unknown. From a limited immunological analysis in ORL1 receptor knockout animals, it would appear that there were no major functional differences between the two animal groups (Nishi *et al.*, 1997). ORL1 receptor mRNA has also been detected in porcine thymus, lymph nodes, spleen, freshly isolated splenocytes and both stimulated and unstimulated cultured splenocytes (Pampusch *et al.*, 1998b; Pampusch *et al.*, 2000).

The data presented indicate that the ORL1 receptor is expressed widely in the immune system and its expression can be up regulated upon cellular activation. Interestingly, the expression of the splice variant of the ORL1 receptor in the immunocompetent cells suggests that the responses obtained from the ligands binding to these receptors may be different from neuronal ORL1 receptors.

1.3.7. General opioid receptor expression control

Sequence analysis of the promoter regions for μ, κ and δ opioid receptors has exposed potential cytokine response elements (reported by Im *et al.* (1999)). In light of the previously

presented reports which demonstrated increased receptor expression following cytokine treatment, Im *et al.* (1999) sought to determine if these sequences were biologically active. They demonstrated that there was no significant cytokine-stimulated opioid receptor gene expression in the U266, RAW264.7, MOLT-4, HSB2, CEM3, Raji, R1.1, NMB, NS20Y and PC12h immune cell lines. In addition, Im *et al.* (1999) concluded that the cytokine response elements (NF-IL6 binding sites) in opioid receptor genes were not functional.

Other studies have reported that the chemokine receptor CCR5 oligomerises with μ, κ and δ opioid receptors expressed on CEM×174 cells (Suzuki *et al.*, 2002). Functional studies revealed that a co-treatment of cells with opioid and CCR5 agonists resulted in cross-talk between the two receptor types on immune cells. Further analysis indicated that this oligomerisation is constitutive and may be altered by ligand binding (Suzuki *et al.*, 2002).

The influence of methadone maintenance and HIV infection on opioid receptor expression has also been investigated. Beck *et al.* (2002) found chronic methadone administration led to a significant increase in the δ opioid receptor expression on lymphocytes, monocytes and granulocytes in HIV-negative intravenous drug users,. However, the antibody used to detect the receptor expression was not used according to the manufacturers directions, therefore clarification of this result is required.

From the available literature on the expression of opioid receptors by immune cells, it is apparent that both classical and non-classical opioid responses occur. A discussion of the effect opioids have on *in vitro* and *in vivo* immune function follows. This is discussed highlighting opioid characteristics of the responses.

1.4. Opioids, infection and immunity

The direct and indirect influence of opioids on immune function has been keenly researched since the publication by Wybran *et al.* (1979). In order to review the large literature base, the research has been divided as follows: *in vitro* and *in vivo* studies, effects by cell type, function, and opioid ligand. This subdivision enables the comparison of opioid ligand effects and provides an opportunity to evaluate the opioid nature of the responses. Where appropriate, summaries of the available data have been formatted into tables including: opioid ligand employed, the direction of the response observed (increased ↑, no effect or no change ↔, decreased ↓), the endpoint quantified and the antagonist sensitivity (~~antagonist~~ indicates the compound was used but was insensitive and blank implies no antagonist data were available). There are also a number of quality reviews in this area if further reading is required (Carr *et al.*, 1996b; Donahoe, 1989, 1991; Donahoe *et al.*, 1985; Eisenstein & Hilburger, 1998; Glasel, 2000; House *et al.*, 1997a; Peterson *et al.*, 1991; Stefano, 1998; Webster, 1998).

1.4.1. *In vitro* evidence for the effect of opioids on infection and immunity

1.4.1.1. Glial cells/brain immune system (*in vitro*)

Glial cells account for more than 85% of the total cell population in the brain (Chao *et al.*, 1996b). Of this, astrocytes account for 85%, with microglial cells making up the other 10-15% (Chao *et al.*, 1996b). Glial cells are the immune system's representatives in the brain and respond to infection or insult. As discussed previously (1.3) glial cells express opioid-like receptors and therefore their function may be modified by endogenous and exogenous opioids. Numerous glial parameters are modified by a variety of opioid ligands including HIV infectivity (Chao *et al.*, 1998a; Chao *et al.*, 1995a; Chao *et al.*, 1994; Li *et al.*, 2002), chemotaxis (Azuma *et al.*, 2001; Hu *et al.*, 2000) and superoxide production (Azuma *et al.*, 2001; Chao *et al.*, 1994; Hu *et al.*, 1998). In each of these cases, classical opioid receptor characteristics were evident, supporting the previously discussed literature for glial cell opioid receptor expression.

The literature is less consistent for other opioid modulated glial functions. For example, morphine and met-enkephalin modulated cytokine and chemokine receptor expression in a classical opioid fashion (Chao *et al.*, 1994; Das *et al.*, 1995; Mahajan *et al.*, 2002), whilst κ opioid receptor ligands behaved differently. Dynorphin A (1-8) very efficiently (10^{-16} - 10^{-12} M) inhibited the lipopolysaccharide-induced production of nitric oxide and TNF-α in a nor-binaltorphimine-insensitive manner (Kong *et al.*, 1997). However, U50488 did not affect the responses. Moreover, similar concentrations (10^{-16} to 10^{-12} M) of dynorphin A (2-17), an analogue of dynorphin that possesses no classical κ opioid receptor activity, exhibited the same inhibitory effects as dynorphin A (1-8). Therefore, it is apparent that these responses were not mediated via classical κ opioid receptors.

A similar situation is apparent for glial cell phagocytosis. Peterson *et al.* (1995) found evidence implicating classical opioid receptors, specifically G_i protein-coupled μ opioid receptors. However, subsequent studies question the direction of the response and the classical opioid nature, due to differing rank order of effect and conflicting opioid antagonist- and toxin-sensitivities (Azuma *et al.*, 2001; Sowa *et al.*, 1997). Sowa *et al.* (1997) suggested that the reason for this discrepancy in the order of potency may be due to DAMGO acting at the μ3 opioid receptor where non-peptide opioids, like morphine, have higher affinity.

Finally, the action of opioids to modulate neurotoxicity has demonstrated conflicting opioid characteristics. Synthetic κ opioid receptor ligands reduce the expression of HIV-1 and its neurotoxic components in a classical κ opioid receptor and quinolinate-dependent manner (Chao *et al.*, 2000). Similarly, Kim *et al.* (2001) and Hu *et al.* (2002) demonstrated classical opioid effects on cell viability. However, naloxone's neuroprotective mechanism of action was non-stereoselective (Liu *et al.*, 2000b). Liu *et al.* (2000a) subsequently found naloxone was able to inhibit lipopolysaccharide-induced activation of microglia thereby inhibiting the release of the pro-inflammatory factors IL-1β, TNF-α and nitric oxide, and inhibiting superoxide production. Interestingly, naloxone blocked the binding of tritiated lipopolysaccharide to cell membranes, and it may be partially via this mechanism that it reduced the neurotoxicity of lipopolysaccharide (Liu *et al.*, 2000a).

The significance of exogenous opioid action on glial cells is amplified by the inducible expression of endogenous opioids. These can act on the originating cell or the neighbouring cells, of neuronal or immune origin (Buzas *et al.*, 2002; Negro *et al.*, 1992). The impact of endogenous opioid production by these cells and the implication of the anatomical location of

the expression are yet to be fully elucidated although there is significant potential for physiological relevance. Moreover, the importance of these endogenous opioid ligands and opioid receptors, all with varying degrees of opioid characteristics, is highlighted by the discovery of these opioid systems in invertebrate glial cells (molluscs *Planorbarius corneus* and *Mytilus edulis*, and the insect *Leucophaea maderae*) (Sonetti *et al.*, 1994). Liu *et al.* (1996) concluded that since morphine acted on immune cells from invertebrates, in a similar fashion to that of vertebrates, the response has been conserved for potentially 500 million years since diverging from the evolution of humans, stressing the importance of this process. Moreover, these findings emphasise the significance of morphine as an endogenous compound.

These data confirm the presence of functional opioid receptors on brain immune cells, agreeing with the receptor expression data discussed previously (1.3). However, the potential involvement of the μ_3 and other non-classical opioid receptors indicates that the responses from these cells will display some ligand selectivity. The role these opioid-like receptors play in brain function needs further investigation, as these sites may be of significant importance for pharmacodynamic effects during opioid exposure of a licit or illicit nature.

1.4.1.2. Thymocytes (*in vitro*)

The thymus is the primary immune organ where T cells develop and mature. The viability of thymocytes cultured in the presence of morphine (Freier & Fuchs, 1993; Luza, 1992) and DPDPE (McCarthy & Rogers, 2001) decreased depending on the cell type examined. McCarthy and Rogers (2001) reported that DPDPE caused a selective decrease in $CD4^+CD8^-$ cell numbers, whilst $CD4^-CD8^-$, $CD4^+CD8^+$ and $CD4^-CD8^+$ numbers were unaffected. They

concluded that apoptosis was the cause of this reduction in cell number. Moreover they suggested that δ opioid receptor agonists are capable of modulating T cell development in the thymus and may participate in negative selection (McCarthy & Rogers, 2001). These data should be considered when investigating other *in vitro* immune functions as alterations in cell viability may mask other opioid effects.

The vital immune functions of chemotaxis and adhesion are also modified by opioids in isolated thymocytes. Thymocytes migrate selectively toward the μ opioid receptor ligand DAMGO, whilst DPDPE and U50488 were without effect (McCarthy *et al.*, 2001a; Szabo & Rogers, 2001). In addition, DAMGO chemotactic and adhesion responses were sensitive to CTAP antagonism, implicating classical opioid receptors (McCarthy *et al.*, 2001a; Szabo & Rogers, 2001). Flow cytometric analysis of the DAMGO responsive cells showed that these cells consist predominantly (45%) of highly immature $CD4^-CD8^-$ T cells. In contrast, cells that migrated toward the chemokine ELC were primarily $CD4^+CD8^-$ or $CD4^-CD8^+$ cells, whilst $CD4^-CD8^-$ were insensitive to ELC (McCarthy *et al.*, 2001a). Moreover, pre-treatment of thymocytes and HaCaT keratinocytes with chemotactic chemokines, such as RANTES, MIP-3b and MCP-1 blocked the DAMGO-induced chemotaxis (Szabo & Rogers, 2001). These results suggest that the receptor through which DAMGO was acting, was involved in chemotaxis and was desensitised by chemokines. McCarthy *et al.* (2001a) and Szabo *et al.* (2001) both concluded that since CTAP antagonised DAMGO-induced chemotaxis, the response was mediated via the μ opioid receptor. However, no μ opioid receptor mRNA expression has been found in thymocytes (Miller, 1996; Pampusch *et al.*, 1998a; Sedqi *et al.*, 1996), and ligand binding evidence suggests that there is no expression (Ovadia *et al.*, 1989; Patrini *et al.*, 1996) or non-classical opioid receptor expression (Roy *et al.*, 1992; Roy *et al.*,

1991). Therefore, it would be difficult to conclude the identity of this receptor from just a single antagonist's effect.

In contrast to thymocyte chemotaxis, studies investigating thymocyte proliferation have hypothesised, and found non-classical opioid receptor involvement. For example, Roy *et al.* (1997) observed, non-classical, naloxone-insensitive morphine inhibition of thymocyte proliferation. Moreover, further increases in naloxone concentration resulted in increased inhibition of proliferation. Interestingly, exogenous supplementation of IL-2 increased the proliferation of the morphine-exposed thymocytes, suggesting cytokine production or signalling may be impaired resulting in reduced proliferation (Roy *et al.*, 1997). Using a different method for quantification of thymocyte proliferation (induction of ornithine decarboxylase), Pignatti *et al.* (1998) found U50488 non-stereoselectively inhibited proliferation in a nor-binaltorphimine-insensitive fashion, whilst U69593 had no effect. Despite the non-opioid characteristics of the U50488 and U69593 responses, it was shown to be pertussis toxin-sensitive implicating a G-protein coupled receptor (Pignatti *et al.*, 1998). Interestingly, Guan *et al.* (1997) only used the active isomer of U50488 and observed inhibition of thymocyte proliferation dependent on the presence of IFN-γ activated macrophages. This discrepancy undoubtedly lies in the methodologies used by the different research groups, and may in fact suggest the results from different methodologies should not be directly compared. Pignatti *et al.* (1998) used induction of ornithine decarboxylase by mitogen-stimulated thymocytes, whilst Guan *et al.* (1997) used incorporation of tritiated thymidine in thymocytes incubated in the presence or absence of IFN-γ-activated macrophages. However, the results of Guan *et al.* (1997) raise an interesting point, that in some situations mixed cultures may be required for opioid immunomodulation, or at least required for opioid receptor expression.

Other instances of more classical opioid activity were noted by Hicks *et al.* (2001), who found classical antagonism of the induction of proliferation by a μ opioid agonist (CGPM-9), in direct contrast to the findings of Roy *et al.* (1997). Both morphine and the novel ligand 4-tyrosylamido-6-benzyl-1,2,3,4 tetrahydroquinoline (CGPM-9) are μ opioid receptor agonists, although at the same concentrations morphine caused nearly a 60% decrease in proliferation (Roy *et al.*, 1997) whilst CGPM-9 caused a 50% increase in proliferation (Hicks *et al.*, 2001). CGPM-9 has a 30-fold greater affinity for the μ opioid receptor than morphine (Wang *et al.*, 1998) and would therefore be expected to have a greater effect than morphine on proliferation. Two possible explanations for the conflicting data are that morphine and CGMP-9 may be acting at different receptors of either opioid or non-opioid classes or, the response caused by CGMP-9 was greater than morphine. This second explanation appears to be contradictory. However, if the response was non-linear and perhaps biphasic, where low stimulation of the receptor by morphine caused inhibition, and high stimulation by CGPM-9 caused proliferation, it would be feasible that greater stimulation of the receptor by CGMP-9 may cause a response in the opposite direction to that of morphine, owing to its higher affinity. However, the naloxone-insensitivity of morphine's effect and the CTOP partial sensitivity of CDMP-9 are difficult to explain.

These data demonstrate that μ opioid receptor ligands increase and decrease thymocyte proliferation, whilst δ ligands increase and κ ligands decrease the proliferative responses. These responses were not all classically opioid due to antagonist insensitivity and non-stereoselectivity, thus implicating a non-classical opioid receptor.

T cells are a vital source of cytokines, therefore the influence opioids have on this immune function has been intensely studied. Guan *et al.* (1997) demonstrated that the presence of stimulated macrophages was necessary for U50488 to inhibit thymocyte IL-2 production. These studies were later supported by Zhang *et al.* (2000) who found staphylococcal enterotoxin B-stimulated thymocytes exposed to U50488 did not alter cytokine production (IL-2, IL-4, IL-5, IL-13, and IFN-γ). However, further investigation of the cytokine receptor expression by these thymocytes revealed a significant, naloxone-sensitive inhibition in the expression of the mRNA of IL-7 receptor α-chain and an increase in the expression of the chemokine receptor CCR2 following exposure to U50488 (Zhang & Rogers, 2000). Curiously, Zhang and Rogers (2000) found naloxone-sensitive U50488 responses that contradicted the naloxone-insensitive morphine effects observed by Roy *et al.* (1997). Moreover, when Roy *et al.* (1997) followed up their discovery that cytokine production may be impaired following morphine exposure they found significant reductions in the levels of IL-2 mRNA and IL-2 transcription rate. Similarly, Roy *et al.* (1997) found reductions in the levels of AP-1 binding proteins following morphine exposure. AP-1 response elements have been shown to be one of the most important elements in regulating IL-2 gene expression (Krummel & Allison, 1995). They also investigated thymocyte expression of c-fos and c-jun and found that c-fos expression was significantly reduced following morphine treatment without changes in c-jun levels (Roy *et al.*, 1997). These changes in cytokine expression contradicted the findings of Zhang *et al.* (2000). Experimental differences may explain the results, since Zhang *et al.* (2000) used male Balb/c mouse thymocytes stimulated with staphylococcal enterotoxin B for 24 hours with no foetal calf serum and investigated U50488 immunomodulation, whilst Roy *et al.* (1997) used outbred IRC mouse thymocytes stimulated with phytohemagglutinin and IL-1 with 15% foetal calf serum for 24 hours and investigated morphine immunomodulation. However, these data seem to suggest that morphine and

U50488 were acting via different receptors due to the different naloxone-sensitivities and cytokine expression patterns. Despite the differences between the two findings, the IL-7 receptor results of Zhang *et al.* (2000) partially agree with Roy *et al.* (1997) since IL-7 causes increased expression of IL-2 and its receptor (Armitage *et al.*, 1990) and support the potential involvement of κ opioid receptors in maturation as IL-7 is critical for this process. Therefore, morphine and possibly U50488 may act to inhibit IL-7 receptor expression thereby inhibiting IL-2 and its receptor expression. This hypothesis does not agree entirely with the work of Zhang *et al.* (2000), however, if the same experimental conditions were used some of these questions may be answered.

Interestingly, Roy *et al.* (1997) went on to suggest that morphine may act through the T cell receptor/CD3 complex since the activation of this complex leads to the induction of c-fos via the p21 ras-raf signal transaction pathway (Pastor *et al.*, 1995). As discussed previously (1.1.1.10) Nguyen *et al.* (2002) found δ opioid receptor mRNA expression was under the strict control of co-stimulation and T cell receptor signalling and Hedin *et al.* (1997) demonstrated that δ opioid receptor agonists augmented IL-2 secretion after α-CD3 and α-CD28 stimulation. These data point to opioids requiring the presence of co-stimulation in order to stimulate proliferation, and potentially, if the co-stimulation is not present and opioids act on the immune cells, inhibition of the response will occur.

As reviewed here, opioids exert a wide range of influences on thymocyte function displaying both classical and non-classical opioid receptor tendencies. The physiological significance of thymocyte opioid modulation may range from an important role in the negative selection of $CD4^+$ cells to altered mobility. If we expand upon the mobility of thymocytes, there is an intriguing link between adhesion and chemotaxis, as increases in each of these may allow

greater extravasation of these cells to access sites where the chemotactic signal originated. Moreover, once the cells have reached this site, opioid-induced chemotaxis would be inhibited due to the increased levels of chemo-attractants. It is tempting to continue this analogy further when focusing on proliferation. Once at the originating chemotactic site, the μ opioid tone in the area may allow increased proliferation. The potential regulatory role endogenous opioids have in thymocyte function must be elucidated. Moreover, the influence of exogenous opioids on this regulation needs to be determined. However, exogenous opioids may not behave as the neuronal data would suggest, due to the presence of non-classical opioid receptors.

1.4.1.3. Splenocytes (*in vitro*)

1.1.1.13.1. Splenocyte proliferation

Splenocyte proliferation is a widely used model to investigate opioid immunomodulation. Table 1.4-1 summarises the results from the available literature for various opioid ligands using this method. The immunomodulatory data generated using some of the compounds provided no definitive response due to the variability in the reported results. For example, the data for met-enkephalin and leu-enkephalin are contradictory and therefore will not be fully discussed (Table 1.4-1).

The data generated for morphine demonstrate that it had an inhibitory effect on splenocyte proliferation. However, the concentration at which the response occurred varied considerably, as did the opioid antagonist selectivity. Jessop *et al.* (1991a) demonstrated morphine dose-dependently inhibited proliferation in mouse splenocytes, whilst others have found *in vitro* incubation with morphine had no effect on splenocyte mitogen-induced

proliferation at all except for the highest concentration tested (100 μM) (Fecho *et al.*, 1996a). The metabolites of morphine, morphine-3-glucuronide and morphine-6-glucuronide were also reported to significantly suppress B cell proliferation at concentrations between 0.01 μM and 100 μM. Although, these occurred only to a small degree and the suppression did not exhibit any obvious dose-response relationship (Thomas *et al.*, 1995a). However, the inactive metabolite of morphine, normorphine, also significantly suppressed cell proliferation at 10 μM which was more potent than morphine (Thomas *et al.*, 1995a), suggesting a non-classical opioid response.

Eisenstein *et al.* (1991) found morphine suppressed Balb/c splenocyte staphylococcal enterotoxin B-, phytohemagglutinin- and phorbol myristic acetate-induced proliferation up to 60%, however at much lower concentrations (10 μM). Naloxone and naltrexone fully antagonised morphine inhibition of staphylococcal enterotoxin B-induced proliferation at 10 μM. This differs with the data of Fecho *et al.* (1996a) and Jessop *et al.* (1991a) which showed naloxone-insensitivity. Rogers *et al.* (1991) reported similar data to Eisenstein *et al.* (1991), however, they found differences in the response between murine strains. They observed naloxone-sensitive and nor-binaltorphimine-insensitive inhibition of proliferation. Balb/c, C57BL/6 and B10.A(5R) strains were sensitive to morphine immunomodulation, whilst B10.BR, C3H/HeJ, C3H/FeJ and CBA/J mice were nearly 10-fold less sensitive to morphine. Interestingly, morphine failed to modify the mitogen-induced proliferation in the μ opioid receptor-deficient mouse strain CxBK/ByJ (Rogers *et al.*, 1991), suggesting that this response was dependent on the μ opioid receptor, in contrast to other reports.

The differences in the reported morphine immune responses are puzzling. Both naloxone-sensitivity and -insensitivity have been observed, combined with differences in response to

morphine. The results observed by Thomas *et al.* (1995a) are intriguing as they demonstrate a non-classical opioid rank order of effect with normorphine more potent than morphine. Despite this lack of classic opioid rank order of effect, responses of a classical opioid nature have been found. Perhaps the differences originate from experimental techniques, for example Jessop *et al.* (1991a) used concanavalin A stimulation of splenocytes, Thomas *et al.* (1995a) reported IL-4 plus α-IgM stimulation of B cells, Fecho *et al.* (1996a) used concanavalin A, phytohemagglutinin or lipopolysaccharide stimulation and Eisenstein *et al.* (1991) used staphylococcal enterotoxin B, phytohemagglutinin and phorbol myristic acetate stimulation. These different methods of proliferative stimulation would result in activation of different cell types from the mixture of splenic cells. Moreover, these cell types, which are targeted by each type of mitogen, may respond differently to morphine. However, this does not negate the significance of the non-classical opioid observations, which therefore implicate non-classical opioid receptors in the responses.

Other synthetic μ opioid receptor agonists such as methadone (Thomas *et al.*, 1995b), diacetylmorphine (heroin) (Thomas *et al.*, 1995b), ohmefentanyl (Wu & Li, 1999), fentanyl (House *et al.*, 1995a) and pethidine (House *et al.*, 1995a) all inhibit splenocyte proliferation (Table 1.4-1). Thomas *et al.* (1995b) found B cell proliferation was significantly inhibited by 100 μM methadone (12% of control response) in a similar manner to the morphine response obtained by Fecho *et al.* (1996a). The high concentrations of methadone required to modulate splenocyte function and hence relative insensitivity of splenocytes to methadone, would suggest that the response is not a classical opioid one since methadone has relatively high affinity for the μ opioid receptor (K_i = 1.7 nM) (Codd *et al.*, 1995). Thomas *et al.* (1995b) also found diacetylmorphine inhibited B cell proliferation, although no dose relationship was apparent. Interestingly, the use of diacetylmorphine in this manner and the results that can be

drawn from such data are limited and will be discussed in a later chapter (Chapter 2). Using ohmefentanyl, which is a high affinity μ opioid receptor agonist, Wu *et al.* (1999) demonstrated naloxone-sensitivity and -insensitivity combined with both induction and inhibition of proliferation within an experimental model, which is a very uncharacteristic opioid effect. Specifically, they found 0.1 pM to 1 nM ohmefentanyl enhanced concanavalin A-induced proliferation, whilst 0.1 and 1 μM caused inhibition (Wu & Li, 1999). Lipopolysaccharide-induced proliferation was unaffected at the low ohmefentanyl concentrations with similar inhibition at the upper ligand concentrations. Naloxone inhibited the induction of concanavalin A proliferation, but had no influence on the inhibition of proliferation with both mitogens (Wu & Li, 1999). These data generated using various synthetic μ opioid ligands, lend support to the presence of, and action via non-classical opioid receptors, whose identity and characteristics are unknown.

Various peptide μ opioid receptor agonists such as DAMGO and demorphin have also been investigated in several studies. Eisenstein *et al.* (1991) found DAMGO inhibited staphylococcal enterotoxin B- and phorbol myristic acetate-induced proliferation only at the two highest concentrations tested (1 and 10 μM), with the inhibition being substantially greater for the phorbol myristic acetate response than staphylococcal enterotoxin B. Interestingly, DAMGO was substantially less inhibitory in C3HeN/FeJ mouse splenocyte preparations (Eisenstein *et al.*, 1991; Rogers *et al.*, 1991). In contrast to the reports of inhibition by DAMGO, Radulescu *et al.* (1991) found DAMGO increased concanavalin A-induced splenocyte proliferation between 1 and 100 nM in a naloxone-sensitive manner (Table 1.4-1). Similarly, Kowalski (1998b) demonstrated concanavalin A and to a lesser degree lipopolysaccharide-induced proliferation were enhanced by DAMGO in a biphasic response that was β-funaltrexamine-sensitive, whilst ICI 174864 and nor-binaltorphimine had

no effect. The difference in response direction of these two ligands, supposedly acting at the same receptor, but still maintaining classical opioid agonist sensitivity, cannot be readily explained. Biphasic immunomodulation was found using demorphin where Caroleo *et al.* (1994) showed that demorphin caused induction of the proliferative response at low (10^{-12} to 10^{-11}M) and inhibition at high (10^{-7} to 10^{-6}M) concentrations. Furthermore, these responses were antagonised by naloxone, in contrast to the findings of Wu *et al.* (1999) using ohmefentanyl (Table 1.4-1). Once again, the dual action of the single ligand was observed with induction and inhibition of proliferation reported, suggesting non-classical opioid receptor involvement.

The endogenous opioid β-endorphin, has been reported to cause enhancement of the proliferation response of splenocytes to concanavalin A and phytohemagglutinin in a naloxone-insensitive fashion (Gilman *et al.*, 1982; Gilmore & Weiner, 1989) (Table 1.4-1). α-Endorphin did not have any effect (Gilman *et al.*, 1982). Neither of the peptides had any effect on B cell mediated lipopolysaccharide or dextran sulphate stimulation (Gilman *et al.*, 1982). Furthermore, Gilmore *et al.* (1989) found the presence of the C-terminal amino acids was required for the increased proliferation by using various β-endorphin fragments. They concluded that β-endorphin was acting via a non-classical opioid receptor with similar characteristics to that previously described (1.3.5), since the C-terminus was vital to the response observed (Gilmore & Weiner, 1989), but is not required for binding to classical opioid receptors. Subsequent studies by Gilmore *et al.* (1990) also found β-endorphin enhanced proliferation of murine splenocytes stimulated by concanavalin A. Interestingly, they found that the magnitude of β-endorphin enhancement was dependent on the state of activation of the cell, since sub-optimal mitogen concentrations gave the greatest β-endorphin response, suggesting the need to reduce the background noise of the experimental model.

These β-endorphin data have also been supported by reports from Jessop *et al.* (1991a), van den Bergh *et al.* (1991) and partially by Lin *et al.* (1994). Lin *et al.* (1994) found β-endorphin depressed the proliferative responses to phytohemagglutinin in rat splenocytes but enhanced it in mice, which is in contrast to the findings of van den Bergh *et al.* (1991) who saw β-endorphin enhanced mitogen-induced proliferation in rats. In contrast to the non-classical opioid receptor responses, Kusnecov *et al.* (1987) demonstrated that splenocytes exposed *in vitro* to β-endorphin (10^{-12} to 10^{-9} M) enhanced concanavalin A-induced proliferation, in a naloxone-sensitive manner. As was observed for most opioid ligands, reports of classical and non-classical opioid responses following β-endorphin also exist, although the majority of the evidence for β-endorphin suggests a non-classical opioid response. If other μ opioid receptor ligands act on isolated splenocytes in a classical opioid manner, why doesn't β-endorphin act in a similar manner? The reasons for this are yet unanswered, however a potential solution to this question incorporates the involvement of a non-classical opioid receptor.

Ligands, other than those of the μ opioid receptor, have also been applied to splenocyte proliferation assays. Using the same experimental model as described earlier, Rogers *et al.* (1991) found the κ opioid agonist U50488 inhibited the proliferative response of murine splenocytes to the mitogenic agents phorbol myristic acetate and staphylococcal enterotoxin B (Table 1.4-1). U50488 was a more potent inhibitor of proliferation than morphine or DAMGO and was sensitive to nor-binaltorphimine and naloxone (Rogers *et al.*, 1991). Moreover, its effect was stereoselective, suggesting classical κ opioid action. U69593 was also shown to cause inhibition of proliferation. As was the case for morphine and DAMGO inhibition, there were U50488-sensitive and -insensitive mouse strains with the same pattern as seen previously. However, the difference between sensitivity and insensitivity was significantly greater for the κ opioid agonist (600 vs 8 times difference). Caroleo *et al.* (1994)

supported these results, by showing that nor-binaltorphimine was able to antagonise U50488-induced inhibition of splenocyte proliferation. Pignatti *et al.* (1998) also found ornithine decarboxylase activity was inhibited by U50488. In contrast to the inhibitory effects of κ opioid agonists, Kowalski (1998b) reported that U50488 enhanced concanavalin A- and lipopolysaccharide-induced proliferation in a bell-shaped concentration response curve with maximal effect at 10^{-7} to 10^{-12} M. The peptide κ opioid agonist dynorphin also enhanced phytohemagglutinin-induced proliferation at sub-optimal mitogen concentrations, but had no effect during intense phytohemagglutinin stimulation (Barreca *et al.*, 1987). Interestingly, the response was insensitive to naloxone suggesting the response may be mediated via a non-classical opioid receptor. In contrast, Ni *et al.* (1999) concluded dynorphin A (1-13) and dynorphin A (1-17) enhanced concanavalin A-stimulated proliferation was via κ opioid receptors since the responses were antagonised by naloxone and nor-binaltorphimine. The majority of these data suggest κ opioid receptor agonists act via κ opioid receptors to modify splenocyte proliferation, which agree with the classical κ opioid receptor binding and expression data, presented previously (1.3.2). However, the response direction and the significant difference in inter-strain sensitivity to κ compared to μ opioid receptor ligands is intriguing and warrants further investigation. Moreover, the difference in sensitivity may be used to confirm the identity of the receptor through which μ and κ opioid receptor ligands act.

Several studies have also investigated the influence of δ opioid receptor agonists on splenocyte proliferation. For example, Caroleo *et al.* (1994) showed using Balb/c splenocytes incubated for 48 hours stimulated with concanavalin A, that the δ selective agonist, deltorphin, only caused induction at a low (10^{-13} M) concentration, which was blocked by naltrindole (Table 1.4-1). Kowalski (1998b) also found a δ opioid agonist caused enhancement of proliferation. DPDPE enhanced concanavalin A- and lipopolysaccharide-

induced lymphocyte proliferation in a bell-shaped concentration response curve, with maximal effect at 10^{-9} to 10^{-12} M. In contrast to induction of proliferation by deltorphin and DPDPE, Shahabi *et al.* (1995b) reported that purified murine $CD4^+$ and $CD8^+$ T cell proliferation were suppressed by deltorphin and DAME in a naltrindole-sensitive manner (Shahabi & Sharp, 1995a). Interestingly, the effect was maximal when cells were pre-incubated with deltorphin prior to activation, and was ineffective when deltorphin was added at the time of activation (Shahabi & Sharp, 1995a). The responses at all concentrations of deltorphin were greater after 48 hours compared to 72 hours in culture. This time course response corresponds to similar differences between δ opioid receptor mRNA levels, with longer incubations reducing levels (Sharp *et al.*, 1997). DPDPE also inhibited the proliferation of $CD4^+$ cells and this was antagonised by naltrindole (Shahabi & Sharp, 1995b), in contrast to the reports of Kowalski (1998b). Other δ opioid receptor ligands have also been investigated by House *et al.* (1996), who found DAME, DTLET, DADLE, deltorphin-1 and DPDPE-TFA had no effect on B cell proliferation, whilst DSLET and DPDPE caused some inhibition at the higher concentrations tested (0.1 to 10 μM). Once again, some data suggest δ opioid receptor agonists caused induction while other data demonstrate inhibition of splenocyte proliferation. Interestingly, the decreased response of proliferation following longer incubations reported by Shahabi *et al.* (1995b) correlates well with the δ opioid receptor mRNA data (Sharp *et al.*, 1997), implicating this classical opioid receptor mRNA in the δ opioid receptor ligand response, although the direction of the response remains unclear, as was the case for κ opioid receptor responses.

In the course of investigating δ opioid agonists, a number of studies found selective δ opioid antagonists produced results that warranted further investigation in their own right. Early studies conducted by Carr *et al.* (1990) found no effect of naltrindole on splenocyte

proliferation (Table 1.4-1). However, subsequent studies reported by House *et al.* (1995b) and Gaveriaux-Ruff *et al.* (2001) found naltrindole caused significant inhibition of mitogen-induced proliferation. House *et al.* (1995b) reported that naltrindole, benzylidene naltrexone and naltriben inhibited B cell proliferation following a 72 hour incubation. Gaveriaux-Ruff *et al.* (2001) also found the same responses. Moreover, the animal model used by Gaveriaux-Ruff *et al.* (2001) was a triple δ/μ/κ opioid receptor deficient strain, thereby implicating a non-classical opioid receptor in the response. When the conclusions for δ opioid receptor antagonists are compared with the data for δ opioid receptor agonists, they suggest that either the δ opioid receptor antagonists were acting at a different receptor from the δ opioid receptor agonists, because of the significantly different response, or both the δ opioid receptor agonists and antagonists were acting at the same receptor, albeit a non-classical opioid site. The reports by Shahabi *et al.* (1995b) and Sharp *et al.* (1997) would suggest that the second hypothesis is not valid, however further work is required to confidently support the first hypothesis. In either case, non-classical opioid receptor activity must be involved. Finally, σ receptor agonists have been shown to modify splenocyte proliferation. Phencyclidine, 1,3-di-(o)-tolylguanidine, haloperidol, and (+)-pentazocine suppressed concanavalin A-induced proliferation at high (10^{-5} M) concentrations while (-)-pentazocine was inactive, thereby displaying stereospecificity (Carr *et al.*, 1991a) (Table 1.4-1). The action of these σ receptor agonists in this fashion is similar to the responses reported for other opioid ligands and reinforces potential for the involvement of non-classical opioid receptors in these responses.

Table 1.4-1 Summary of opioid effect on splenocyte proliferation.

Compound	Effect	End point	Antagonist	Study
(-)-Pentazocine	⇔	Con A-stimulated splenocyte proliferation		Carr *et al.* (1991a)
(+)-Pentazocine	↓	Con A-stimulated splenocyte proliferation		Carr *et al.* (1991a)
α-endorphin	⇔	Stimulated splenocyte proliferation		Gilman *et al.* (1982)
α-endorphin	⇔	LPS or dextran sulphate splenocyte proliferation		Gilman *et al.* (1982)
β-endorphin	↑	Splenocyte proliferation		Jessop *et al.* (1991a)

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Splenocyte proliferation		van den Bergh *et al.* (1994a; 1991)
β-endorphin	↑	Stimulated splenocyte proliferation	~~Naloxone~~	Gilman *et al.* (1982)
β-endorphin	↔	LPS or dextran sulphate splenocyte proliferation		Gilman *et al.* (1982)
β-endorphin	↑	Stimulated splenocyte proliferation	~~Naloxone~~	Gilmore *et al.* (1989)
β-endorphin	↑	Stimulated splenocyte proliferation		Gilmore *et al.* (1989)
β-endorphin	↓	PHA-stimulated rat splenocytes		Lin *et al.* (1994)
β-endorphin	↑	PHA-stimulated mouse splenocytes		Lin *et al.* (1994)
β-endorphin	↑	Stimulated splenocyte proliferation	Naloxone	Kusnecov *et al.* (1987)
1,3-di-(o)-tolylguanidine	↓	Con A-stimulated splenocyte proliferation		Carr *et al.* (1991a)
Benzylidene	↓	Splenic B cell proliferation		House *et al.* (1995b)
DADLE	↔	Splenic B cell proliferation		House *et al.* (1996)
DAME	↔	Splenic B cell proliferation		House *et al.* (1996)
DAME	↓	Purified murine $CD4^+$ and $CD8^+$ T cell proliferation	Naltrindole	Shahabi *et al.* (1995b)
DAMGO	↓	Staphylococcal enterotoxin B		Rogers *et al.* (1991)
DAMGO	↑	Con A stimulate splenocyte proliferation	Naloxone	Radulescu *et al.* (1991)
DAMGO	↑	Con A & LPS stimulate splenocyte proliferation	β-funaltrexamine ~~ICI 174864~~ ~~nor-BNI~~	Kowalski (1998b)
DAMGO (only high concentrations)	↓	Staphylococcal enterotoxin B, PHA and phorbol myristic acetate-induced proliferation	Naloxone Naltrexone	Eisenstein *et al.* (1991)
Deltorphin	↓	Purified murine $CD4^+$ and $CD8^+$ T cell proliferation	Naltrindole	Shahabi *et al.* (1995b)
Deltorphin (low)	↑	Con A-stimulated splenocyte proliferation	Naltrindole	Caroleo *et al.* (1994)
Deltorphin-1	↔	Splenic B cell proliferation		House *et al.* (1996)
Demorphin (high)	↓	Con A-stimulated splenocyte proliferation	Naloxone	Caroleo *et al.* (1994)
Demorphin (low)	↑	Con A-stimulated splenocyte proliferation	Naloxone	Caroleo *et al.* (1994)
Diacetylmorphine	↓ (no dose response relationship)	Splenic B cell proliferation		Thomas *et al.* (1995b)
DPDPE	↑	Con A & LPS stimulate splenocyte proliferation		Kowalski (1998b)
DPDPE	↓	Purified murine $CD4^+$ and $CD8^+$ T cell proliferation	Naltrindole	Shahabi *et al.* (1995b)
DPDPE (high concentrations only)	↓	Splenic B cell proliferation		House *et al.* (1996)
DPDPE-TFA	↔	Splenic B cell proliferation		House *et al.* (1996)
DSLET	↓	Splenic B cell proliferation		House *et al.* (1996)
DTLET	↔	Splenic B cell proliferation		House *et al.* (1996)
Dynorphin	↑	Sub-optimal mitogen-stimulated proliferation	~~Naloxone~~	Barreca *et al.* (1987)
Dynorphin	↔	Intense mitogen-stimulated proliferation		Barreca *et al.* (1987)
Dynorphin A (1-13)	↑	Stimulated splenocyte proliferation	Naloxone Nor-BNI	Ni *et al.* (1999)
Dynorphin A (1-17)	↑	Stimulated splenocyte proliferation	Naloxone Nor-BNI	Ni *et al.* (1999)
Fentanyl	↓	Splenic B cell proliferation		House *et al.* (1995a)
Haloperidol	↓	Con A-stimulated splenocyte proliferation		Carr *et al.* (1991a)

Compound	Effect	End point	Antagonist	Study
Leu-enkephalin	↓	PHA-stimulated mouse splenocyte proliferation		Lin *et al.* (1990)
Leu-enkephalin	↑	PHA-stimulated rat splenocyte proliferation		Lin *et al.* (1990)
Leu-enkephalin	↔	Splenocyte proliferation		van den Bergh *et al.* (1994a; 1991)
Leu-enkephalin	↑	Ornithine decarboxylase activity	Naloxone	Pignatti *et al.* (1998)
Leu-enkephalin (high concentrations)	↓	PHA-stimulated splenocyte proliferation		Plotnikoff (1982)
Leu-enkephalin (low concentrations)	↑	PHA-stimulated splenocyte proliferation		Plotnikoff (1982)
Met-enkephalin	↔	Splenocyte proliferation		Das *et al.* (1997)
Met-enkephalin	↑	T cell mitogen-induced splenocyte proliferation		Yang *et al.* (1990)
Met-enkephalin	↓	LPS-induced splenocyte proliferation		Yang *et al.* (1990)
Met-enkephalin	↑	LPS-induced splenocyte proliferation		Li (1998)
Met-enkephalin	↓	PHA-stimulated mouse splenocyte proliferation		Lin *et al.* (1990)
Met-enkephalin	↑	PHA-stimulated rat splenocyte proliferation		Lin *et al.* (1990)
Met-enkephalin	↔	Splenocyte proliferation		van den Bergh *et al.* (1994a; 1991)
Met-enkephalin (high concentrations)	↑	PHA-stimulated splenocyte proliferation		Plotnikoff (1982)
Met-enkephalin (low concentrations)	↓	PHA-stimulated splenocyte proliferation		Plotnikoff (1982)
Methadone (100 μM only)	↓	Splenic B cell proliferation		Thomas *et al.* (Thomas *et al.*, 1995b)
Morphine	↓	Splenocyte proliferation	~~Naltrexone~~ ~~Naloxone~~	Jessop *et al.* (1991a)
Morphine	↓	Staphylococcal enterotoxin B, PHA and phorbol myristic acetate-induced proliferation	Naloxone Naltrexone	Eisenstein *et al.* (1991)
Morphine	↓	Staphylococcal enterotoxin B	Naloxone Nor-NBI	Rogers *et al.* (1991)
Morphine (100 μM only)	↓	Splenic B cell proliferation		Thomas *et al.* (1995a)
Morphine (100 μM only)	↓	Splenocyte proliferation	~~Naloxone~~	Fecho *et al.* (1996a)
Morphine-3-glucuronide	↓	Splenic B cell proliferation		Thomas *et al.* (1995a)
Morphine-6-glucuronide	↓	Splenic B cell proliferation		Thomas *et al.* (1995a)
Naloxone	↑	Con A-induced splenocyte proliferation		Yang *et al.* (1990)
Naloxone	↑	Con A-stimulated splenocyte proliferation		Wu *et al.* (1999)
Naloxone	↔	LPS-stimulated splenocyte proliferation		Wu *et al.* (1999)
Naltrexone (100 μM only)	↓	Splenocyte proliferation		Fecho *et al.* (1996a)
Naltrexone naltriben	↓	Splenic B cell proliferation		House *et al.* (1995b)
Naltrindole	↔	Splenocyte proliferation		Carr *et al.* (1990)
Naltrindole	↓	Splenic B cell proliferation		House *et al.* (1995b)
Naltrindole	↓	Splenocyte proliferation		Gaveriaux-Ruff *et al.* (2001)
Normorphine	↓	Splenic B cell proliferation		Thomas *et al.* (1995a)
Ohmefentanyl (high concentrations)	↓	Con A-stimulated splenocyte proliferation	~~Naloxone~~	Wu *et al.* (1999)
Ohmefentanyl (high concentrations)	↓	LPS-stimulated splenocyte proliferation	~~Naloxone~~	Wu *et al.* (1999)
Ohmefentanyl (low concentrations)	↑	Con A-stimulated splenocyte proliferation	Naloxone	Wu *et al.* (1999)
Ohmefentanyl (low concentrations)	↔	LPS-stimulated splenocyte proliferation		Wu *et al.* (1999)

Compound	Effect	End point	Antagonist	Study
Pethidine	↓	Splenic B cell proliferation		House *et al.* (1995a)
Phencyclidine	↓	Con A-stimulated splenocyte proliferation		Carr *et al.* (1991a)
Pro-enkephalin	↔	Splenocyte proliferation		Das *et al.* (1997)
Pro-enkephalin A antisense oligodeoxynucleotides	↑	Splenocyte proliferation		Fulford *et al.* (2000)
Pro-opiomelanocortin antisense oligodeoxynucleotides	↓	Splenocyte proliferation		Fulford *et al.* (2000)
U50488	↑	Con A- & LPS-stimulated splenocyte proliferation		Kowalski (1998b)
U50488	↓	Ornithine decarboxylase activity		Pignatti *et al.* (1998)
U50488	↓	Con A-stimulated splenocyte proliferation	Nor-BNI	Caroleo *et al.* (1994)
U50488 (stereoselective)	↓	Staphylococcal enterotoxin B	~~Naloxone~~ ~~Nor-BNI~~	Rogers *et al.* (1991)
U69593	↓	Staphylococcal enterotoxin B		Rogers *et al.* (1991)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

These splenocyte proliferation data are indicative of the complexity and controversy of opioid immunomodulation. Whilst the data for μ and δ opioid effects display few classical opioid characteristics, κ opioid receptor involvement in modulation of splenocyte proliferation is likely, however the direction of these responses is unclear. Nonetheless, whilst some classical opioid activity has been reported, the non-classical opioid responses cannot be ignored and the identity and characteristics of these receptors must be determined.

1.1.1.13.2. Splenocyte cytokine production

As discussed previously (1.2.3) cytokines are integral to the propagation of immune activation signals, therefore in light of the effect opioids have on splenocyte proliferation, these influence on cytokine expression and release has also been investigated, with the data summarised in Table 1.4-2. Morphine has been found to have a mixed influence on cytokine production. For example, morphine had no effect on IL-2 secretion or receptor expression (Saini & Sei, 1993), however it displayed naloxone- and naltrexone-insensitive dose-

dependent inhibition of production of IL-2 and IL-4 (Jessop & Taplits, 1991a), μ opioid receptor-dependent (determined by knockout studies) inhibition of IFN-γ and IL-4 protein synthesis (Roy *et al.*, 2001a), and minor suppression of IL-2 production (Thomas *et al.*, 1995a). These data suggest morphine's action is via a non-classical opioid receptor, which is supported by the non-classical rank order of effect of its metabolites morphine-3-glucuronide, morphine-6-glucuronide and normorphine (Thomas *et al.*, 1995a). Data generated using other synthetic μ opioid ligands, methadone and diacetylmorphine also highlight the non-classical opioid effect on splenocyte cytokine production (Thomas *et al.*, 1995b).

Cytokine modulation by peptide μ opioid receptor agonists is varied, with DAMGO (Jessop & Taplits, 1991a) and β-endorphin (Jessop & Taplits, 1991b) shown to have no effect, whilst van den Bergh *et al.* (1991) found β-endorphin increased the production of IL-2 in a naloxone-insensitive fashion. Subsequent studies found that the presence of α- and β-endorphin in *in vitro* cultures of $CD4^+$ T cells, stimulated with concanavalin A or α-CD3 resulted in a substantial increase of IL-2, IL-4, IL-6 and IFN-γ production but not IL-1 (van den Bergh *et al.*, 1994a). As was concluded for the non-peptide μ opioid receptor ligands, these affects appear not to be mediated via the classical opioid receptor since cytokine modulation was preserved using a fragment of β-endorphin that lacks the N-terminal opioid receptor binding section (van den Bergh *et al.*, 1994a; van den Bergh *et al.*, 1994b). Interestingly, naloxone by itself enhanced cytokine synthesis, which van den Bergh *et al.* (1994a) suggested indicates the endogenous production by lymphocytes of inhibitory opioid peptides. In contrast to the non-classical opioid receptor conclusions for β-endorphin, Ni *et al.* (1999) found dynorphin A (1-13) and dynorphin A (1-17) enhanced concanavalin A IL-2 production in a dose-dependent, naloxone- and nor-binaltorphimine-sensitive fashion,

implicating a κ opioid receptor in this response, which agrees with the influence of κ opioid receptor agonists on proliferation discussed previously.

δ opioid receptor agonists also modulate cytokine production, with deltorphin found to enhance IL-2 levels at low concentrations (10^{-11} M) and to inhibit production at high concentrations (10^{-9} to 10^{-7} M) in purified $CD4^+$ or $CD8^+$ splenocytes from C57BL/6 and CD1 mice (Shahabi & Sharp, 1995a). House *et al.* (1996) found significantly increased IL-2 production by DPDPE, DPDPE-trifluoroacetate, and deltorphin-1, with no clear effect of DADLE, DSLET or DTLET. Interestingly, DAME caused substantial increases in IL-2 production at low concentrations and significant inhibition at high concentrations as Shahabi *et al.* (1995a) reported for deltorphin. On the other hand, IL-4 production was increased by DAME, DPDPE and DPDPE-trifluoroacetate, whilst the remaining δ opioid peptides were without significant effect on this response (House *et al.*, 1996). These cytokine data agree with splenocyte proliferation data mentioned previously, where deltorphin caused induction of proliferation at low concentrations (Caroleo *et al.*, 1994). Furthermore, the reports of deltorphin inhibiting proliferation (Shahabi & Sharp, 1995b) may be explained by the inhibition of cytokine production at high concentrations. However, the influence of δ opioid receptor agonists and antagonists on cytokine receptor expression is unclear (Carr *et al.*, 1990).

As was found for splenocyte proliferation, the action of δ opioid receptor antagonists on splenocyte cytokine production uncovered interesting results. House *et al.* (1997b) found IL-4 levels were significantly suppressed by low concentrations of either TIPP or D-TIPP, and by 10 μM ICI 174864, which agreed with their previous reports of increased IL-4 production by δ opioid receptor agonists (House *et al.*, 1996). They suggest a δ opioid receptor

excitatory tone in splenocyte activity, or the action of δ agonists and antagonists at two distinct receptors, as discussed previously. The stimulation and inhibition of cytokine production by the opioid ligands investigated agrees well in most cases with the previously discussed proliferation data. The nature of the receptor that mediates the μ and δ opioid receptor ligands responses is still unclear, as both classical and non-classical opioid responses were observed, whilst the small amount of available κ opioid receptor ligand data implicate a more classical κ opioid receptor in altered splenocyte cytokine production.

Table 1.4-2 Summary of the opioid effects on splenocyte cytokine production.

Compound	Effect	End point	Antagonist	Study
Azidophenazocine (high concs only)	↓	Stimulated IFN-γ production		Garza *et al.* (1993)
DADLE	⇔	IL-2 production		House *et al.* (1996)
DADLE	⇔	IL-4 production		House *et al.* (1996)
DAME	↑	IL-4 production		House *et al.* (1996)
DAME (high)	↓	IL-2 production		House *et al.* (1996)
DAME (low)	↑	IL-2 production		House *et al.* (1996)
DAME	↓	IL-2 production		Shahabi *et al.* (1995a)
DAMGO	⇔	Cytokine production		Jessop *et al.* (1991a)
Deltorphin (high)	↓	IL-2 production		Shahabi *et al.* (1995a)
Deltorphin (low)	↑	IL-2 production		Shahabi *et al.* (1995a)
Deltorphin-1	↑	IL-2 production		House *et al.* (1996)
Deltorphin-1	⇔	IL-4 production		House *et al.* (1996)
Diacetylmorphine	↓	Splenic T cell production of IL-2 and IL-4		Thomas *et al.* (1995a)
DPDPE	↑	IL-2 production		House *et al.* (1996)
DPDPE	↑	IL-4 production		House *et al.* (1996)
DPDPE-trifluoroacetate	↑	IL-2 production		House *et al.* (1996)
DPDPE-trifluoroacetate	↑	IL-4 production		House *et al.* (1996)
DSLET	⇔	IL-2 production		House *et al.* (1996)
DSLET	⇔	IL-4 production		House *et al.* (1996)
D-TIPP	⇔	IL-2 production		House *et al.* (1997b)
D-TIPP	↓	IL-4 production		House *et al.* (1997b)
DTLET	⇔	IL-2 production		House *et al.* (1996)
DTLET	⇔	IL-4 production		House *et al.* (1996)
Dynorphin A (1-13)	↑	Con A-stimulated IL-2 release	Naloxone Nor-BNI	Ni *et al.* (1999)
Dynorphin A (1-17)	↑	Con A-stimulated IL-2 release	Naloxone Nor-BNI	Ni *et al.* (1999)
α-endorphin	↑	Stimulated IL-2, IL-4, IL-6 and IFN-γ production		van den Bergh *et al.* (1994a)
α-endorphin	⇔	Stimulated IL-1 production		van den Bergh *et al.* (1994a)
β-endorphin	⇔	IL-2 and IL-4 production		Jessop *et al.* (1991a)
β-endorphin	↑	IL-2 production	~~Naloxone~~	van den Bergh *et al.* (1991)
β-endorphin	↑	Stimulated IL-2, IL-4, IL-6 and IFN-γ production		van den Bergh *et al.* (1994a)
β-endorphin	⇔	Stimulated IL-1 production		van den Bergh *et al.* (1994a)

Compound	Effect	End point	Antagonist	Study
Haloperidol (high concentrations only)	↓	Stimulated IFN-γ production		Garza *et al.* (1993)
ICI 174864	↓	IL-2 production		House *et al.* (1997b)
ICI 174864	↓	IL-4 production		House *et al.* (1997b)
Leu-enkephalin	↔	Stimulated cytokine release		Jessop *et al.* (1991a)
Met-enkephalin	↑	IL-2 & IL-6 production		Li (1998)
Met-enkephalin	↔	Stimulated IL-1, IL-2, IL-4, IL-6 or IFN-γ production		van den Bergh *et al.* (1994a; 1991; 1994b)
Methadone	↓	Splenic T cell production of IL-2 and IL-4		Thomas *et al.* (1995a)
Morphine	↓	Production of IL-2 and IL-4	~~Naloxone~~ ~~Naltrexone~~	Jessop *et al.* (1991a)
Morphine	↓	IFN-γ and stimulated IL-4 protein synthesis		Roy *et al.* (2001a)
Morphine	↔	Stimulated IL-2 production		Saini *et al.* (1993)
Morphine	↓	IL-2 production		Thomas *et al.* (1995a)
Morphine	↔	IL-4 production		Thomas *et al.* (1995a)
Morphine-3-glucuronide	↔	IL-2 production		Thomas *et al.* (1995a)
Morphine-3-glucuronide	↔	IL-4 production		Thomas *et al.* (1995a)
Morphine-6-glucuronide (100μM only)	↓	IL-2 production		Thomas *et al.* (1995a)
Morphine-6-glucuronide (100μM only)	↔	IL-4 production		Thomas *et al.* (1995a)
Naloxone	↑	Cytokine production		van den Bergh *et al.* (1994a)
Normorphine	↔	IL-2 production		Thomas *et al.* (1995a)
Normorphine	↔	IL-4 production		Thomas *et al.* (1995a)
Pentazocine (high concs only)	↓	Stimulated IFN-γ production		Garza *et al.* (1993)
TIPP	↔	IL-2 production		House *et al.* (1997b)
TIPP	↓	IL-4 production		House *et al.* (1997b)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.1.1.13.3. Splenocyte functional analysis

Many different aspects of splenocyte function have been investigated including immunoglobulin production, mixed lymphocyte response, cytotoxic T cell production, phagocytosis, protein synthesis, viability and toxicity, potassium conduction, cAMP production and calcium entry. As the research in this thesis does not directly involve any of these functions they will not be discussed in detail, instead reference tables summarising the available literature have been included in Chapter 8.

1.1.1.13.4. Summary

The fact that opioids modulate splenocyte function is unquestionable, however the direction of the response (i.e. inhibition or induction) and manner (i.e. classical opioid or non-classical opioid responses) in which the effect occurs remains unclear. It is evident that μ and δ opioid receptor ligands do not alter splenocyte function in a similar fashion to that which neuronal data would have us hypothesise, with non-classical rank order of effect, antagonist insensitivity and uncharacteristic ligand selectivity. These functional data are supported by the previously discussed μ and δ opioid receptor data (1.3.1 & 1.3.3). However, the splenocyte data for κ opioid receptor ligands more closely reflect that which one would expect when extrapolating the anticipated effect from neuronal data, although the presence of functional κ opioid receptor splice variants is apparent (Alicea *et al.*, 1998; McCarthy *et al.*, 2001b) or a κ opioid-like receptor.

1.4.1.4. Natural Killer cells (*in vitro*)

Particular interest in the effect of opioids on Natural Killer cell function has arisen due to the prevalence of HIV and AIDS in the intravenous injecting drug user population. Furthermore, the potential of cancer patients maintained on morphine to have reduced immune response to cancerous cells has also triggered research. A summary of the data available from the literature is found in Table 1.4-3. It is evident that the opioid system significantly modifies Natural Killer cell function, in some cases in a non-classically opioid manner, with

antagonist-insensitivity (House *et al.*, 1995a), non-stereoselectivity (Adelson *et al.*, 1994; Ochshorn *et al.*, 1989; Ochshorn *et al.*, 1990) and uncharacteristic rank order of effect (Thomas *et al.*, 1995a).

Table 1.4-3 Summary of the opioid effects on Natural Killer cell function.

Compound	Effect	End point	Antagonist	Study
(+)-PPP	⇔	Natural Killer cell function		Carr *et al.* (1992)
(R)-(-)- and (S)-(+)-Methadone (high concentrations only)	↓	Natural Killer cell function		Ochshorn *et al.* (1989; 1990)
(+/-)-Naloxone	↓	Natural Killer cell function		Adelson *et al.* (1994)
(+/-)-pentazocine	⇔	Natural Killer cell function		Carr *et al.* (1992)
β-endorphin	↓	Natural Killer cell function	Naloxone	Prete *et al.* (1986)
β-endorphin	↑	Natural Killer cell function	Naloxone	Kay *et al.* (1984)
β-endorphin	↑	Natural Killer cell function	Donor dependent	Morley *et al.* (1985)
β-endorphin	↑	Natural Killer cell function	Naloxone	Mathews *et al.* (1983)
β-endorphin	↑	Natural Killer cell function	Naloxone	Mandler *et al.* (1986)
β-endorphin	↑	Natural Killer cell function and granzyme B expression		Wakao *et al.* (2000)
β-endorphin	⇔	Natural Killer cell perforin and Fas ligand expression		Wakao *et al.* (2000)
1,3-di-(o)-tolylguanidine	↓	Natural Killer cell function		Carr *et al.* (1992)
Benzylidene naltrexone	↓	Natural Killer cell function		House *et al.* (1995b)
DADLE	↓	Natural Killer cell function		House *et al.* (1996)
DAME	↑	Natural Killer cell function		House *et al.* (1996)
Deltorphin-1	↑	Natural Killer cell function		House *et al.* (1996)
Diacetylmorphine	↓	Natural Killer cell function		Thomas *et al.* (1995a)
DPDPE	↑	Natural Killer cell function		House *et al.* (1996)
DSLET	↑	Natural Killer cell function		House *et al.* (1996)
D-TIPP	⇔	Natural Killer cell function		House *et al.* (1997b)
DTLET	↑	Natural Killer cell function		House *et al.* (1996)
Dynorphin	↓	Natural Killer cell function	Naloxone	Prete *et al.* (1986)
Enkephalins	↑	Natural Killer cell function		Zhong *et al.* (1996)
Enkephalins	↑	Natural Killer cell function	Naloxone	Mathews *et al.* (1983)
Enkephalins	↑	Natural Killer cell function	Donor dependent	Faith *et al.* (1984)
Enkephalins	↑	Natural Killer cell function	Post-traumatic stress disorder	Mosnaim *et al.* (1993)
Enkephalins	↑	Natural Killer cell function		Prete *et al.* (1986)
Enkephalins	↑	Natural Killer cell function		Bajpai *et al.* (1995)
Fentanyl	↓	Natural Killer cell function	~~Naltrexone~~	House *et al.* (1995a)
Fentanyl	⇔	Natural Killer cell function		Jacobs *et al.* (1999)
Haloperidol	↓	Natural Killer cell function		Carr *et al.* (1992)
ICI 174864	⇔	Natural Killer cell function		House *et al.* (1997b)
Pethidine	↓	Natural Killer cell function	~~Naltrexone~~	House *et al.* (1995a)
Methadone (high concentrations only)	↓	Natural Killer cell function		House *et al.* (1995a)
Methadone (high concentrations only)	↓	Natural Killer cell function		Ochshorn *et al.* (1989)
Morphine	↓	Natural Killer cell function		Condevaux *et al.* (2001)
Morphine	↓	Natural Killer cell respiratory burst		Molitor *et al.* (1992b)
Morphine	⇔	Natural Killer cell function		Thomas *et al.* (1995a)
Morphine-3-glucuronide	⇔	Natural Killer cell function		Thomas *et al.* (1995a)
Morphine-6-glucuronide	↑	Natural Killer cell function		Thomas *et al.* (1995a)
Naloxone	↑⇔↓	Natural Killer cell function	Donor dependent	Martin-Kleiner *et al.* (1993)
Naloxone	↓	Natural Killer cell function		Kay *et al.* (1990)

Compound	Effect	End point	Antagonist	Study
Naltriben	↓	Natural Killer cell function		House *et al.* (1995b)
Naltrindole	↓	Natural Killer cell function		House *et al.* (1995b)
Normorphine	↔	Natural Killer cell function		Thomas *et al.* (1995a)
TIPP	↔	Natural Killer cell function		House *et al.* (1997b)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.4.1.5. Peripheral blood mononuclear cells (*in vitro*)

In human studies, the most accessible source of immunocompetent cells is from peripheral blood. Therefore, many opioid immunopharmacology studies have been performed using isolated peripheral blood mononuclear cells. In fact it was using this cell type that Wybran *et al.* (1979) first demonstrated opioid modulation of immune function. It is important to note that whilst these isolated immune cells are not fully differentiated and functioning as they would in peripheral tissues or organs, they do provide a good indication of the status of the immune system. Moreover, with additional stimulation *in vitro*, they can differentiate into their other forms, and the function of the cells in these states can be quantified. Due to the clinical significance of human research, a variety of functional immune assays have been used to quantify opioid modulation of peripheral blood mononuclear cells. These include oxygen production, mixed lymphocyte response, cytotoxic T lymphocyte response, proliferation, cytokine expression, rosette formation, amino peptidase activity, chemotaxis, HIV infectivity, phagocytosis, degranulation and immunoglobulin production. Other more cellular based assays such as viability, nuclear factor-κB binding, cell rounding and nitric oxide production, inducible form of NOS expression and substance P expression have also been used. The immune functions that are directly related to this research project are reviewed, with summaries of the other available data in Chapter 8.

Wybran *et al.* (1979) was the first to clearly demonstrate opioid immunomodulation. Specifically, morphine and dextromoramide inhibited the percent of active T rosettes in a

naloxone-sensitive fashion. Interestingly met-enkephalin increased the percent of active rosettes in a naloxone-sensitive fashion. Moreover, the response was demonstrated to be stereoselective since dextromoramide significantly inhibited the response whilst levomoramide had no effect. Subsequent studies have expanded these findings, although not all have displayed such opioid characteristics. For example, Molitor *et al.* (1992b) found morphine exposure to peripheral blood mononuclear cells decreased oxygen species generation naloxone-insensitively, whilst methadone's response was naloxone-sensitive. The use of a gradually expanding range of opioid ligands also highlighted non-classical opioid rank order of effect, for instance codeine and codeine-6-glucuronide both inhibited a mixed lymphocyte reaction to a similar extent as morphine and morphine-6-glucuronide despite their significantly reduced μ opioid receptor binding affinity. As observed by Molitor *et al.* (1992b) for methadone, several examples of classical opioid receptor activity have also been demonstrated. The significance of these findings, with reference to classical and non-classical opioid characteristics, will now be discussed in greater detail.

1.1.1.13.5. Peripheral blood mononuclear cell proliferation

Human peripheral blood immune cells, commonly known as white blood cells, comprise 60-70% neutrophils, 2-4% eosinophils, 1% basophils, 20-25% lymphocytes and 3-8% monocytes. Of these cells, lymphocyte and monocytes are mononuclear cells which are often isolated from peripheral blood using iso-osmotic density gradients that are denser than mononuclear cells and lighter than granulocytes, with erythrocytes aggregated or preferentially lysed. A summary of the data for opioid modulation of peripheral blood mononuclear cell proliferation can also be found in Table 1.4-4.

Some studies show morphine, has no effect on cell proliferation (Hole, 1984; Palm *et al.*, 1996; Saito *et al.*, 1975), causes a concentration-dependent inhibition (Deitch *et al.*, 1988; Guarnaccia *et al.*, 1990; Makman *et al.*, 1995a; Sergeeva *et al.*, 1993; Srinivasan *et al.*, 1996) or induces the response (Bocchini *et al.*, 1983; Stefano *et al.*, 1995). As observed for other immune cell types and functions, the opioid nature of the responses also varies. Classical opioid antagonism and stereoselectivity have been reported by Makman *et al.* (1995a), whilst Srinivasan *et al.* (1996) found non-classical rank order of effect as they had observed for mixed lymphocyte reactions. In the majority of cases, morphine was a very effective modulator of peripheral blood mononuclear cell proliferation, with maximal effect at physiological concentrations (nM to low μM). However, potent inhibition of proliferation was not reported by Fecho *et al.* (1996a), who found i*n vitro* incubation with morphine had no effect on mitogen-induced proliferation at all concentrations except for the highest (100 μM), with naltrexone unable to antagonise the response. Instead, naltrexone had the same effect at 100 μM when tested alone. These effects of morphine, once again appear to be non-classical opioid responses. This conclusion for μ opioid receptor agonists is reinforced by the significant inhibition by methadone above 50 μM (Singh, 1980) and the ligand selectivity reported for DAMGO inactivity (Hucklebridge *et al.*, 1990; Morgan, 1996).

Modulation of peripheral blood mononuclear cell proliferation by β-endorphin is primarily via a naloxone-insensitive receptor, however the direction of the response varies. Ientile *et al.* (1997a; 1997b) established that β-endorphin was able to enhance spermidine transport and dose-dependently enhanced the incorporation of radioactive spermidine and spermine in a naloxone-insensitive manner. Similarly, Sulowska *et al.* (2002) and Fontana *et al.* (1987) also observed increases in quantified proliferation. Conversely, McCain *et al.* (1986; 1982) and Deitch *et al.* (1988) found β-endorphin to be a suppressor of phytohemagglutinin-induced T

cell proliferation, while Owen *et al.* (1998) reported minimal β-endorphin effect. Despite the lack of a clear β-endorphin response direction the effects appear to be mediated via the naloxone-insensitive β-endorphin receptor discussed previously (1.3.5)

Studies using δ opioid receptor ligands found a variety of compounds were able to induce proliferation, including DSLET, DPDPE and met-enkephalin (Bajpai *et al.*, 1995; Bajpai *et al.*, 1997; Hucklebridge *et al.*, 1990; Sulowska *et al.*, 2002). Some evidence of classical opioid antagonism was also seen for the met-enkephalin response which was blocked by ICI 174864 (Hucklebridge *et al.*, 1990) and naloxone (Bajpai *et al.*, 1997). Other methods to modulate δ opioid tone have also been employed. For example, using an anti-sense oligonucleotide strategy, Kamphuis *et al.* (1998) blocked the production of met-enkephalin and enkephalin-containing intermediary peptides, which resulted in enhancement of the proliferative T cell response and inhibition of monocyte IL-6 secretion. *In vitro* reconstitution of the anti-sense treated cultures with exogenous met-enkephalin or deltorphin reversed the inhibition of monocyte IL-6 production. However, the addition of exogenous met-enkephalin or deltorphin to the anti-sense treated T cell cultures was not enough to have an effect on T cell proliferation.

These data for opioid modulation of peripheral blood mononuclear cell function agree with previously discussed splenocyte μ opioid ligand data and provide further evidence of the action of these ligands via a non-classical opioid receptor. In contrast to previous cell types and immune functions, the δ opioid response displayed more classical receptor characteristics, although fewer studies have been conducted. The characteristics of κ opioid ligand effects on peripheral blood mononuclear cell proliferation are unclear, as minimal data are available. The use of human immune cells emphasises the clinical significance of opioid

immunomodulation. Moreover, the data highlights the significant role endogenous opioids have in controlling immune function and the potential pharmacodynamic impact of exogenous opioid administration. In light of the inhibition of immune function by opioid exposure, further research may be required to ascertain the impact of opioid exposure in immunosuppressed and chronically opioid-maintained patients. The research conducted thus far on *in vivo* immune status in humans following opioid exposure is discussed (1.4.2 and 1.4.4).

Table 1.4-4 Summary of opioid effects on peripheral blood mononuclear cell proliferation.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Proliferation	~~Naloxone~~	Ientile *et al.* (1997a; 1997b)
β-endorphin	↑	Proliferation		Sulowska *et al.* (2002)
β-endorphin	⇔	PHA-induced proliferation		Fontana *et al.* (1987)
β-endorphin	↑	Con A-induced proliferation	~~Naloxone~~	Fontana *et al.* (1987)
β-endorphin	↓	Proliferation	~~Naloxone~~	McCain *et al.* (1986; 1982)
β-endorphin	↓	Proliferation		Deitch *et al.* (1988)
β-endorphin	⇔	Proliferation		Owen *et al.* (1998)
Codeine	↓	Proliferation		Srinivasan *et al.* (1996)
Codeine-6-glucuronide	↓	Proliferation		Srinivasan *et al.* (1996)
DAMGO	⇔	Proliferation		Morgan (1996)
DAMGO	⇔	Proliferation		Hucklebridge *et al.* (1990)
Deltorphin-1 + naltrindole	↓	Proliferation		Noviello *et al.* (1997)
Deltorphin-I	↑	Proliferation		Noviello *et al.* (1997)
DPDPE	↑	Proliferation		Hucklebridge *et al.* (1990)
DPDPE	⇔	Proliferation		Hucklebridge *et al.* (1990)
DPDPE	⇔	Proliferation		Morgan (1996)
DSLET	↑	Proliferation		Hucklebridge *et al.* (1990)
Fentanyl	⇔	Proliferation		Hole (1984)
Leu-enkephalin	⇔	Proliferation		Owen *et al.* (1998)
Levorphanol	↓	Peripheral blood granulocyte activation induced by TNF-α	Naloxone	Makman *et al.* (1995a)
Met-enkephalin	↑	Proliferation	ICI 174864	Hucklebridge *et al.* (1990)
Met-enkephalin	↑	Proliferation	Naloxone	Bajpai *et al.* (1997)
Met-enkephalin	↑	Proliferation		Bajpai *et al.* (1995)
Met-enkephalin	↑	Proliferation		Sulowska *et al.* (2002)
Met-enkephalin	⇔	Proliferation		Owen *et al.* (1998)
Met-enkephalin anti-sense oligonucleotide	↑	Proliferation		Kamphuis *et al.* (1998)
Methadone (high concentrations)	↓	Proliferation		Singh (1980)
Morphine	↓	Peripheral blood granulocyte activation induced by TNF-α	Naloxone	Makman *et al.* (1995a)
Morphine	↓	IL-1-induced activation granulocytes	Naloxone	Makman *et al.* (1995a)
Morphine	↓	Proliferation		Srinivasan *et al.* (1996)
Morphine	↓	Proliferation		Sergeeva *et al.* (1993)
Morphine	↓	Proliferation		Guarnaccia *et al.* (1990)
Morphine	↓	Proliferation		Deitch *et al.* (1988)

Compound	Effect	End point	Antagonist	Study
Morphine	⇔	Proliferation		Saito *et al.* (1975)
Morphine	⇔	Proliferation		Hole (1984)
Morphine	⇔	Proliferation		Palm *et al.* (1996)
Morphine	↑	Proliferation	Naloxone	Stefano *et al.* (1995)
Morphine (high concentrations)	↓	Proliferation		Bocchini *et al.* (1983)
Morphine (highest concentration only)	↓	Proliferation	~~Naltrexone~~	Fecho *et al.* (1996a)
Morphine (low concentrations)	↑	Proliferation		Bocchini *et al.* (1983)
Morphine-3-glucuronide	⇔	Proliferation		Palm *et al.* (1996)
Morphine-6-glucuronide	↓	Proliferation		Srinivasan *et al.* (1996)
Morphine-6-glucuronide	⇔	Proliferation		Palm *et al.* (1996)
MPF (Lys-Lys-Gly-Glu)	↑	Proliferation		Owen *et al.* (1998)
Naloxone (high concentration)	↓	Proliferation		Bocchini *et al.* (1983)
Naloxone (low concentration)	↑	Proliferation		Bocchini *et al.* (1983)
Naltrexone	↓	Proliferation		Fecho *et al.* (1996a)
Naltrindole	↑	Proliferation		Noviello *et al.* (1997)
Nociceptin/orphanin FQ	↓	Proliferation		Peluso *et al.* (2001)
Peptidomimetic compound (Tyr-NH-CH2-CH2-O-Phe-NH2)	↓	Proliferation	Naloxone	Narayan *et al.* (2002)
U50488 (high concentrations)	↓	Proliferation		Morgan (1996)

⇔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.1.1.13.6. Peripheral blood mononuclear cell signalling molecules and receptor expression

Cytokine production by peripheral blood mononuclear cells is an integral step in the activation of a successful immune response, and therefore modulations in their expression and release may be involved in opioid immunomodulations. A summary of the data for opioid modulation of peripheral blood mononuclear cell cytokine expression can be found in Table 1.4-5, with a discussion of the results to follow.

Morphine has been shown to selectively increase the release of TGF-β without altering the release of TNF-α or IL-6 in a naloxone-sensitive manner (Chao *et al.*, 1992). Although, Zhou *et al.* (1992) found that morphine did not alter TGF-β1 mRNA levels, potentially indicating

that the increase of TGF-β release observed by Chao *et al.* (1992) may involve post-transcriptional mechanisms. Chao *et al.* (1993) also reported that morphine treated peripheral blood mononuclear cells released less TNF-α when subsequently stimulated with lipopolysaccharide and phytohemagglutinin. Interestingly, naloxone antagonised the inhibitory effect of morphine on TNF-α release in phytohemagglutinin, but not lipopolysaccharide-stimulated cells (Chao *et al.*, 1993). Moreover, TGF-β antibodies enhanced morphine inhibition of TNF-α release (Chao *et al.*, 1993). A possible explanation for this may be drawn from a study conducted by Stefano *et al.* (1994), who observed a reduced response of human granulocytes to TNF-α following morphine exposure (Stefano *et al.*, 1994). These authors established that there was a parallel increase in expression of neutral endopeptidase 24.11 caused by morphine, in a naloxone-sensitive manner, which resulted in a down regulation of the stimulatory effect of TNF-α since neutral endopeptidase 24.11 degrades TNF-α. The physiological significance of opioid-induced changes in endopeptidase 24.11 expression is unclear, however it strengthens the hypothesis of opioid involvement in immune system function and control. Finally, Roy *et al.* (2001a) found that morphine treatment of human peripheral blood mononuclear cells decreased IL-2 and IFN-γ and increased IL-4 and IL-5 in a concentration-dependent manner. These authors went on to suggest that this profile of cytokine expression induced by morphine exposure pushed the immune system to initiate a T_H2 type response (which was discussed previously 1.2.3). The physiological significance of this switch in cytokine production and resultant changes in immune function is discussed later in this chapter (1.4.2.1). The opioid characteristics of morphine's cytokine modulatory response displayed some non-classical opioid features, once again emphasising the likelihood of novel receptors mediating these responses.

The μ opioid peptide, DAMGO, behaved differently to morphine and significantly inhibited the human peripheral blood mononuclear cell production of IL-6 in a CTAP-sensitive fashion (Morgan, 1996). DAMGO also increased the expression of chemokines and their receptors MCP-1, RANTES, IFN-γ-inducible protein-10 (IP-10) and CCR5 (Szabo *et al.*, 2001). Wetzel *et al.* (2000) extended these findings and examined the influence of DAMGO on chemokine expression in HIV-infected cells and reported significant increases in RANTES and IP-10 expression, whilst MCP-1 protein levels remained unaffected. These data highlight the role opioids have in chemotactic signalling which has been discussed previously (1.4.1.2).

The function δ opioid ligands have in modulation of immune signalling has also been studied. Naloxone-insensitive increases in IFN levels (Brown & Van Epps, 1986) and naloxone-sensitive increases in IL-2 concentrations by met-enkephalin were found (Bajpai *et al.*, 1997). In contrast, ICI 174864-sensitive inhibition of IL-6 production by met-enkephalin and DPDPE has also been found (Morgan, 1996). Interestingly, δ opioid receptor agonists also appear to be involved in chemotaxis (Brown & Van Epps, 1985)

These data demonstrate the significant impact opioids have on cytokine production; however the physiological significance of some of these changes at this stage is not apparent. Nonetheless, the data suggesting a re-direction of the immune response to T_H2 type signalling is significant. These cytokine data are slightly different to the previously presented splenocyte cytokine production results. This is to be expected as cell type and compartment specificity of opioid response have been reported, and circulating immune cells have slightly different characteristics to their organ residing counterparts.

Table 1.4-5 Summary of the effect of opioids on peripheral blood mononuclear cell cytokine and chemokine release and mRNA expression and receptor expression.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	IFN production	~~Naloxone~~	Brown *et al.* (1986)
β-endorphin	↓	T cell chemotactic factors		Brown *et al.* (1985)
DAMGO	↓	IL-6 production	CTAP	Morgan (1996)
DAMGO	↑	MCP-1 expression		Szabo *et al.* (2001)
DAMGO	↑	RANTES		Szabo *et al.* (2001)
DAMGO	↑	CCR5		Szabo *et al.* (2001)
DAMGO	↑	IP-10 expression		Wetzel *et al.* (2000)
DAMGO	↑	RANTES		Wetzel *et al.* (2000)
DAMGO	↔	MCP-1 expression		Wetzel *et al.* (2000)
Deltorphin-1	↑	IFN-γ production		Noviello *et al.* (1997)
Deltorphin-1 + naltrindole	↔	IFN-γ production		Noviello *et al.* (1997)
DPDPE	↓	IL-6 production	ICI 174864	Morgan (1996)
Fentanyl	↔	IL-1β, TNF-α or TNF-α soluble receptor levels		McBride *et al.* (1996)
Fentanyl	↑	IL-1Ra release		McBride *et al.* (1996)
Met-enkephalin	↑	IFN production	~~Naloxone~~	Brown *et al.* (1986)
Met-enkephalin	↑	IL-2 production	Naloxone	Bajpai *et al.* (1997)
Met-enkephalin	↓	T cell chemotactic factors	Indomethacin	Brown *et al.* (1985)
Methadone	↔	TGF-β1		Zhou *et al.* (1992)
Morphine	↑	Stimulated TGF-β release	Naloxone	Chao *et al.* (1992)
Morphine	↔	Stimulated TNF-α and IL-6 release		Chao *et al.* (1992)
Morphine	↔	TGF-β1 mRNA levels		Zhou *et al.* (1992)
Morphine	↓	IFN-α and IFN-β levels	~~Naloxone~~	Nair *et al.* (1997)
Morphine	↓	IFN-α production		Stoll-Keller *et al.* (1997)
Morphine	↓	IFN-γ production	Naloxone	Peterson *et al.* (1987)
Morphine	↓	PHA-stimulated TNF-α production	Naloxone	Chao *et al.* (1993)
Morphine	↓	LPS-stimulated TNF-α production	~~Naloxone~~	Chao *et al.* (1993)
Morphine	↓	IL-2 and IFN-γ release		Roy *et al.* (2001a)
Morphine	↑	IL-4 and IL-5 release		Roy *et al.* (2001a)
Morphine	↑	IL-4 mRNA levels		Roy *et al.* (2001a)
Naltrindole	↑	IFN-γ production		Noviello *et al.* (1997)
Peptidomimetic compound (Tyr-NH-CH2-CH2-O-Phe-NH2)	↓	IL-9 and IL-10 production		Narayan *et al.* (2002)
Peptidomimetic compound (Tyr-NH-CH2-CH2-O-Phe-NH2)	↔	IL-2, IL-15 and IFN-γ production		Narayan *et al.* (2002)
U50488	↓	IL-6 production	Nor-BNI	Morgan (1996)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.1.1.13.7. Peripheral blood mononuclear cell chemotaxis

The effect of opioids on chemotaxis has been intensely researched. Interestingly, unlike the majority of all the other literature available on opioid immunomodulation of peripheral blood mononuclear cells, there is considerable lack of consensus in the results as summarised in Table 1.4-6. Morphine, methadone and DAMGO all readily caused reduced chemotaxis, with several groups finding a classical opioid mechanisms of action for morphine. However, β-endorphin enhanced chemotaxis is naloxone-sensitive. This difference between μ opioid receptor ligands is perplexing and suggests different naloxone-sensitive receptors are involved in the response. Chemotaxis following met-enkephalin exposure the only opioid investigated where a consensus was not apparent with naloxone-sensitivity and -insensitivity of the enhanced and inhibited responses observed (Table 1.4-6). Zhang *et al.* (2003) proposed a possible mechanism by which met-enkephalin caused a decrease in chemokine receptor affinity and coupling efficiency between receptors and G-protein. Therefore, this heterologous desensitisation resulted in reduced chemotactic signalling, blocking chemotactic movement. This finding has significant implications as it suggests direct intra-cellular communication between opioid receptors and chemotactic receptors. Moreover, it explains the previously described chemotactic behaviour of opioids by different cell types.

Table 1.4-6 Summary of the effects of opioid exposure on peripheral blood mononuclear cell chemotaxis.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↔	Chemotaxis or random migration		Deitch *et al.* (1988)
β-endorphin	↑ (Dependent on peptide NH2-terminal sequence)	Chemotaxis	Naloxone	Heagy *et al.* (1990)
β-endorphin	↑	Chemotaxis	Naloxone (stereoselective)	Ruff *et al.* (1985)
β-endorphin	↑	Chemotaxis		Pasnik *et al.* (1999)
DADLE	↑	Chemotaxis		Heagy *et al.* (1990)
DAMGO	↓	Chemotaxis (IL-8 and RANTES)		Miyagi *et al.* (2000)
DAMGO	↓	Chemotaxis	Naloxone	Choi *et al.* (1999)
DAMGO	↑	Chemotaxis		Heagy *et al.* (1990)
Dextrallorphan	↔	Chemotaxis		Ruff *et al.* (1985)
DPDPE	↑	Chemotaxis		Heagy *et al.* (1990)
Dynorphin	↓	Chemotaxis (IL-8 and RANTES)		Miyagi *et al.* (2000)
Endomorphin	↓	Chemotaxis	Naloxone	Choi *et al.* (1999)
Leu-enkephalin	↑	Chemotaxis		Heagy *et al.* (1990)
Leu-enkephalin	↑	Chemotaxis	Naloxone (stereoselective)	Ruff *et al.* (1985)
Levallorphan	↑	Chemotaxis	Naloxone (stereoselective)	Ruff *et al.* (1985)
Met-enkephalin	↓	Chemotaxis or random migration		Deitch *et al.* (1988)
Met-enkephalin	↓	Chemotaxis (MIP1 α or IL-8)	Naloxone	Grimm *et al.* (1998)
Met-enkephalin	↔	Chemotaxis (NAP-2)		Grimm *et al.* (1998)
Met-enkephalin	↓	Chemotaxis (IL-8 and RANTES)	~~Naltrindole~~ ~~Nor-BNI~~ ~~Naloxone~~	Miyagi *et al.* (2000)
Met-enkephalin	↑	Chemotaxis		Heagy *et al.* (1990)
Met-enkephalin	↑	Chemotaxis		Pasnik *et al.* (1999)
Met-enkephalin	↓	Chemotaxis (MIP-1a)		Zhang *et al.* (2003)
Met-enkephlamide	↑	Chemotaxis		Sich *et al.* (1987)
Met-enkephlamide	↔	Chemotaxis (optimal FMLP)		Sich *et al.* (1987)
Met-enkephlamide	↑	Chemotaxis (low FMLP)		Sich *et al.* (1987)
Methadone	↓	Chemotaxis	Naloxone	Choi *et al.* (1999)
Morphine	↓	Chemotaxis		Deitch *et al.* (1988)
Morphine	↓	Chemotaxis (MIP1 α or IL-8)	Naloxone	Grimm *et al.* (1998)
Morphine	↓ Minimally affected	Chemotaxis PBMC from patients with histiocytic medullary reticulosis		Fricchione *et al.* (1997)
Morphine	↓	Chemotaxis (DAMA and IL-1)		Stefano *et al.* (1998)
Morphine	↓	Chemotaxis (IL-8 and RANTES)		Miyagi *et al.* (2000)
Morphine	↓	Chemotaxis	Naloxone	Choi *et al.* (1999)
Naloxone (highest concentration only)	↓	Chemotaxis		Deitch *et al.* (1988)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.1.1.13.8. Summary

There is no doubt that opioids significantly alter peripheral blood mononuclear cell function in various species, including humans. The role classical opioid-like receptors play in mediating these effects appears to be limited to κ opioid ligands, with μ and δ opioid receptor compounds behaving in non-classical opioid fashions in many cases. The reasons for both classical and non-classical opioid effects being observed using μ and δ ligands are unclear. However, this does not reduce the significance of the effects. Interestingly, there are numerous reports of interindividual variability in the response of peripheral blood mononuclear cells to opioid exposure (Balog *et al.*, 2001; Balog *et al.*, 1999; Brown & Van Epps, 1986; Fontana *et al.*, 1987). The reasons for this variability are unknown, but could involve factors such as genetic polymorphisms in receptor expression, activity or signalling, or variations in immune activation status. Nonetheless, the clinical significance of such variability should be elucidated, as the impact of interindividual variations in immunocompetent cell response to opioids could be wide ranging. For example, the expression of substance P has been shown to be up-regulated by morphine in human mononuclear phagocytes and lymphocytes at both the mRNA and the protein level in a naloxone-sensitive manner (Li *et al.*, 2000), with increases in the expression of the substance P receptor (NK-1R) also observed (Li *et al.*, 2000). This has extreme physiological relevance as increases in substance P expression are associated with hyperalgesia (Zimmermann, 1991); therefore, possibly implicating the immune system in the reduction of efficacy after prolonged morphine exposure and interindividual variations in pharmacodynamic response to opioids.

1.4.1.6. Other cells of the immune system (*in vitro*)

Many other cell types of the immune system have been isolated and exposed *in vitro* to opioid ligands. These cell types include: Peyer's patch cells, keratinocytes, alveolar macrophages, Langerhans cells, dendritic cells, peritoneal neutrophils and macrophages, bone marrow cells, lymph node cells, basophils and mast cells. As these cells are not directly related to this thesis, they will not be discussed. Summaries of the available literature are Chapter 8.

1.4.1.7. Cell lines (*in vitro*)

1.1.1.13.9. Cancer cells

The influence of methadone on the growth and survival of cancerous cells has revealed non-stereoselective binding (Maneckjee & Minna, 1997) and inhibition of growth (Maneckjee & Minna, 1992). However, despite these non-classical opioid effects, naltrexone was capable of antagonising methadone's effects (Maneckjee & Minna, 1992). The mechanism by which methadone reduced cancer cell growth required *de novo* mRNA and protein synthesis, implying the response was not merely non-specific necrosis of the cells. These methadone data strongly implicate a non-classical opioid receptor, however, the naltrexone-sensitivity of the response confuses the issue, as other non-classical opioid actions of methadone discussed previously displayed non-stereoselectivity and antagonist-insensitivity. Morphine and a synthetic derivative KT-90 have also been shown to reduce cancerous cell growth (Sueoka *et al.*, 1998). Further studies are required to investigate these results and the mechanisms involved in the responses, as the clinical application of opioids in this fashion may be beneficial in combination chemotherapy for treating cancer and cancer associated pain.

1.1.1.13.10. Other cell lines

The development of knowledge and expertise in culturing cell lines has allowed numerous groups to utilised these models to further characterise opioid-induced immunomodulation. Table 1.4-7 summarises the immunomodulation of a number of different cell lines. In contrast to many primary tissue culture data, studies using cell lines demonstrated many classical opioid receptor characteristics, especially for μ and κ opioid receptor agonists. However, some non-classical opioid responses were evident. Roy *et al.* (1996) concluded that the Bac 1.2 F5 macrophage cell line expresses two receptors types: a classical δ opioid receptor that was responsible for the increased proliferation; and a non-classical opioid receptor through which morphine inhibited proliferation. In contrast, Iuvone *et al.* (1995) suggested that μ and κ opioid receptors were the prominent modulatory forces in J774 macrophage nitric oxide production, while δ opioid receptors played a minimal role. They came to this conclusion on the basis of the rank order of effect: morphine > DAMGO > U50488 > DPDPE = Deltorphin II. However, it would be expected that DAMGO would be more potent than morphine if a classical opioid effect were apparent. The use of cell lines has allowed more detailed biochemical characterisation of the opioid-induced modulations. Due to the "purity" of cells used in cell line experiments there may be a lack of co-stimulation signals that would impact on the results, as discussed previously. This may explain some of the differences between the results from primary tissue culture and cell lines.

Table 1.4-7 Summary of the effects of opioids on several immune cell line functions.

Compound	Effect	End point	Antagonist	Study
VARIOUS IMMUNE CELL LINES				
β-endorphin	↑	Stimulated IL-2 production (EL-4 and LBRM33-1A5 cells)	Naloxone	Bessler *et al.* (1990)
γ-endorphin	↑	Stimulated IL-2 production (EL-4 and LBRM33-1A5 cells)		Bessler *et al.* (1990)
Enkephalins	↑	Stimulated IL-2 production (EL-4 and LBRM33-1A5 cells)		Bessler *et al.* (1990)

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Cellular DNA synthesis and the cellular population growth of mitogen-induced peripheral blood mononuclear cells, B-lymphoma Namalva cells and EBV-transformed lymphocytes		Sergeeva *et al.* (1993)
Morphine	↑	GHR mRNA levels (IM-9 cells)	Naloxone	Henrohn *et al.* (1997)
Morphine	↑	Proliferation (IM-9 cells)		Henrohn *et al.* (1997)
Morphine	↑	Expression ERK1, ERK2, MEK1 and MEKK	Naloxone	Chuang *et al.* (1997)
T CELL LINES				
DADLE	↑	Intracellular calcium (DOR-Jul.1)	Pertussis toxin EGTA	Sharp *et al.* (1998a)
DALDE	↓	Stimulated cAMP production		Sharp *et al.* (1998a)
DAMGO	↑	Apoptosis (Jurkat)		Singhal *et al.* (1999)
Deltorphin	↑	Intracellular calcium (DOR-Jul.1)	Pertussis toxin EGTA	Sharp *et al.* (1998a)
DPDPE	↑	cAMP levels in NALM 6 and (Jurkat)	Naloxone	Heagy *et al.* (1999)
Met-enkephalin	↑	cAMP levels in NALM 6 and (Jurkat)	Naloxone	Heagy *et al.* (1999)
Met-enkephalin	↓	Chemotaxis to RANTES (Jurkat)		Rogers *et al.* (2000)
Met-enkephalin	⇔	Chemotaxis to SDF-1α (Jurkat)		Rogers *et al.* (2000)
Morphine	↑	Proliferation of myeloid K562 and T-lymphoma (Jurkat)		Sergeeva *et al.* (1993)
Morphine	↑	IL-4 promoter activity (Jurkat)		Roy *et al.* (2001a)
Morphine	↑	NFAT binding to response element (Jurkat)		Roy *et al.* (2001a)
Morphine	⇔	Protein synthesis and cAMP levels		Jessop *et al.* (1991b)
Morphine	↑	Apoptosis and necrosis (Jurkat)	Naloxone	Singhal *et al.* (1999)
Morphine	↑	Bax expression (Jurkat)		Singhal *et al.* (1999)
Morphine	↓	Bcl-2 expression (Jurkat)		Singhal *et al.* (1999)
Morphine	↑	Activation of caspase-3		Singhal *et al.* (1999)
U50488	⇔	Stimulated or unstimulated intracellular calcium levels (R1.1)		Martin-Kleiner *et al.* (2002)
U50488	↓	κ opioid receptor expression (mouse thymoma cell lines R1.G1 and R1EGO)		Martin-Kleiner *et al.* (2001)
U50488	↓	IL-2 production and expression of CD25 (DPK)		Guan *et al.* (1998)
U50488	↓	Differentiation (DPK)		Guan *et al.* (1998)
U69593	↓	Unstimulated intracellular calcium levels (R1.1)	~~Nor-BNI~~	Martin-Kleiner *et al.* (2002)
U69593	↑	Stimulated intracellular calcium levels (R1.1)		Martin-Kleiner *et al.* (2002)
MACROPHAGE CELL LINES				
DADLE	↑	Proliferation (Bac 1.2 F5)	Naloxone	Roy *et al.* (1996)
DAMGO	↓	Nitric oxide production (J774)	Naloxone	Iuvone *et al.* (1995)
Deltorphin II	⇔	Nitric oxide production (J774)		Iuvone *et al.* (1995)
DPDPE	⇔	Nitric oxide production (J774)		Iuvone *et al.* (1995)
Endomorphin –1 & -2	↓	Stimulated IL-10 and IL-12 production (THP-1 cells)		Azuma *et al.* (2002)
Endomorphin –1 & -2	↑	NF-κB binding (THP-1 cells)		Azuma *et al.* (2002)
Endomorphin –1 & -2	↓	Chemotaxis (THP-1 cells)		Azuma *et al.* (2002)

Compound	Effect	End point	Antagonist	Study
Morphine	↑	P38 MAPK phosphorylation	Naltrexone α-TGF-β	Singhal *et al.* (2002)
Morphine	↑	p53 and Nitric oxide synthase expression	α-TGF-β	Singhal *et al.* (2002)
Morphine	↑	Apoptosis (J774)	α-TGF-β antibody	Singhal *et al.* (2000)
Morphine	↑	Expression of bax (J774)	α-TGF-β antibody	Singhal *et al.* (2000)
Morphine	↑	p53 MAPK phosphorylation and Bax expression	LNAME	Singhal *et al.* (2002)
Morphine	↑	Fas and FasL expression	Naltrexone	Singhal *et al.* (2002)
Morphine	↑	Apoptosis	Caspase-3 inhibitor	Singhal *et al.* (2002)
Morphine	↓	Proliferation (Bac 1.2 F5)	~~Naloxone~~ (only 10%)	Roy *et al.* (1996)
Morphine	↓	Nitric oxide production (J774)	Naloxone	Iuvone *et al.* (1995)
U50488	↓	Nitric oxide production (J774)	Naloxone	Iuvone *et al.* (1995)
U50488	↓	Stimulated IL-1 and TNF-α production	Naloxone Nor-BNI	Belkowski *et al.* (1995a)
U50488	↔	Stimulated IL-6 production		Belkowski *et al.* (1995a)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.4.2. *In vivo* evidence for the affect of opioids on infection and immunity peripheral administration

The consistent dilemma for researchers conducting experiments on a drug *in vitro* is that when the drug is evaluated *in vivo* the responses may be completely different. This can arise from the drug acting at sites that were not present in the *in vitro* model. Moreover, metabolic conversion of the parent drug can produce metabolites with significantly different properties, which may be of decreased or increased activity, and can act at the original site of action studied *in vitro*, or at new sites. These same dilemmas face researchers who investigate the immunomodulatory behaviour of opioids, as metabolic conversion of some opioids to active metabolites occurs. The central action of opioids produce many changes in hormone release and neuronal activity that can alter immune function. Therefore, numerous studies have investigated both the *in vitro* and *in vivo* immunomodulatory effects of opioid exposure. There are numerous reviews on this area (Adler *et al.*, 1993; Bryant *et al.*, 1990; Plotnikoff, 1985), with the relevant available literature reviewed here.

1.4.2.1. *In vivo* immune functions

General functions of the immune system have been studied for altered activity following opioid exposure *in vivo*. Some examples of these are the antibody response, tumour growth, viral mortality, opioid cellular toxicity, cell surface receptor expression, anaphylactic shock, graft survival, cytokine production, endorphin production and hypersensitivity reactions. These data are summarised in tables in Chapter 8. Of the results which relate to the current project, Lockwood *et al.* (1996) concluded the suppression of the antibody response by morphine was due to central opioid receptors, since naloxone methobromide, a peripherally acting antagonist did not have any affect on morphine's response. However, methadone (de Waal *et al.*, 1998; LeVier *et al.*, 1995) and buprenorphine (Van Loveren *et al.*, 1994) did not influence the antibody responses in the same fashion as morphine, suggesting they were acting via sites other than classical opioid receptors and mechanisms. Similar non-classical opioid effects were observed by Carr *et al.* (1996c) who investigated the influence morphine, fentanyl, mirfentanil and OHM3507 (a derivative of fentanyl) had on Natural Killer cell function and survival following tumour inoculation. As discussed previously (1.4.1.4), morphine and fentanyl suppress Natural Killer cell function, while mirfentanil and OHM3507 have little effect. In contrast, *in vivo* morphine surprisingly increased survival following tumour inoculation, whilst OHM3507 decreased it. These findings contradicted the hypothesised outcome, as it was anticipated that Natural Killer cell function would determine tumour survivability. Furthermore, the variable efficacy of similar μ opioid ligands to alter these responses suggests a more complex relationship than merely one receptor, one affect, and thereby implicate additional non-classical opioid receptors. Similar to tumour growth, viral-induced mortality was also reduced by morphine (Alonzo & Carr, 1999). Meanwhile, bacterial-induced death increased, although this may have been due to reduced gastric motility

and therefore greater contact time to increase the bacterial load (Eisenstein *et al.*, 2001). In contrast to viral mortality, graft rejection was reduced by β-endorphin, leu- and met-enkephalin, morphine and naltrindole, but increased by naloxone (Arakawa *et al.*, 1992; Bryant & Roudebush, 1990; Maric & Jankovic, 1987; Sacerdote *et al.*, 1998; Sacerdote *et al.*, 2000b). On one hand, we see the increased killing of mutated self and invading pathogens, and yet on the other, reduced rejection of foreign tissues results from opioid agonist exposure. The action of naltrindole is curious, as it behaved in a similar fashion to opioid agonists, despite the disparity in opioid characteristics, although this is in keeping with its non-classical opioid activity observed *in vitro*. The reduced rejection of grafts may be due to the switch of T cells from T_H1 to T_H2 immune responses observed *in vitro* (discussed previously), that results in a reduction of cytotoxic T cell activity, which is closely related to the detection and elimination of non-self. Following on from the altered cytokine production is the modification of delayed type hypersensitivity responses. Delayed type hypersensitivity is predominately a T_H1 type response associated with T_H1 type cytokines. Opioids reduce the prevalence of these cytokines, which may explain the reduced severity of most of the delayed type hypersensitivity responses reported (Bryant & Roudebush, 1990; Hendrickson *et al.*, 1989; Kasahara *et al.*, 1995; Pellis *et al.*, 1986; Schoolov *et al.*, 1995; Virsik & Bussiere, 1995; Wu *et al.*, 1999).

In the review of *in vivo* immune functions, one can see some of the *in vitro* opioid immunomodulatory results potentially explaining some of the *in vivo* immune responses. However, what is unclear from many of these *in vivo* studies is the location of the receptors that mediated these responses following *in vivo* exposure, as they could be centrally located or expressed on the effected cells, or a combination of the two, resulting in modified immune function. These questions are discussed further later in this chapter (1.5).

1.4.2.2. Thymocytes (*in vivo* peripheral)

The influence of *in vivo* exposure to opioids, primarily that of morphine, on thymus weight, thymocyte numbers and cell ratios, proliferation and DNA damage are summarised in the following section (Table 1.4-8). Thymus weight and cell numbers in the thymus are reduced following *in vivo* exposure to morphine, although in some cases some individual cell populations seem to be influenced to a greater degree than others. These responses appear to be initiated via classical opioid receptors, due to the classical opioid antagonism, and mediated by glucocorticoids. However, buprenorphine had the opposite effect to morphine, and actually increased thymic weight (Van Loveren *et al.*, 1994), implying that the receptor that initiates the response may not be identical to that which mediates morphine's effect, thereby suggesting ligand selectivity and non-classical opioid characteristics. The ability of thymocytes to proliferate once isolated is also significantly reduced by *in vivo* opioid treatment. However, the treatment length and dose appear to influence this response, although little is understood about this relationship for this cell type. The conclusions for peripheral blood mononuclear cells may provide further directions for research (1.4.1.5). As observed *in vitro*, thymocytes are modified by opioid exposure. However, *in vivo* indirect modification of thymocyte function also occurs, with glucocorticoids responsible for some of these effects (Bryant *et al.*, 1991). The direct effects of opioids on thymic cells *in situ* is still to be elucidated, as is the relative contribution of direct and indirect immunomodulation on the final response.

Table 1.4-8 Summary of the *in vivo* opioid effect on various thymocyte parameters.

Compound	Effect	End point	Antagonist	Study
THYMOCYTE ORGAN WEIGHT				
Buprenorphine	↑	Thymic weight		Van Loveren *et al.* (1994)
Dihydroetorphine	↓	Thymic weight		Wu *et al.* (1999)
Leu-enkephalin	⇔	Thymic weight		Maric *et al.* (1987)

Compound	Effect	End point	Antagonist	Study
Met-enkephalin	↑	Thymic weight		Jankovic *et al.* (1991)
Met-enkephalin	↔	Thymic weight		Maric *et al.* (1987)
Morphine	↓	Thymic weight		Arora *et al.* (1990)
Morphine	↓	Cellularity of the thymus	Naltrexone	Freier *et al.* (1993)
Morphine	↓	Cellularity of the thymus		Bryant *et al.* (1987)
Morphine	↓	Cellularity of the thymus	Naltrexone	Bhargava *et al.* (1995)
Morphine	↓	Thymic weight		LeVier *et al.* (1994)
Morphine	↓	Thymic weight	Adrenalectomy RU-486	Bryant *et al.* (1991)
Naloxone	↔	Thymic weight		Maric *et al.* (1987)
THYMOCYTE CELL NUMBERS AND RATIOS				
Morphine	↓	Thymic Cell number		Gaveriaux-Ruff *et al.* (1998)
Morphine	↓	$CD4^+CD8^+$ thymic cells	Naltrexone	Freier *et al.* (1993)
Morphine	↑	Thymic $CD4^+/CD8^+$ ratio		Arora *et al.* (1990)
Morphine	↓	Thymocytes recovered		Carr *et al.* (1995)
Morphine	↔	Percentage of $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^+CD8^+$ thymocytes		Carr *et al.* (1995)
THYMOCYTE PROLIFERATION				
Morphine	↓	Proliferation	Naloxone	Roy *et al.* (1995)
Morphine	↓	Proliferation (long time after implantation)		Bryant *et al.* (1988)
Morphine	↔	Proliferation (short time after implantation)		Bryant *et al.* (1988)
Morphine	↔	Proliferation		Mellon *et al.* (1999)
Morphine	↓	Proliferation	Naloxone	Ishikawa *et al.* (1993)
Morphine (large pellets)	↓	Proliferation		Bryant *et al.* (1987)
Morphine (small pellets)	↑	Proliferation		Bryant *et al.* (1987)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.4.2.3. Splenocytes (*in vivo* peripheral)

Similar to opioid-induced changes in thymic weight, splenic weights were also reduced by the majority of opioids in a naloxone-sensitive, glucocorticoid-dependent manner. Once again, buprenorphine behaved differently to morphine and did not affect organ weight (Van Loveren *et al.*, 1994). Interestingly, Bussiere *et al.* (1992) concluded that strain differences in response to morphine and naltrexone treatment suggest that morphine may be acting through both classical and non-classical opioid receptors, which supports the dissimilar data with morphine and buprenorphine (Table 1.4-9). In contrast to the almost conclusive results for opioid modulation of thymocyte numbers and ratios, the splenocyte data are less clear (Table

1.4-10). Morphine has been found to have less impact on cell numbers and ratios, despite the significant effect on organ weight and cell viability and toxicity (Table 1.4-10).

Table 1.4-9 Summary of the *in vivo* opioid effects on splenocyte organ weight.

Compound	Effect	End point	Antagonist	Study
Buprenorphine	⇔	Spleen weight		Van Loveren *et al.* (1994)
Dihydroetorphine	↓	Spleen weight		Wu *et al.* (1999)
Leu-enkephalin	⇔	Spleen weight		Maric *et al.* (1987)
Met-enkephalin	↑	Spleen weight		Jankovic *et al.* (1991)
Met-enkephalin	⇔	Spleen weight		Maric *et al.* (1987)
Morphine	↓	Spleen weight		Bhargava *et al.* (1995; 1994)
Morphine	↓	Spleen weight		LeVier *et al.* (1994)
Morphine	↓	Spleen weight	μ opioid receptor knockout model	Bussiere *et al.* (1992)
Morphine	↓	Spleen weight		Bryant *et al.* (1987)
Morphine	↓	Spleen weight	Nalorphine Naloxone	Lefkowitz *et al.* (1975)
Morphine	↓	Spleen weight	Adrenalectomy RU-486	Bryant *et al.* (1991)
Morphine	↓	Spleen weight		Arora *et al.* (1990)
Morphine	↓	Spleen weight		Portoles *et al.* (1995)
Naloxone	⇔	Spleen weight		Maric *et al.* (1987)

⇔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-10 Summary of the *in vivo* opioid effects on splenocyte cell numbers and ratios.

Compound	Effect	End point	Antagonist	Study
β-endorphin	⇔	Lymphocyte trafficking		Panerai *et al.* (1995)
β-endorphin	↑	Frequency of micronucleated binuclear splenocytes	Adrenalectomy RU-486 Naloxone	Couch *et al.* (1995)
DAMGO	↑	Percent of T cells		Kowalski (1998b)
Diacetylmorphine	↓	Cell number		Fecho *et al.* (2001)
Diacetylmorphine	↓	Percent of a splenic granulocyte subset, the $CD11b/c^+$ HIS48(hi) cells		Fecho *et al.* (2000)
Diacetylmorphine	⇔	Relative numbers of $CD4^+$ $CD3^+$ T cells, $CD8^+$ $CD3^+$ T cells, $CD45^+$ B cells, NKR-P1A(lo) $CD3^+$ T cells, $CD11b/c^+$ $ED1^+$ (or $CD11b/c^+$ $HIS48^-$) monocytes/macrophages or $CD11b/c^+$ $ED1^-$ (or $CD11b/c^+$ $HIS48^+$) total granulocytes in the spleen		Fecho *et al.* (2000)
Diacetylmorphine	↑	Apoptosis		Fecho *et al.* (2000)
Diacetylmorphine	⇔	Necrosis		Fecho *et al.* (2001)
Diacetylmorphine	↑	Apoptosis		Fecho *et al.* (2001)
DPDPE	⇔	Percent of T cells		Kowalski (1998b)
Methadone	↓	Absolute number of $CD4^-CD8^+$ and $CD4^+CD8^+$ cells		LeVier *et al.* (1995)
Methadone	↓	Natural Killer cell number		van der Laan *et al.* (1996)
Morphine	⇔	Cell ratios		Gaveriaux-Ruff *et al.* (1998)
Morphine	↓	Proportion of B cells		Eisenstein *et al.* (1998)
Morphine	⇔	$CD4^+/CD8^+$ ratio		Eisenstein *et al.* (1998)

Compound	Effect	End point	Antagonist	Study
Morphine	⇔	Numbers of Ig^+, $CD3^+$, $CD4^+$ and $CD8^+$ splenocytes		Portoles *et al.* (1995)
Morphine	↑	$CD4^+/CD8^+$ ratio		Arora *et al.* (1990)
Morphine	⇔	$CD4^+/CD8^+$ ratio		Hilburger *et al.* (1997a)
Morphine	↓	Number of macrophages	Naltrexone	Hilburger *et al.* (1997a)
Morphine	↑	Proportion of T cells	Naltrexone	Chang *et al.* (1998a)
Morphine	↑	Frequency of micronucleated binuclear splenocytes	Adrenalectomy RU-486 Naloxone	Couch *et al.* (1995)
Morphine	↑	Frequency of micronucleated cells	Adrenalectomy RU-486 ~~Metyrapone~~	Sawant *et al.* (2001)
Morphine	↑	Apoptosis		Singhal *et al.* (1997)
Morphine	↑	Frequency of micronucleated binuclear splenocytes	Naloxone	Sawant *et al.* (1995)
Naloxone	⇔	Frequency of micronucleated binuclear splenocytes	Naloxone	Sawant *et al.* (1995)
N-methylmorphine	↑	Frequency of micronucleated cells		Sawant *et al.* (2001)
U50488	⇔	Percent of T cells		Kowalski (1998b)

⇔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Splenocyte proliferation following *in vivo* opioid exposure (primarily morphine) has been extensively analysed over the last 15 years (Table 1.4-11). Morphine has been consistently shown to induce significant suppression of stimulated splenocyte proliferation. This response is considered to be initiated via central opioid receptors and mediated in a mitogen dependent manner by sympathetic signalling and is associated with increased release of nitric oxide by splenic macrophages (Fecho *et al.*, 1994; Fecho *et al.*, 1995a). Differences in the responses by opioid receptor ligands types have been observed with DAMGO, β-endorphin, buprenorphine, hydromorphone, codeine, oxycodone and tramadol influencing the response differently to morphine (Table 1.4-11). Sacerdote *et al.* (1997b) found that the structural features that were associated with enhanced antinociception did not correlate with enhanced immunosuppression, suggesting that the receptor that mediated these responses was not of the same type that mediates antinociception, and therefore may be of a non-classical opioid nature. Specifically, they demonstrated that the immunosuppressive effects of opioids is independent of the antinociceptive effect (Sacerdote *et al.*, 1997b). Interestingly, substitution of the C_3 hydroxyl group decreased the antinociception, whilst immunosuppression was

unchanged (Figure 1.1-1). In contrast, modifications at the C_6 and bonds between C_{7-8} resulted in minimal nociceptive changes but abolished the immunosuppressive effects (Sacerdote *et al.*, 1997b). This is of great interest as the immunomodulatory responses observed by Sacerdote *et al.* (1997b) undoubtedly had some central components which had previously been assumed to be initiated via classical opioid receptors. Therefore, non-classical opioid immunomodulation occurs not only at the cell surface, but also at much higher central levels at unknown receptors.

Table 1.4-11 Summary of the *in vivo* opioid effects on splenocyte proliferation.

Compound	Effect	End point	Antagonist	Study
α-β-endorphin antibodies	↑	Proliferation		Panerai *et al.* (1995)
(-)-Tramadol	↔	Proliferation		Sacerdote *et al.* (1999)
(+)-Tramadol	↑	Proliferation	Metergoline	Sacerdote *et al.* (1999)
β-endorphin	↑	Proliferation	Naloxone	Kusnecov *et al.* (1987)
2-n-pentyloxy-2-phenyl-4-methyl-morpholine	↓	Proliferation		Hadjipetrou-Kourounakis *et al.* (1989)
Buprenorphine	↑	Proliferation		Van Loveren *et al.* (1994)
Codeine	↔	Proliferation		Sacerdote *et al.* (1997b)
DAMGO (4 injections)	↑	Proliferation		Kowalski (1998b)
DAMGO (single injection)	↑	Proliferation		Kowalski (1998b)
Diacetylmorphine	↓	Proliferation	Naltrexone	Fecho *et al.* (1999; 2000)
Dihydroetorphine	↓	Proliferation	Naloxone Phentolamine ~~Propranolol~~	Wu *et al.* (1998)
DPDPE (4 injections)	↑	Proliferation		Kowalski (1998b)
DPDPE (single injection)	↔	Proliferation		Kowalski (1998b)
Hydromorphone	↔	Proliferation		Sacerdote *et al.* (1997b)
Met-enkephalin	↑	Proliferation	Naloxone	Kowalski (1998a)
Met-enkephalin	↑	Proliferation	~~Adrenalectomy~~	Marotti *et al.* (1992)
Methadone	↓	Proliferation		LeVier *et al.* (1995)
Morphine	↓	B cell proliferation	Naltrexone	Bhargava *et al.* (1995)
Morphine	↓	B cell proliferation		Bhargava *et al.* (1994)
Morphine	↓	Con A-induced proliferation	Chlorisondamine Adrenalectomy	Fecho *et al.* (1996b)
Morphine	↔	LPS proliferation		Hamra *et al.* (1996)
Morphine	↑	Nitric oxide production	L-NAME	Fecho *et al.* (1994)
Morphine	↑	Nitric oxide production		Fecho *et al.* (1995a)
Morphine	↓	PHA-induced proliferation	Chlorisondamine ~~Adrenalectomy~~	Fecho *et al.* (1996b)
Morphine	↓	PHA, pokeweed and Con A proliferation	Naltrexone	Hamra *et al.* (1996)
Morphine	↓	Proliferation	β-adrenergic antagonists	Fecho *et al.* (1993)
Morphine	↓	Proliferation		Portoles *et al.* (1995)
Morphine	↓	Proliferation	Adrenalectomy	Bryant *et al.* (1991)
Morphine	↓	Proliferation		Tsai *et al.* (2000)
Morphine	↓	Proliferation		Mellon *et al.* (2001)

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Proliferation		West *et al.* (1998)
Morphine	↔	Proliferation		Mellon *et al.* (1999)
Morphine	↔	Proliferation		Bayer *et al.* (1990)
Morphine	↓	Proliferation	~~N-methylnaltrexone~~	Fecho *et al.* (1996a)
Morphine	↓	Proliferation	IL-1 IL-2	Lu *et al.* (1996)
Morphine	↓	Proliferation	Naloxone	Ishikawa *et al.* (1993)
Morphine	↓	Proliferation	Naltrexone	Lysle *et al.* (1993a)
Morphine	↓	Proliferation		West *et al.* (1997)
Morphine	↓	Proliferation		Nelson *et al.* (1997; 1998)
Morphine	↓	Proliferation		Fecho *et al.* (1999)
Morphine	↓	Proliferation	*Ex vivo* haemoglobin	Fecho *et al.* (1995b)
Morphine	↑	Proliferation (end of pellet)		Bryant *et al.* (1988)
Morphine	↓	Proliferation (soon after pellet implantation)		Bryant *et al.* (1988)
Morphine	↓	Proliferation (withdrawal)	Clonidine	West *et al.* (1999)
Morphine (large pellet)	↓	Proliferation		Bryant *et al.* (1987)
Morphine (small pellet)	↑	Proliferation		Bryant *et al.* (1987)
Morpine-6-glucuronide	↓	Proliferation		Carrigan *et al.* (2001)
Nalorphine	↓	Proliferation	Nor-BNI	Sacerdote *et al.* (1997b)
Naloxone	↓	Proliferation		Kusnecov *et al.* (1987)
Naloxone	↑	Proliferation		Sacerdote *et al.* (1997b)
Naloxone	↑	Proliferation		Panerai *et al.* (1995)
Naltrexone	↔	Proliferation		Hamra *et al.* (1996)
Naltrexone	↑	Proliferation		Sacerdote *et al.* (1997b)
Oxycodone	↔	Proliferation		Sacerdote *et al.* (1997b)
Tramadol	↓	Proliferation		Tsai *et al.* (2001)
Tramadol	↑	Proliferation		Sacerdote *et al.* (1997a)
U50488 (4 injections)	↑	Proliferation		Kowalski (1998b)
U50488 (single injection)	↔	Proliferation		Kowalski (1998b)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

As mentioned previously, the primary influence of opioids on cytokine production by T cell splenocytes is to switch from a T_H1 to T_H2 type response (Table 1.4-12). T_H1 responses primarily result in activation of cytotoxic cells, such as macrophages and cytotoxic T cells, while T_H2 responses support B cell responses. These data were expanded by Eisenstein *et al.* (1993) who found inhibition of cytokine production by morphine could be restored by the replenishment of macrophage cytokines, whilst those that act on T and B cells had no effect. They concluded that *in vivo* morphine treatment specifically targeted macrophage function, which in turn caused suppression of other immune functions (Eisenstein *et al.*, 1993). The opioid antagonist naloxone increased T_H1 cytokines, suggesting that the opioid system has a

regulatory role in suppressing T_H1 responses. Many of these responses were sensitive to classical opioid antagonism. However, Roy *et al.* (1999) reported that the response to high doses of morphine could not be antagonised by naloxone, implicating a non-classical opioid receptor, as Sacerdote *et al.* (1997b) concluded from an analysis of *in vivo* structure-effect relationships discussed previously.

Table 1.4-12 Summary of the *in vivo* opioid effects on cytokine production by splenocytes.

Compound	Effect	End point	Antagonist	Study
Codeine	↓	IL-2 production		Sacerdote *et al.* (1997b)
Diacetylmorphine	↓	IL-1β, IL-2 and IFN-γ production (24 hours after treatment)		Pacifici *et al.* (2000)
Diacetylmorphine	↑	IL-1β, IL-2 and IFN-γ production (up to 40 minutes after treatment)		Pacifici *et al.* (2000)
Diacetylmorphine	↑	TGF-β1 and IL-10 production (2 to 24 hours after treatment)		Pacifici *et al.* (2000)
Dihydroetorphine	↓	IL-2 production		Wu *et al.* (1999)
Dihydroetorphine	↓	IL-2 production	Naloxone Phentolamine ~~Propranolol~~	Wu *et al.* (1998)
Hydromorphone	⇔	IL-2 production		Sacerdote *et al.* (1997b)
Morphine	↓	Cytokine production	IL-1 IL-6 IFN-γ ~~IL-2~~ ~~IL-4~~ ~~IL-5~~	Eisenstein *et al.* (1993)
Morphine	↓	IFN-γ production		West *et al.* (1998; 1997)
Morphine	↓	IFN-γ production		Nelson *et al.* (1997; 1998)
Morphine	↓	IFN-γ production (withdrawal)	Clonidine	West *et al.* (1999)
Morphine	↓	IL-1β, IL-2 and IFN-γ production (24 hours after treatment)		Pacifici *et al.* (2000)
Morphine	↑	IL-1β, IL-2 and IFN-γ production (up to 40 minutes after treatment)		Pacifici *et al.* (2000)
Morphine	↓	IL-2 and IFN production	Naltrexone	Lysle *et al.* (1993a; 1993b)
Morphine	↓	IL-2 and IL-4 production		Bhargava *et al.* (1994)
Morphine	↓	IL-2 and IL-4 production		Casalinuovo *et al.* (2000)
Morphine	↓	IL-2 production		Sacerdote *et al.* (1997b)
Morphine	↓	IL-2 synthesis		Roy *et al.* (1999)
Morphine (low dose)	↑	IL-6 and TNF-α synthesis	Naloxone	Roy *et al.* (1999)
Morphine (high dose)	↑	IL-6 and TNF-α synthesis	~~Naloxone~~	Roy *et al.* (1999)
Morphine	⇔	INF-γ production		Casalinuovo *et al.* (2000)
Morphine	↑	TGF-β1 and IL-10 production (2 to 24 hours after treatment)		Pacifici *et al.* (2000)
Morphine	↓	T_H1 type cytokines		Roy *et al.* (1999)
Morphine	↑	T_H2 type cytokines		Roy *et al.* (1999)
Morphine	⇔	TNF-α production		Bhargava *et al.* (1994)
Morphine-6-glucuronide	↓	IFN-γ production		Carrigan *et al.* (2001)
Nalorphine	↓	IL-2 production	Nor-BNI	Sacerdote *et al.* (1997b)
Naloxone	↑	IL-2 and IFN-γ production		Sacerdote *et al.* (2000b)

Compound	Effect	End point	Antagonist	Study
Naloxone	↑	IL-2 and IFN-γ production		Sacerdote *et al.* (2000c)
Naloxone	↑	IL-2 production		Sacerdote *et al.* (1997b)
Naloxone	↓	IL-4 production		Sacerdote *et al.* (2000b)
Naloxone	↓	IL-4 production		Sacerdote *et al.* (2000c)
Naloxone	↑	T_H1 cytokines		Sacerdote *et al.*(2001a)
Oxycodone	↔	IL-2 production		Sacerdote *et al.* (1997b)
SCN 80	↑	TNF-α and nitric oxide production		Gomez-Flores *et al.* (2001)
Tramadol	↑	IL-2 production		Sacerdote *et al.* (1997a)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Other functional analysis of splenocyte function following *in vivo* opioid exposure has been conducted using cell surface receptor expression, cytotoxic T cell activity, rosette formation, phagocytic activity, immunoglobulin production, calcium influx and nitric oxide synthase production. As these functions are not directly related to this research project they will not be discussed, but summary tables are available in Chapter 8.

These *in vivo* opioid immunomodulatory data implicate indirect mechanisms of action on splenocytes, which are dependent on glucocorticoid release (discussed in detail later in this chapter 1.5), however the classical opioid nature of these responses is unclear, as was found *in vitro*. Therefore, further studies characterising the non-classical opioid receptors that initiate opioid immunomodulation *in vivo* must be conducted.

1.4.2.4. Natural Killer cells (*in vivo* peripheral)

As was discussed previously for *in vitro* influences of opioids on Natural Killer cell function (1.4.1.4), the function of Natural Killer cells is considered very important. Therefore, any detrimental alteration in their function is seen as detrimental to the function of the immune system. Many studies have been conducted (Table 1.4-13), with the majority using morphine, and all except one reported either no effect or inhibition of Natural Killer cell activity. These data suggest that opioid modification of Natural Killer cell function is initiated via naltrexone-

and naloxone-sensitive receptors and mediated by glucocorticoids (Freier & Fuchs, 1994) and both α and β adrenergic systems (Carr *et al.*, 1993). Interestingly, Jacobs *et al.* (1999) found significant interindividual variability in the naloxone-sensitivity of the Natural Killer cell numbers following fentanyl exposure. They reported that in two subjects naloxone completely blocked the fentanyl-induced increase in Natural Killer cell numbers, whilst in two other individuals the increase in Natural Killer cell numbers was not blocked by naloxone. However, they observed no change in Natural Killer cell function.

Table 1.4-13 Summary of the *in vivo* opioid effects on Natural Killer cell function.

Compound	Effect	End point	Antagonist	Study
(-)-Naloxone	↑	Natural Killer cell activity		Carr *et al.* (1991)
(-)-Tramadol	⇔	Natural Killer cell activity		Sacerdote *et al.* (1999)
(+)-Naloxone	⇔	Natural Killer cell activity		Carr *et al.* (1991)
(+)-Tramadol	↑	Natural Killer cell activity	Metergoline	Sacerdote *et al.* (1999)
Buprenorphine	⇔	Natural Killer cell activity		Van Loveren *et al.* (1994)
Codeine	↓	Natural Killer cell activity		Sacerdote *et al.* (1997b)
Diacetylmorphine	↓	Natural Killer cell activity		Nair *et al.* (1986)
Diacetylmorphine	↓	Natural Killer cell activity		Nair *et al.* (1989a)
Diacetylmorphine	↓	Natural Killer cell activity	Naltrexone	Fecho *et al.* (2000)
Fentanyl	↓	Natural Killer cell activity		Beilin *et al.* (1996)
Fentanyl	⇔	Natural Killer cell activity		Jacobs *et al.* (1999)
Fentanyl	↑	Natural Killer cell numbers		Jacobs *et al.* (1999)
Fentanyl	↑	Natural Killer cell activity		Yeager *et al.* (2002)
Hydromorphone	⇔	Natural Killer cell activity		Sacerdote *et al.* (1997b)
Met-enkephalin	↑	Natural Killer cell activity		Zhong *et al.* (1996)
Met-enkephalin	↑	Natural Killer cell activity	Naloxone	Kowalski (1998a)
Methadone	↓	Natural Killer cell activity		van der Laan *et al.* (1996)
Methadone	⇔	Natural Killer cell activity		Pacifici *et al.* (1994)
Mirfentanil	↓	Natural Killer cell activity		Carr *et al.* (1996c)
Mirfentanil	⇔	Natural Killer cell activity		Carr *et al.* (1996a)
Morphine	⇔	Natural Killer cell activity		Sacerdote *et al.* (2000a)
Morphine	↓	Splenic Natural Killer cell activity	Naltrexone	Carr *et al.* (1994)
Morphine	↓	Natural Killer cell activity	Naloxone Naltrexone ~~Naloxone methiodide~~ RU 486	Freier *et al.* (1994)
Morphine	↓	Natural Killer cell activity		Shavit *et al.* (1998)
Morphine	↓	Natural Killer cell activity		Yeager *et al.* (1995)
Morphine	↓	Natural Killer cell activity		West *et al.* (1998)
Morphine	↓	Natural Killer cell activity		Tsai *et al.* (2000)
Morphine	⇔	Splenic Natural Killer cell activity		Carr *et al.* (1995)
Morphine	⇔	Splenic Natural Killer cell activity		Carpenter *et al.* (1995; 1995)
Morphine	↓	Natural Killer cell activity		Bhargava *et al.* (1994)
Morphine	↓	Natural Killer cell activity	Naltrexone	Bayer *et al.* (1990)
Morphine	↓	Natural Killer cell activity	Naltrexone	Lysle *et al.* (1993a; 1993b)
Morphine	↓	Splenic Natural Killer cell activity (withdrawal)	Clonidine	West *et al.* (1999)

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Natural Killer cell activity	24 hours after injection	Nelson *et al.* (1998)
Morphine	↓	Natural Killer cell activity		Carr *et al.* (1993b)
Morphine	↓	Natural Killer cell activity	~~Bromocriptine~~	Provinciali *et al.* (1996)
Morphine	↑	Natural Killer cell activity		Yeager *et al.* (1992)
Morphine	↓	Natural Killer cell activity	Phentolamine Propranolol Prazocin ~~Yohimbine~~ β-funaltrexamine ~~Naloxonazine~~ ~~Naltrindole~~ ~~Nor-BNI~~	Carr *et al.* (1993)
Morphine	↓	Natural Killer cell activity		Sacerdote *et al.* (1997b)
Morphine	↓	Natural Killer cell activity		LeVier *et al.* (1994)
Morphine	↓	Natural Killer cell activity	AZT	Pacifici *et al.* (1992)
Morphine	↓	Natural Killer cell activity	Naltrexone	Bhargava *et al.* (1995)
Morphine	↓	Natural Killer cell activity		Fecho *et al.* (1993)
Morphine	↓	Natural Killer cell activity	Chlorisondamine	Mellon *et al.* (2001)
Morphine	↓	Natural Killer cell activity		van der Laan *et al.* (1996)
Morphine	↓	Natural Killer cell activity		Lysle *et al.* (1993a)
Morphine	↓	Natural Killer cell activity		Carr *et al.* (1996c)
Morphine	↓	Natural Killer cell activity	Mirfentanil	Carr *et al.* (1996a)
Morphine (acute)	↓	Natural Killer cell activity		West *et al.* (1997)
Morphine (chronic)	↔	Natural Killer cell activity		West *et al.* (1997)
Morphine (chronic)	↔	Natural Killer cell activity		Carpenter *et al.* (1995; 1995)
Morphine-6-glucuronide	↓	Natural Killer cell activity		Carrigan *et al.* (2001)
Nalorphine	↓	Natural Killer cell activity		Sacerdote *et al.* (1997b)
Naloxone	↔	Natural Killer cell activity		Sacerdote *et al.* (1997b)
Naltrexone	↔	Natural Killer cell activity		Bayer *et al.* (1990)
Naltrexone	↔	Natural Killer cell activity		Sacerdote *et al.* (1997b)
OHM3507	↓	Natural Killer cell activity		Carr *et al.* (1996c)
Oxycodone	↔	Natural Killer cell activity		Sacerdote *et al.* (1997b)
Remifentanil	↓	Natural Killer cell activity		Sacerdote *et al.* (2001b)
Tramadol	↑	Natural Killer cell activity		Sacerdote *et al.* (2000a)
Tramadol	↑	Natural Killer cell activity		Sacerdote *et al.* (1997a)
Tramadol	↓	Natural Killer cell activity		Tsai *et al.* (2001)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.4.2.5. Peripheral blood mononuclear cells (*in vivo* peripheral)

Peripheral blood mononuclear cell numbers and their ratios are significantly altered by opioid exposure (Table 1.4-14). The primary influence of *in vivo* μ opioid receptor agonist exposure is to cause a reduction in the number of T cells. Although, there are conflicting reports on the influence of the individual T cell subpopulations. The opioid nature of this response is also unclear, as few studies have investigated this characteristic. Interestingly, Donahoe *et al.*

(2001) related some of the cell number changes to the stress levels experienced by the animals in the study, and therefore concluded that observations obtained from humans will undoubtedly not be able to control for the stress level of patients, especially addicts or ex-addicts.

Table 1.4-14 Summary of the *in vivo* opioid effects on peripheral blood mononuclear cell ratios.

Compound	Effect	End point	Antagonist	Study
Diacetylmorphine	↑	Null lymphocyte number		McDonough *et al.* (1980)
Diacetylmorphine	↓	Total percentage of T cells	Naloxone	McDonough *et al.* (1980)
Diacetylmorphine	↔	White blood cell counts		McDonough *et al.* (1980)
Fentanyl	↑	Natural Killer cell numbers ($CD16^+CD56^+$)		Jacobs *et al.* (1999)
Fentanyl	↑	Numbers of $CD16^+$ and $CD8^+$ cells		Yeager *et al.* (2002)
Met-enkephalin	↓	CD4 cell number		Maric *et al.* (1987)
Morphine	↓	$CD4^+CD62L^+$ and $CD4^+CD62L^-$ cell numbers		Donahoe *et al.* (2001)
Morphine	↔	Cell distribution		Flores *et al.* (1995)
Morphine	↔	Cell subpopulation numbers		Flores *et al.* (1995)
Morphine	↑	Lymphopenia	Adrenalectomy	Flores *et al.* (1995)
Morphine	↔	Number of $CD19^+$ cells		Carr *et al.* (1993b)
Morphine	↓	Numbers of $CD8^+CD16^+$ cells		Carr *et al.* (1993b)
Morphine	↓	Percent of CD4 cells		Carr *et al.* (1993a)
Morphine	↓	Percent of CD4 cells		Kimes *et al.* (1992)
Morphine	↓	Percent of CD4, $CD4^+CD29^+$ and $CD4^+CD45RA^+$cells		Carr *et al.* (1993b)
Morphine	↑	Percent of $CD4^+CD29^+$ cells		Carr *et al.* (1993a)
Morphine	↑	Percent of CD8 cells		Carr *et al.* (1993a)
Morphine	↓	Percent of CD8 cells		Kimes *et al.* (1992)
Morphine	↑	Percent of CD8 cells		Carr *et al.* (1993b)
Morphine	↔	Total lymphocyte counts and the distribution of lymphocyte subpopulations		Palm *et al.* (1998)
Naloxone	↑	Immune cell numbers		Maric *et al.* (1987)
Oxymorphone	↔	Phenotypic expression of T cell surface antigens		Kimes *et al.* (1992)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

The *in vivo* effect of opioid exposure on peripheral blood immune cell proliferation is summarised in Table 1.4-15. In all studies, except for one human study, morphine has been shown to suppress the proliferation of isolated peripheral blood immune cells. Interestingly, the length of exposure has a significant impact on the response. In chronic opioid treatment studies, there are sustained elevations of glucocorticoid levels, while in contrast, acute morphine administration only produces a transient increase in corticosterone concentration

which returns to baseline within six hours (Flores *et al.*, 1996). Therefore, a transient increase in steroids may not be sufficient to significantly suppress the proliferative responses of immune cells, and the length of exposure to elevated glucocorticoids may be the key factor in determining the overall contribution of the effect on the immune system. The morphine-induced inhibition of lymphocyte activity appears to have both a glucocorticoid-dependent and a glucocorticoid-independent component. The acute treatment data reported by Flores *et al.* (1996) suggests that only a minor role is played by a glucocorticoid-dependent mechanism, on the other hand the results from chronic morphine treatment suggests that a major component of the total suppression is glucocorticoid-dependent. It is interesting to note that in the report by Bryant *et al.* (1991), who conducted a chronic morphine experiment, that although adrenal activation was a critical component, other mechanisms must also contribute to the overall splenocyte immunosuppression, since a significant degree of immunosuppression remained even after adrenalectomy and RU486 treatment.

Table 1.4-15 Summary of the *in vivo* opioid effects on peripheral blood immune cell proliferation.

Compound	Effect	End point	Antagonist	Study
Diacetylmorphine	↓	Proliferation		Nair *et al.* (1986)
Fentanyl	↓	Proliferation	Naltrexone	Flores *et al.* (1995)
Fentanyl	⇔	Proliferation		Yeager *et al.* (2002)
Methadone	↓	Proliferation		Lysle *et al.* (1993a; 1993b)
Morphine	↓	Proliferation	Naltrexone	Bayer *et al.* (1990)
Morphine	↓	Proliferation	Naltrexone	Mellon *et al.* (1999)
Morphine	↓	Proliferation	Tolerance developed	Bayer *et al.* (1996)
Morphine	↓	Proliferation	~~RU 486~~ ~~Adrenalectomy~~ ~~Hypophysectomy~~	Flores *et al.* (1994)
Morphine	↓	Proliferation		Flores *et al.* (1995)
Morphine	⇔	Proliferation		Palm *et al.* (1998)
Morphine	⇔	Proliferation		Palm *et al.* (1996)
Morphine	↓	Proliferation		Sacerdote *et al.* (2000a)
Morphine	↓	Proliferation	~~Naloxone~~	Hernandez *et al.* (1993)
Morphine	↓	Proliferation	Naltrexone	Lysle *et al.* (1993a; 1993b)
Morphine	↓	Proliferation	~~N-methylnaltrexone~~	Fecho *et al.* (1996a)
Morphine	↓	Proliferation	Chlorisondamine	Mellon *et al.* (2001)
Morphine	↓	Proliferation	Chlorisondamine ~~Atropine methylbromide~~ ~~Phentolamine~~ ~~Nadolol~~	Flores *et al.* (1996)
Morphine	↓	Proliferation		Fecho *et al.* (1993)

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Proliferation	Adrenalectomy ~~Chlorisondamine~~	Fecho *et al.* (1996b)
Morphine-3-glucuronide	↔	Proliferation		Palm *et al.* (1996)
Morphine-6-glucuronide	↔	Proliferation		Palm *et al.* (1996)
Naloxone	↑	Proliferation		Manfredi *et al.* (1993)
Naltrexone	↑	Proliferation		Manfredi *et al.* (1993)
N-methylmorphine	↔	Proliferation		Hernandez *et al.* (1993)
Remifentanil	↓	Proliferation		Sacerdote *et al.* (2001b)
Tramadol	↔	Proliferation		Sacerdote *et al.* (2000a)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Many other functions of peripheral blood mononuclear cells have been investigated, with the available literature for cytokine production, cell membrane receptor expression, cell rolling, superoxide production, phagocytosis and cytotoxicity, HIV infectivity, genetic damage, rosette formation, chemotaxis, immunoglobulin production and cAMP production being presented in summarised tables in Chapter 8. The opioid immunomodulation of peripheral blood mononuclear cells demonstrates similar properties to those of thymocytes, splenocytes and Natural Killer cells. Although, it is interesting to note the effect exposure length has on peripheral blood mononuclear cell function. The opioid characteristics of these responses are still to be fully investigated, and therefore warrant further attention in light of the results for other immune cell types.

1.4.2.6. Other cells of the immune system (*in vivo* peripheral)

Many other cell types of the immune system have been isolated following *in vivo* exposure to opioids. These cell types include: bone marrow, peritoneal macrophages, mast cells, lymph node cells, lung immune cells and glial cells. These cell types are not directly related to the work in this thesis, summaries of the available literature are presented in Chapter 8.

1.4.3. *In vivo* evidence for the affect of opioids on peripheral infection and immunity (central administration)

The influence of opioids delivered in very small quantities directly into the brain on peripheral immune function has been increasingly investigated, as the methodology becomes more standardised and robust. The results of the studies using this method have demonstrated that opioids delivered centrally are capable of modulating peripheral immune function, despite not directly coming in contact with the effected cell, confirming the role of indirect mechanisms of action. Mu opioid receptor agonists, such as morphine and DAMGO primarily have an inhibitory influence on the immune cells residing in immune organs (Table 1.4-16 and Table 1.4-17), peripheral circulation (Table 1.4-18 and Table 1.4-19) or resident in peripheral tissues (Table 1.4-20). These responses appear to be initiated via classical opioid receptors and mediated via nitric oxide and non-glucocorticoid-dependent pathways. The central administration of leu- and met-enkephalin was found to increase some immune responses and Dimitrijevic *et al.* (2000) concluded that central μ opioid receptors are responsible for the central immunomodulatory action of leu-enkephalin. In contrast, the central immunoenhancing effect of met-enkephalin appeared to be mediated independently of μ opioid receptors, instead being mediated via δ opioid receptors. These data provide very strong evidence of a brain to immune signalling pathway, although some of the non-classical opioid effects that were observed following peripheral administration were not observed, suggesting further investigation of these non-classical opioid responses is required.

Table 1.4-16 Summary of the central *in vivo* effects of opioids on thymocytes.

Compound	Effect	End point	Antagonist	Study
Buprenorphine	⇔	Proliferation		Gomez-Flores *et al.* (2000)
DAMGO	⇔	Proliferation		Mellon *et al.* (1998b)
Morphine	⇔	Proliferation		Mellon *et al.* (1998b)
Morphine	↓	Proliferation		Gomez-Flores *et al.* (2000)
Morphine	↓	Proliferation	~~RU 486~~	Liang-Suo *et al.* (2002)
SCN 50	⇔	Proliferation and cytokine production		Nowak *et al.* (1998)

⇔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-17 Summary of the central *in vivo* effects of opioids on splenocytes.

Compound	Effect	End point	Antagonist	Study
γ globulins	↑	Proliferation		Panerai *et al.* (1995)
β-endorphin	↑	Proliferation	Naltrindole	Boyadjieva *et al.* (2002)
β-endorphin	↓	Proliferation		Panerai *et al.* (1995)
β-funaltrexamine	⇔	Splenic PFC		Dimitrijevic *et al.* (2000)
β-funaltrexamine	⇔	Splenic PFC		Radulovic *et al.* (1996)
Buprenorphine	⇔	T cell and macrophage function		Gomez-Flores *et al.* (2000)
DAMGO	↑	Stimulated IgM levels and rosette forming cell number	Bilateral electrolytic lesioning of the nucleus accumbens	Devoino *et al.* (2002)
DAMGO	↓	Proliferation	N-methylnaltrexone	Nelson *et al.* (2000)
DAMGO	↑	Stimulated nitric oxide release	N-methylnaltrexone	Schneider *et al.* (1998)
DAMGO	⇔	Proliferation		Mellon *et al.* (1998b)
DAMGO	↓	Proliferation	L-NAME	Schneider *et al.* (1996)
DPDPE	⇔	Proliferation		Nelson *et al.* (2000)
DPDPE	⇔	Stimulated nitric oxide release		Schneider *et al.* (1998)
DPDPE	⇔	Proliferation		Schneider *et al.* (1996)
Endomorphin-1	⇔	Immunomodulation		Carrigan *et al.* (2000)
Enkephalins	↓	Proliferation		Jankovic *et al.* (1987)
ICI 174864	↓	Splenic PFC		Dimitrijevic *et al.* (2000)
ICI 174864	↓	Splenic PFC		Radulovic *et al.* (1996)
ICI 174864	↑	PFC response		Radulovic *et al.* (1994)
Leu-enkephalin	↑	Splenic PFC	Naltrindole	Dimitrijevic *et al.* (2000)
Leu-enkephalin	↑	Splenic PFC	Naltrindole ICI 174864 β-funaltrexamine ~~nor BNI~~	Radulovic *et al.* (1996)
Met-enkephalin	↑	Splenic PFC	Naltrindole ICI 174864 ~~β-funaltrexamine~~ ~~nor BNI~~	Dimitrijevic *et al.* (2000)

Compound	Effect	End point	Antagonist	Study
Met-enkephalin	↑	PFC response	ICI 174864	Radulovic *et al.* (1994)
Morphine	↓	Proliferation, IL-2 and IFN-γ production	N-methylnaltrexone	Lysle *et al.* (1996)
Morphine	↓	Proliferation	~~N-methylnaltrexone (icv or ip)~~	Fecho *et al.* (1996a)
Morphine	↔	IL-6 and TNF-α production by splenic macrophages	Naloxone	Bian *et al.* (1995)
Morphine	↑	IL-2 and TNF-β production and T cell proliferation	Naloxone	Bian *et al.* (1995)
Morphine	↓	IL-2 production		Gomez-Flores *et al.* (1999)
Morphine	↓	Proliferation	~~RU 486~~	Liang-Suo *et al.* (2002)
Morphine	↓	T cell and macrophage function		Gomez-Flores *et al.* (2000)
Morphine	↔	Proliferation		Mellon *et al.* (1998b)
Morphine	↓	Proliferation		Hoffman *et al.* (1995)
Morphine	↓	Stimulated nitric oxide production by stimulated macrophages		Gomez-Flores *et al.* (1999)
Morphine	↓	Phagocytosis of bacteria		Gomez-Flores *et al.* (1999)
Morphine (i.t.)	↔	Proliferation and expression of cell surface markers		Hamra *et al.* (1996)
Morphine-6-glucuronide	↓	Proliferation and IFN-γ production		Carrigan *et al.* (2001)
MR 2034	↓	Splenic PFC	Quaternary naltrexone (icv or ip)	Radulovic *et al.* (1995)
MR 2034	↑	PFC response	MR 2266	Radulovic *et al.* (1994)
Naloxone	↑	Humoral immune response		Jankovic *et al.* (1987)
Naltrexone	↓	Stimulated splenic iNOS expression		Lysle *et al.* (1999)
Naltrexone	↓	Stimulated nitric oxide levels		Lysle *et al.* (1999)
Naltrindole	↔	Splenic PFC		Dimitrijevic *et al.* (2000)
Nor-BNI	↑	Splenic PFC	β-funaltrexamine	Dimitrijevic *et al.* (2000)
Nor-BNI	↑	Splenic PFC		Radulovic *et al.* (1996)
SNC 80	↑	Proliferation		Nowak *et al.* (1998)
SNC 80	↔	Stimulated nitric oxide levels		Nowak *et al.* (1998)
U69593	↔	Proliferation		Nelson *et al.* (2000)
U69593	↔	Stimulated nitric oxide release		Schneider *et al.* (1998)
U69593	↔	Proliferation		Schneider *et al.* (1996)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-18 Summary of the central *in vivo* effects of opioids on Natural Killer cells.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Natural Killer cell activity		Hsueh *et al.* (1995)
Buprenorphine	↔	Natural Killer cell activity		Gomez-Flores *et al.* (2000)
DAMGO	↓	Natural Killer cell activity		Nelson *et al.* (2000)
DAMGO	↔	Natural Killer cell activity		Mellon *et al.* (1998b)
DPDPE	↔	Natural Killer cell activity		Nelson *et al.* (2000)
Dynorphin	↔	Natural Killer cell activity		Hsueh *et al.* (1995)

Compound	Effect	End point	Antagonist	Study
Endomorphine-1	↔	Natural Killer cell activity		Carrigan *et al.* (2000)
Leu-enkephalin (96 hours after injection)	↑	Natural Killer cell activity		Kowalski *et al.* (1995)
Leu-enkephalin (immediately after injection)	↓	Natural Killer cell activity		Kowalski *et al.* (1995)
Met-enkephalin	↑	Natural Killer cell activity		von Horsten *et al.* (1998)
Met-enkephalin (immediately after injection)	↓	Natural Killer cell activity		Kowalski *et al.* (1995)
Met-enkephalin (96 hours after injection)	↑	Natural Killer cell activity		Kowalski *et al.* (1995)
Morphine	↓	Natural Killer cell activity	N-methylnaltrexone	Lysle *et al.* (1996)
Morphine	↓	Natural Killer cell activity	Naltrexone	Shavit *et al.* (1986)
Morphine	↓	Natural Killer cell activity		Gomez-Flores *et al.* (1999)
Morphine	↓	Natural Killer cell activity		Gomez-Flores *et al.* (2000)
Morphine	↔	Natural Killer cell activity		Mellon *et al.* (1998b)
Morphine	↓	Natural Killer cell activity	~~RU 486~~	Liang-Suo *et al.* (2002)
Morphine	↓	Natural Killer cell activity	Naloxone	Bian *et al.* (1995)
Morphine	↓	Natural Killer cell activity	Naloxone	Zhang *et al.* (1998)
Morphine	↓	Natural Killer cell activity	Naltrexone	Weber *et al.* (1989)
Morphine	↓	Natural Killer cell activity		Hoffman *et al.* (1995)
Morphine (i.t.)	↓	Natural Killer cell activity		Yokota *et al.* (2000)
Morpine-6-glucuronide	↓	Natural Killer cell activity		Carrigan *et al.* (2001)
Naltrexone	↔	Natural Killer cell activity		Weber *et al.* (1989)
SNC 80	↔	Natural Killer cell activity		Nowak *et al.* (1998)
U69593	↔	Natural Killer cell activity		Nelson *et al.* (2000)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-19 Summary of the central *in vivo* effects of opioids on peripheral blood mononuclear cells.

Compound	Effect	End point	Antagonist	Study
DAMGO	↓	Proliferation	Naltrexone	Mellon *et al.* (1998b)
DPDPE	↓	Proliferation		Mellon *et al.* (1998b)
Morphine	↓	Proliferation		Hoffman *et al.* (1995)
Morphine	↓	Proliferation	CTOP ~~Naltrindole~~ ~~Nor-BNI~~	Mellon *et al.* (1998b)
Morphine	↓	Proliferation	~~Adrenalectomy~~	Houghtling *et al.* (2000)
Morphine	↓	Proliferation		Hernandez *et al.* (1993)
U50488	↓	Proliferation		Mellon *et al.* (1998b)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-20 Summary of the central *in vivo* effects of opioids on peritoneal macrophages.

Compound	Effect	End point	Antagonist	Study
Met-enkephalin	↑	Superoxide production	α-met-enkephalin antibody	Vujic-Redzic *et al.* (2000)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-21 Summary of the central *in vivo* effects of opioids on cytokine production.

Compound	Effect	End point	Antagonist	Study
β-funaltrexamine	↑	Stimulated IL-6 levels		Bertolucci *et al.* (1997)
Fentanyl	↓	IL-6 levels		Shirouzu *et al.* (1998)
Met-enkephalin	↑	IL-6 levels		von Horsten *et al.* (1998)
Morphine	↓	IL-6 levels	Naltrexone Adrenalectomy	Houghtling *et al.* (2000)
Morphine (i.t.)	↓	IL-2 production		Zhang *et al.* (1998)
Naltrindole	↓	Stimulated IL-6 levels		Bertolucci *et al.* (1997)
Nor-BNI	↑	Stimulated IL-6 levels		Bertolucci *et al.* (1997)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.4.4. *In vivo* affect on infection defence

Many of the studies conducted to date have been primarily focused on, and concerned with, the influence opioids have on the very clinically relevant outcome of infection defence. As this is not the focus of this research project, only a short summary of the literature will be presented. It should be obvious from the review of the opioid immunomodulatory literature that opioids alter almost every facet of immune function. It is therefore inevitable that *in vivo* opioid exposure will alter infection defence. The majority of the animal data suggests that increased susceptibility to infection occurs (Table 1.4-22, and discussed previously). When opioids are used clinically to treat pain, there are few adequate alternatives, presenting a dilemma to the clinicians of providing inadequate pain relief or potentially increasing their patients susceptibility to infection. The immunology associated with opioid maintenance therapy has been drawn into the political arena, as there is considerable debate whether opioid

maintenance therapies improve immune function. This debate is heightened when focusing on the treatment of opioid addiction. The potential for opioid addicts to have altered immune function was acknowledged some time ago (Brown *et al.*, 1974; Steinmuller *et al.*, 1979). Since then numerous studies have analysed the immune status of chronic opioid users (Table 1.4-23), with a number of quality reviews also written (Donahoe, 1988; Kreek, 1988; Novick *et al.*, 1991b; Weber, 1988; Yahya & Watson, 1987). There is consensus that opioid abuse causes a suppression of the immune system, although it is unclear if this is a direct effect of the opioid use, as lifestyle also plays a major role (Donahoe *et al.*, 1986; Govitrapong *et al.*, 1998; Lazzarin *et al.*, 1984). However, some researchers believe immune function is normalised by opioid maintenance treatment (Novick & Kreek, 1992; Novick *et al.*, 1989b; Novick *et al.*, 1991a; Schalm *et al.*, 1983), whilst others believe it remains a concern by potentially increasing the risk of infection (Confalonieri *et al.*, 1992; Klimas *et al.*, 1991). The influence of factors such as lifestyle may significantly affect the results of research conducted in the opioid addicted population (Novick *et al.*, 1991a), as well as other factors such as nutritional status and general health (Heathcote & Taylor, 1981; Hebert *et al.*, 1990; Huggins *et al.*, 1991). Nonetheless, the potential immune modification by opioid maintenance therapy does not invalidate the relevance of effective maintenance treatment in those subjects who are unable to remain drug-free (Confalonieri *et al.*, 1992).

The debate surrounding the use of opioids to treat opioid addiction gained more momentum when HIV infection was associated with injecting drug use and maintenance therapy. Some researchers even called for the removal of methadone from use as a maintenance therapy (Carballo-Dieguez *et al.*, 1994), as there was evidence for increased risk of HIV infection with continued abuse (Chao *et al.*, 1995b; Chuang *et al.*, 1993; Nair *et al.*, 1997; Quang-Cantagrel *et al.*, 2001), while others believed maintenance reduced the risk (Chang *et al.*, 1998a; Rager, 1990). Interestingly, Peterson *et al.* (1999) postulated that a non-classical

opioid receptor was involved in the detrimental effects of opioids on HIV disease progression as has been suggested for other opioid immunomodulations.

Whilst this is a very important and clinically relevant area of research, it is not the focus of this research project. For additional information refer to the several quality reviews and research articles which are available in this area (Ayuso, 1994; Chao *et al.*, 1994; Donahoe, 1996; Donahoe & Vlahov, 1998; Hubbard *et al.*, 1988; Kreek, 1990; Kreek *et al.*, 1986; Zolla-Pazner *et al.*, 1987).

Table 1.4-22 Summary of the *in vivo* opioid effects on infection defence in animals.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	Susceptibility to sub-lethal endotoxin challenge	Naltrexone	Hilburger *et al.* (1997b)
Morphine	↑	Oral infection	Naltrexone	MacFarlane *et al.* (2000)
Morphine	↑	Mortality induced by sepsis	Naltrexone (low dose morphine only)	Roy *et al.* (1999)
Morphine	↓	Immune response to SRBC	Naloxone	Gungor *et al.* (1980)
Morphine	↔	Humoral response to bacterial and viral antigens		Molitor *et al.* (1992a)
Morphine	↑	Lethality of murine toxoplasmosis		Chao *et al.* (1991)
Morphine	↓	Viral-induced mortality	Naloxone	Alonzo *et al.* (1999)
Morphine	↑	Toxoplasma gondii-induced mortality	Naltrexone	Chao *et al.* (1990)
Morphine	↑	Salmonella replication		Eisenstein *et al.* (2001)
Morphine (chronic low dose)	↔	Viral infection		Starec *et al.* (1991)
Morphine (single high dose)	↑	Mortality		Starec *et al.* (1991)
Naloxone	↓	Viral-induced mortality		Alonzo *et al.* (1999)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

Table 1.4-23 Summary of the *in vivo* opioid effects on infection defence in humans.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	T cell function	Naloxone	Donahoe *et al.* (1991)
Diacetylmorphine	↓	T helper/T suppressor ratio		Donahoe *et al.* (1991)
Diacetylmorphine	↓	E-rosette formation	Cocaine	Donahoe *et al.* (1991)
Methadone	↔	Elevated IgG and IgM levels in IDU		Lazzarin *et al.* (1984)
Injecting drug use	↑	Sexually transmitted disease		Lazzarin *et al.* (1984)
Injecting drug use	↑	Oral and cutaneous bacterial infections		Lazzarin *et al.* (1984)

↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

1.4.5. Summary

Since the first study of Wybran *et al.* (1979), who identified opioid modulation of immune function, a new area of immunology and pharmacology research was grafted and a myriad of studies have been conducted. The variety of opioid ligands and the multitude of immune functions that have been found to be affected are also continually growing. The influence of opioids on immune function *in vivo* is not only via cell surface receptors, but includes central processes and signalling pathways which will be reviewed next. However, the receptors that initiate opioid immunomodulation, whether centrally or peripherally located, do not appear to be entirely classically opioid and hence have not been fully characterised. Therefore, these non-classical opioid effects need to be further investigated.

1.5. Brain-immune communication

There is now significant evidence that suggests there is bi-directional communication between the central nervous system and the immune system. Blalock (1984) went as far to call the immune system a sensory organ, which expands the role of the immune system from the traditionally conceived role of host defence (1.2). There are a number of other quality reviews on this topic from which some of the following concepts were drawn (Lutgendorf, 2003; Maier & Watkins, 1998; Maier *et al.*, 1994; Webster *et al.*, 2002).

1.5.1. Brain to immune communication

The central nervous system, in some cases, does not have to signal long distances to find immune targets. In fact, the immune cells of the brain, glia, comprise more than 85% of the total population of brain cells (Chao *et al.*, 1996b), and provide structural support to the neurons. Moreover, there are more similarities than dissimilarities between the signalling molecules of the immune system and the nervous system (Smith & Blalock, 1981). The following sections will briefly introduce some of the mechanisms through which communication between these two systems can occur.

1.1.1.14. Hypothalamus Pituitary Adrenal (HPA) axis

The HPA axis, together with the systemic sympathetic and adrenomedullary systems, are the peripheral limbs of the stress system, whose main function is to maintain basal and stress-related homeostasis (Chrousos, 1995), and to prepare the body for the fight or flight response. The HPA axis interacts with the immune system, sensing inflammatory signals, such as TNF-α, IL-1 and IL-6, and modulating the activity of this system primarily via its product,

glucocorticoids (e.g. cortisol). The brain monitors and integrates these communications and, when appropriate, activates secretion of corticotropin-releasing hormone from the hypothalamus. These hormones stimulate the pituitary gland to secrete pro-opiomelanocortin products, which include adrenocorticotropic hormone and β-endorphin, which stimulates the adrenal cortex to secrete glucocorticoids. Substances that promote corticotropin-releasing hormone secretion include stimulatory neurotransmitters, cytokines, and corticotropin-releasing hormone itself. Corticotropin-releasing hormone secretion is decreased by inhibitory neurotransmitters; in addition, endogenous opioids provide continuous background inhibition of corticotropin-releasing hormone secretion. Furthermore, cortisol modulates its own release, dampening potentially excessive HPA responses. This system is summarised in the left side of Figure 1.5-1. Pro-inflammatory cytokines stimulate the HPA axis to release anti-inflammatory glucocorticoids to rebalance the system, summarised in the right side of Figure 1.5-1. Any disturbance in the functioning of the HPA axis at any level can cause an imbalance in the system, leading to potential susceptibility to infection if over stimulation occurs, or immunological dysfunction resulting in damage to self when under stimulation eventuates (Webster *et al.*, 2002).

Acute administration of exogenous opioids, such as morphine, cause an elevation in ACTH, β-endorphin and corticosterone (Zhou *et al.*, 1999), whereas chronic administration attenuates their production (Fuertes *et al.*, 2000; Mellon & Bayer, 1998a). These chronic effects of morphine are associated with significant changes in immune function (as discussed previously) partially due to alterations in the homeostasis of the HPA axis. House *et al.* (2001) suggested that some of these immunomodulations may be the result of selective tolerance of some systems to the effects of opioids, leading to uncontrolled activation of others (Figure 1.5-2). The effect is believed to be mediated via opioid receptors as opioid

receptor knockout animal models have shown no effect of morphine on glucocorticoid levels (Roy *et al.*, 2001c).

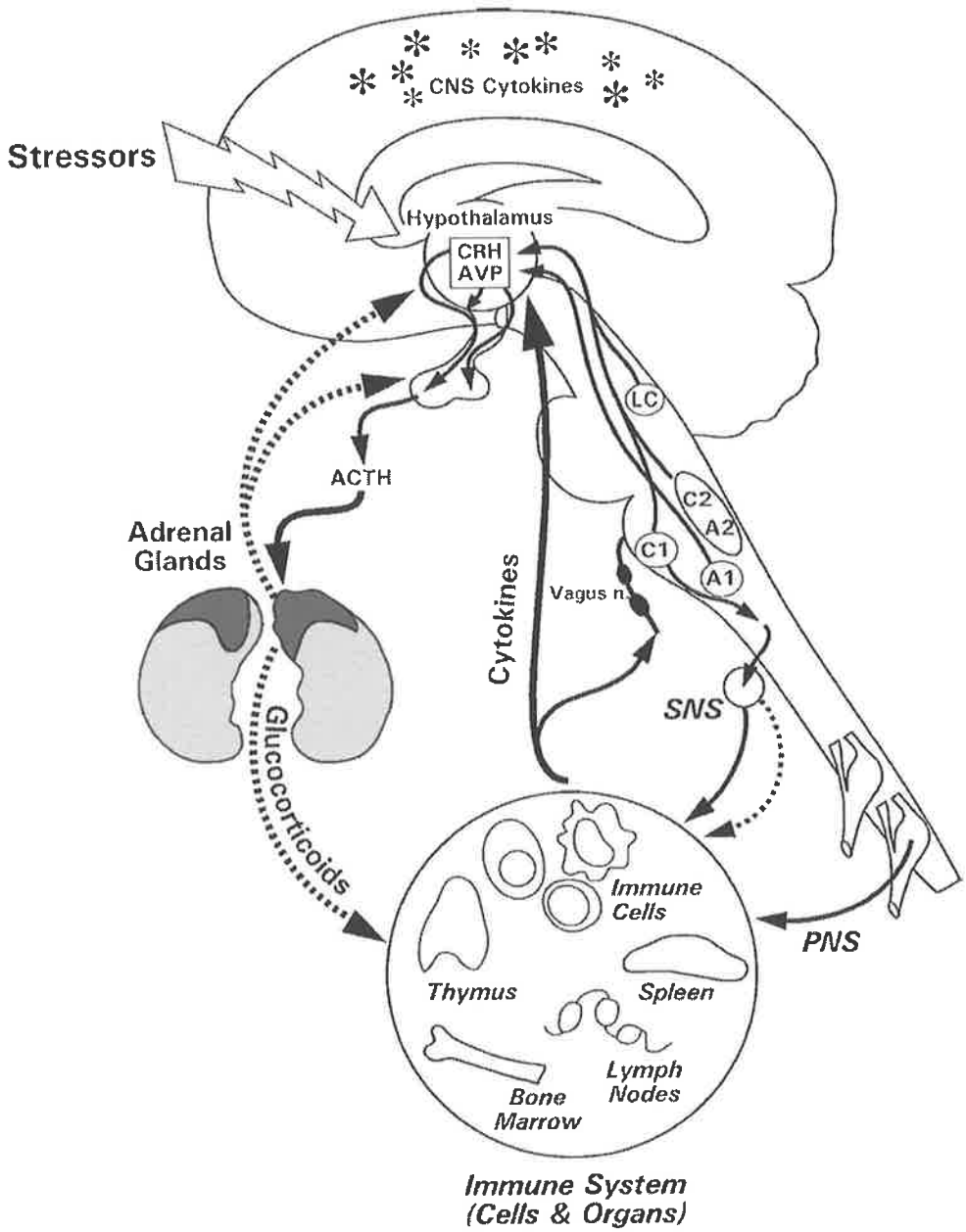


Figure 1.5-1 Diagram of the routes of communication between the brain and the immune system, including the HPA axis, sympathetic (SNS) and peripheral nervous systems (PNS), vagus nerve and cytokine feedback to the brain.

Webster *et al.* (2002) reprinted, with permission, from the Annual Review of Immunology, Volume 20 © 2002 by Annual Reviews www.annualreviews.org

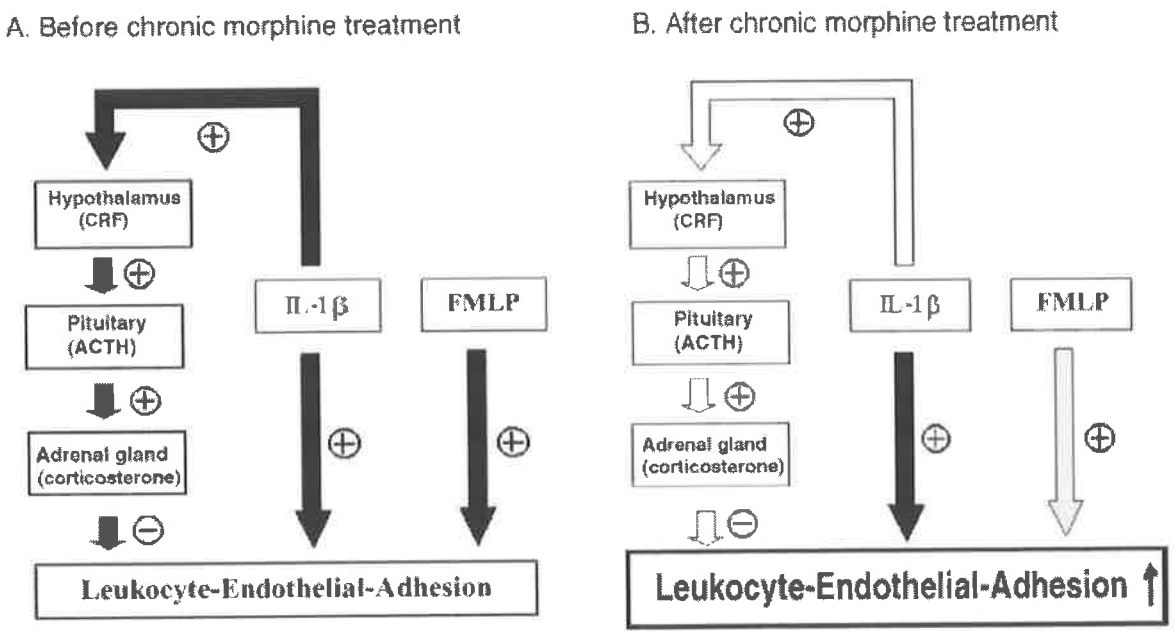


Figure 1.5-2 An example of the selective tolerance of some pathways in the HPA axis following chronic morphine treatment.

House *et al.* (2001) reprinted with permission from the Journal of Neuroimmunology, Volume 118 © 2001 by Elsevier.

1.1.1.15. Peripheral neuronal innervation

The second way the brain can communicate with the immune system is via the autonomic nervous system. Sympathetic neurons innervate and terminate in the major organs of the immune system (Felten *et al.*, 1985; Felten *et al.*, 1981). These noradrenergic fibres make synapse-like connections with splenocytes and release noradrenaline and other neurotransmitters, which then act on their respective receptors expressed by immunocompetent cells (Mellon & Bayer, 1998a; O'Dorisio *et al.*, 1985; Payan & Goetzl, 1985). These include α- and β-adrenergic receptors, with the activation of β_2 receptors resulting in modulation of the immune response (induction and inhibition), whilst α-adrenergic receptors activate different signalling pathways causing different effects, allowing for extremely tightly controlled responses (Heijnen & Kavelaars, 1999; Hori *et al.*, 1995;

Madden *et al.*, 1995). Activation of the sympathetic nervous system has compartmental specificity, with thymic responses modulated, whilst the antibody response of splenic and lymph node cells are enhanced (Felten & Felten, 1988). Interestingly, this system is differentially affected by morphine and buprenorphine, with decreased or no effect on bioamine release following buprenorphine, in contrast to morphine-induced increases of bioamine release (Hall *et al.*, 1998). These data suggest that morphine and buprenorphine are not acting at the same receptor sites, casting doubt over the role of classical opioid receptors in activation of the autonomic nervous system.

1.5.2. Examples of brain-immune cross talk and the role of opioids

1.1.1.16. Influence of stress on the immune system

As already mentioned psychological and physical stress are able to modulate the immune system via activation of the HPA axis and sympathetic nervous system. When these systems are activated following surgery, patient recovery is hampered, meaning that reduction of such stressors associated with surgery would be beneficial. Therefore, this is a significant and very productive area of research with many studies reporting normalisation of the stress response reducing such symptoms as post-operative pain (Beilin *et al.*, 2003), thereby implicating the immune system in pain perception. Furthermore, the type of analgesic and anaesthetic used in the surgery has also been associated with differential modification of the immune response (Procopio *et al.*, 2001).

Other stressors such as experimental administration of endotoxins and cytokines associated with endotoxin exposure have been shown to result in immune-brain communication. As shown in Figure 1.5-1, the vagal nerve is involved in the transmission of inflammatory signals back to the brain resulting in elevated central pro-inflammatory cytokine levels (Fleshner *et*

al., 1998). However, this only occurs under some conditions (Hansen *et al.*, 2000; Van Dam *et al.*, 2000) as some central responses are independent of this pathway. Of interest is that a number of the stressful stimuli cause analgesic responses that can be attenuated by classical opioid antagonists (Bianchi *et al.*, 1999; Stein, 1993), whilst other stressors induce non-classical opioid analgesia (Mizoguchi *et al.*, 1997). The analgesic responses resulting from electric foot shock were found to depend on the extent of shock; 2 and 80-100 shocks induced naltrexone reversible analgesia mediated via spinal κ opioid receptors (Watkins *et al.*, 1992b), whilst 5-40 shocks were insensitive to naltrexone antagonism (Sutton *et al.*, 1994). The non-opioid analgesia was further investigated and found to be blocked by combinations of selective opioid receptor antagonists: μ, κ and δ antagonists, μ and δ antagonists, and μ and κ antagonists but not by κ and δ antagonist combinations (Watkins *et al.*, 1992a). Distinctions between front and hind paw shock were also made, with front paw shock attenuated by naloxone, whilst hind paw shock was not (Watkins *et al.*, 1994).

1.1.1.17. Immune involvement in pain control

Our understanding of the role that the immune system plays in the control of pain is constantly expanding. Inflammation is well accepted as a modulator of pain-sensitivity, however, the subtle involvement of other immune derived factors in this process is still being unravelled. Cytokines, such as IL-2, IL-4, IL-10, IL-13 and IL-1 receptor antagonist (IL-1Ra) have been shown to directly induce analgesia with some displaying naloxone-sensitivity (Jiang *et al.*, 2000b; Kanaan *et al.*, 1998; Maier *et al.*, 1993; Sommer *et al.*, 2001; Song *et al.*, 2002a; Vale *et al.*, 2003; Wang *et al.*, 1997; Yao *et al.*, 2002). There is considerable debate as to the receptor that mediates interferon-induced analgesia, as some believe it to be an opioid receptor because of classical opioid antagonist-sensitivity (Jiang *et al.*, 2000a; Wang *et al.*,

2000), while others found no such potential for opioid involvement (Reyes-Vazquez *et al.*, 1984), and some have found the combination of both opioid and non-opioid systems (Song *et al.*, 2002b; Song & Zhao, 2000). Interestingly, Wang *et al.* (2001; 2002) have even reported characterisation of the analgesic domain of IFN and hypothesised a residue that binds to the μ opioid receptor.

Further interactions occur between immune signalling molecules and opioid receptors. Szabo *et al.* (2002) demonstrated chemokines attenuated opioid analgesia by heterologous desensitisation of opioid receptors by chemokine receptors demonstrating another feedback pathway to return the system to homeostasis. Some cytokines such as IL-1, IL-6 and TNF-α have been shown to induce hyperalgesia (Junger & Sorkin, 2000; Maier *et al.*, 1993; Oka *et al.*, 1995; Tadano *et al.*, 1999). However, the dose administered is very important, as analgesia can result from IL-1 administration (Bianchi *et al.*, 1998; Bianchi *et al.*, 1992; Hori *et al.*, 1998; Ji *et al.*, 2002a; Ji *et al.*, 2002b).

Cytokines have also been shown to alter the binding of opioid ligands to opioid receptors with increases (Wiedermann, 1989) and decreases observed (Ahmed *et al.*, 1985), although a recent study has not confirmed these results (Roy *et al.*, 1992). Further research into the influence of cytokine modulation of opioid signalling is required in light of the potential link with nociception and analgesia. The immune system also contributes to pain relief by the release of endogenous opioids (Sharp & Yaksh, 1997; Stein *et al.*, 1990). During inflammation, β-endorphin containing lymphocytes, primarily activated memory cells (Mousa *et al.*, 2001), selectively home toward inflamed tissues (Cabot *et al.*, 1997a) which then release their opioid load preferentially in the inflamed area (Cabot *et al.*, 2001).

Spinal glial cells also appear to play a role in pain control as Milligan *et al.* (2003) found these cells were responsible for mirror-image inflammatory neuropathy pain, created both acutely and chronically through glial and pro-inflammatory cytokine actions. Furthermore, these glial cells appear to be responsible for the generation of pro-inflammatory cytokines which in turn cause tolerance to morphine (Raghavendra *et al.*, 2002). Bianchi *et al.* (1999) demonstrated that IL-6 knockout mice had reduced analgesic response to the restraint stress and to the administration of low-dose morphine. Tolerance to morphine developed more rapidly in IL-6 knockout mice, and these mice had a decreased number of opioid receptors in the midbrain but not in the hypothalamus, therefore the density of μ opioid receptors diminished while that of δ opioid receptors was unchanged. Hence, IL-6 is necessary for correct development of the opioid system and is involved in the response to both endogenous and exogenous opioids (Bianchi *et al.*, 1999). These data are supported by others who suggest cytokines are also able to control the expression of opioid receptors. For example, the combination of IL-1α and IL-1β causes the increase in μ opioid receptor expression (Chang *et al.*, 1998b; Vidal *et al.*, 1998), while IL-6 is capable of reducing κ opioid receptor expression (Jenab & Morris, 2000).

The link between the immune system, pain perception and opioids has been strengthened by the use of animal models with altered sensitivity to morphine. For example, diabetic mice have reduced antinociceptive effect following morphine administration (Gul *et al.*, 2000; Kamei *et al.*, 1992; Kamei *et al.*, 1993). Furthermore, splenectomy restores morphine antinociception, whilst transfer of splenocytes from diabetic mice to healthy controls produces a non-responsive animal (Kamei *et al.*, 1992). Similar results were found for the morphine tolerant Beige-J mouse strain, including adoptive transfer of tolerance (Kimball & Raffa,

1989; Raffa *et al.*, 1988). The response in diabetic mice was found to be dependent on elevated levels of IL-1β (Gul *et al.*, 2000).

These data suggest a substantially expanded role for the immune system than merely host defence. Moreover, the data implicate the immune system in the delicate control of the sensory system. Therefore, further research into this developing area is required to fully appreciate the role the immune system plays.

1.1.1.18. Opioid Withdrawal

All of the links between the immune system, opioids and central nervous system process, sparked research into the influence the immune system has on opioid dependence and withdrawal. IFN-α, cyclosporine A, gamma irradiation, cyclophosphamide, cortisol, muramyl peptide, tuftsin and dietary immune suppression all inhibited the precipitation of opioid withdrawal (Aronowski *et al.*, 1985; Dafny, 1983a, b, 1984; Dafny *et al.*, 1989a, b; Dafny *et al.*, 1988; Dafny & Pellis, 1986; Dafny & Reyes-Vazquez, 1985, 1987; Dafny *et al.*, 1985; Dafny *et al.*, 1983; Dougherty *et al.*, 1986a; Dougherty & Dafny, 1988; Dougherty *et al.*, 1987a; Dougherty *et al.*, 1986b; Dougherty *et al.*, 1987b; Dougherty *et al.*, 1990; Hall *et al.*, 1991; Kulkarni *et al.*, 1997; McVaugh *et al.*, 1989; Montgomery & Dafny, 1987; Pellis *et al.*, 1987). Furthermore, Dougherty *et al.* (1990) demonstrated that re-establishment of adherent cells (predominantly macrophages) into immunosuppressed animals allowed precipitation of withdrawal. In contrast to these findings, others were unable to demonstrate reduced opioid withdrawal as a result of immunosuppression (Berthold *et al.*, 1989; Dantzer *et al.*, 1987). Instead, other environmental factors and the sickness of the animals were suggested to have caused the observed results. The secondary effects of cyclosporine to

inhibit nitric oxide production were investigated and were found to attenuate antinociception and withdrawal via a mechanism dependent on reduced nitric oxide levels (Homayoun *et al.*, 2002a; Homayoun *et al.*, 2002b). However, the use of other agents which act to suppress the immune system and increase nitric oxide levels (Angulo *et al.*, 2000), suggest that while this mechanism may be involved, it is not the only pathway. Interestingly, Fecho *et al.* (1994; 1995a) concluded that macrophage-derived nitric oxide was required for morphine-induced immunosuppression, thereby implicating immune-derived nitric oxide in the withdrawal process, hence satisfying both hypotheses.

1.6. Summary and Aims

The complexity of the immune system is undoubtedly greater than we currently appreciate. There is substantial evidence that exogenous and endogenous opioids are capable of modulating the immune system, although, the physiological reason and significance of these effects is not yet apparent. Nonetheless, the extent of the immunomodulatory effects suggests that opioids are an extremely important mode of communication for the immune system, with the preservation of the morphine response on immune cells from invertebrates and vertebrates conserved for potentially 500 million years since diverging from the evolution of humans. Immunocompetent cell expression of receptors that bind opioids is well accepted, however the characteristics of these receptors appear to be different to their classical counterparts expressed by neuronal cells, as are the effects that are mediated via these receptors. Furthermore, some *in vivo* data have suggested non-classical opioid effects that originated at central levels, implying the expression of non-classical opioid receptors also extends to these neuronal cells. This is not to dismiss the data demonstrating the expression of classical opioid receptors and effects by immunocompetent cells, but instead to acknowledge that both varieties of receptors may be present and to recognise the need to characterise the novel effects. An inhibiting fact that has confused the research is the disparity of results using similar methodologies, making comparisons between opioid ligands extremely difficult. Furthermore, the use of a single antagonist to determine classical opioid characteristics has also led to potentially misleading conclusions. Soon after Wybran *et al.* (1979) demonstrated the link between the immune system and opioids, others began investigating the novel roles the immune system could play in opioid pharmacodynamics and systems such as pain perception. This research has been very fruitful, with our understanding of the immune system changing from one of purely host defence, to a more wondrous system capable of sensing and communicating the presence of infection. These expanded roles of the immune

system have also extended into modulating pain perception. The role opioids and non-classical opioid receptors have in the immune systems modulation of pain is unknown. These unanswered questions opened the door for a research project into opioid immunopharmacology. Therefore, the aims of this PhD project were

Aim 1: To develop an experimental system in which the direct immunomodulation of immune function by the drug applied to the system could be quantified rapidly and safely. This aim is addressed and discussed in Chapter 2. The experimental system that was optimised was a splenocyte mitogenesis assay, with special attention taken to quantify the proliferative response within pharmacologically relevant experimental conditions. Furthermore, the instability of opioid ligands in cell culture was also addressed.

Aim 2: To quantify the *in vitro* immunomodulatory effects of a wide variety of 4,5- and non-4,5-epoxymorphinans using the experimental system developed. This aim is addressed and discussed in Chapter 3 for 4,5-epoxymorphinans and in Chapter 4 for non-4,5-epoxymorphinans. Using this large number of compounds within the one experimental system allowed for comparison of the inter-ligand differences in response, and the development of a structure-effect relationship for 4,5-epoxymorphinan immunomodulation. Moreover, the use of non-4,5-epoxymorphinans allowed further clarification of the non-classical opioid nature of the direct immunomodulation by opioids.

Aim 3: To quantify the *in vivo* immunomodulatory effects of opioids on *ex vivo* splenocyte proliferation with respect to the involvement of non-classical opioid receptors. This aim is addressed and discussed in Chapter 5. Using racemic methadone and its individual isomers, a comparison of the antinociceptive and immunosuppressive efficacy of these compounds was

undertaken. This *in vivo* study allowed quantification of both direct and indirect action of opioids on splenocyte immune function, with unexpected results.

Aim 4: To examine the relationship between pain, opioids and the immune system in humans. This aim is addressed and discussed in Chapter 6. Using isolated peripheral blood mononuclear cells from healthy subjects the *in vitro* immunomodulatory capacity of several opioids (both 4,5-epoxymorphinans and non-4,5-epoxymorphinans) was quantified and related to *in vivo* pain responses.

Chapter 2. Cell culture methods development

2.1. Introduction

Research into the direct influence of opioids on immunocompetent cell function has become increasingly important due to numerous studies implicating the immune system in the pharmacodynamic effects of opioids. For example, it has been established that the expression and release of endogenous opioid agonist peptides by activated immune cells during inflammation (Cabot *et al.*, 1997a) and stress (Rittner *et al.*, 2001) is responsible for analgesia resulting from these insults. Moreover, recent evidence demonstrating that immune-derived cytokines are integral to the development of opioid tolerance (Raghavendra *et al.*, 2002) and previous research suggesting that immune status alters the analgesic efficacy of morphine (Kamei *et al.*, 1992) and the severity of opioid withdrawal (Dougherty *et al.*, 1990) all imply a substantial role for the immune system, and in particular peripheral immunocompetent cells in opioid pharmacodynamic effects. It is therefore imperative to understand, not only *in vivo* influences, but also the *in vitro* consequences of opioid exposure, as the mechanisms via which opioids alter immunocompetent cells directly and indirectly differ greatly and *in vitro* responses are important to understand from a mechanism perspective as they help in interpreting *in vivo* effects.

Numerous *in vitro* cell culture models have been utilised to investigate the immunomodulatory effects of morphine and a small number of other 4,5-epoxymorphinans and non-4,5-epoxymorphinans, as well as a number of endogenous and synthetic peptides. Various types of immune tissues have been investigated (Table 2.1-1) with a number of immune functions quantified (Table 2.1-2). Quantification of the cellular functions has also differed, for instance, proliferation has been quantified using tritiated thymidine incorporation (Bocchini *et al.*, 1983), MTT assays (Sulowska *et al.*, 2002) or XTT assays (Thomas *et al.*, 1995b) just as some examples. There are numerous other parameters which have varied

between experiments and models used by different research groups including total incubation time, pre-exposure of drug prior to stimulation, total exposure time (including quantification step), quantification time, cell number, foetal calf serum concentration and stimulation strength (mitogen, cytokine or antibody concentration). It is clear that the variety of methods that have been used to investigate opioid immunomodulation is large, and this review will only focus on those that have been used for *in vitro* experiments. The results gathered from the plethora of experiments conducted have been conflicting and hence have led to confusion surrounding the physiological affect and significance of *in vitro* opioid immunomodulation. It should be reiterated that the direct effects of opioids depend on the cell type and stimulation used.

Table 2.1-1 Experimental models used to investigate opioid immunomodulation

Experimental model	Type	Study
Tissue Cultured	Peripheral blood mononuclear cells	Wybran (1985)
	Thymocytes	Roy *et al.* (1991)
	Splenocytes	Barreca *et al.* (1987)
	Peritoneal macrophages	Foster *et al.* (1987)
	Lymph node cells	Hemmick *et al.* (1991)
	Glial cells	Chao *et al.* (1994)
	Bone marrow	Loh *et al.* (1993)
	Peyer's patch	Carr *et al.* (1990)
	Keratinocytes	Bigliardi *et al.* (2002)
	Alveolar macrophages	Zhou *et al.* (1992)
	Dendritic cells	Esche *et al.* (1999)
	Langerhans cells	Hosoi *et al.* (1999)
	Purified cell populations	Wybran *et al.* (1979)
	Tumour cell lines	Iuvone *et al.* (1995)
Organism	Human	Wybran *et al.* (1979)
	Monkey	Donahoe (1991)
	Mouse	Carr *et al.* (1986)
	Rat	Mediratta *et al.* (1988)
	Swine	Molitor *et al.* (1992b)
Strain		Eisenstein *et al.* (1995)

Table 2.1-2 Experimental functions quantified to investigate opioid immunomodulation

Function	Cell type	Study
Cytotoxicity	Natural Killer cell	Prete *et al.* (1986)
	T cell	Prete *et al.* (1986)
Phagocytosis		Casellas *et al.* (1991)
Chemotaxis		Peterson *et al.* (1995)
Mitogen-induced proliferation	Concanavalin A	Jessop *et al.* (1991a)
	Lipopolysaccharide	Thomas *et al.* (1995a)
	Phytohemagglutinin	Roy *et al.* (1991)
	Toxic shock syndrome toxin	West *et al.* (1999)
	Pokeweed	Martinez *et al.* (1990)
Other mitogenic stimulation	Cytokine	Roy *et al.* (1991)
	Antibody	Riley *et al.* (1998)
	Cytokine & antibody	Riley *et al.* (1998)
Toxicity		Falek *et al.* (1991)
Viability		Peterson *et al.* (1989)
Cytokine production		Chang *et al.* (1996)
Receptor expression		Shahabi *et al.* (1995a)
mRNA levels		Bessler *et al.* (1990)
Protein synthesis		Jessop *et al.* (1991a)

2.1.1. Methods: Method Optimisation

It is my belief that the methods used to investigate opioid immunomodulation must be rigorously optimised in order to detect changes in cell function in a reproducible and reliable manner. Therefore, the splenocyte mitogenesis model was employed, as this was a widely used and reported method in the literature for opioid immunomodulation. It can be a simple, rapid, cost effective technique that reflects the activity of numerous cellular functions allowing for a broad spectrum analysis of cellular function. Balb/c mice were chosen as the animal source of immune tissue due to availability, cost and previous published data demonstrating that this strain shows a robust opioid immunomodulation (Eisenstein *et al.*, 1995). Other strains investigated by Eisenstein *et al.* (1995) did not display significant

opioid-induced alterations and were therefore less sensitive to immunomodulation, therefore making the opioid-induced changes more difficult to detect and quantify. The parameters which were identified that required optimisation included: exposure time (incubation with opioids), total incubation time (including quantification), mitogen concentration, cell number, foetal calf serum concentration and the method used for quantification of proliferation, since some of these parameters have previously been reported to influence the response to opioids (Gilmore *et al.*, 1990). AlamarBlue was chosen to quantify proliferation as opposed to tritiated thymidine incorporation, as quantification takes hours, rather than days, reducing the total incubation time (Ahmed *et al.*, 1994). Moreover, AlamarBlue can be added to the stimulated cells earlier, before large amounts of proliferation have occurred due to its sensitivity detecting proliferation (Nakayama *et al.*, 1997).

AlamarBlue incorporates a fluorometric/colorimetric growth indicator based on the detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction indicator which fluoresces in response to chemical reduction of growth media resulting from cell growth. Minimising the total incubation time was important as previous reports demonstrated that responses to opioids are not as great after longer incubation times (Shahabi & Sharp, 1995a) and in fact there was down regulation of receptor expression after long incubations (Sharp *et al.*, 1997). AlamarBlue has been shown to produce reliable quantification of proliferation, with results that are comparable to those responses obtained from tritiated thymidine (Ahmed *et al.*, 1994; Zhi-Jun *et al.*, 1997) or MTT assays (Guarino *et al.*, 1999). It has also been shown not to interfere with cytokine mRNA synthesis or expression, expression of cell surface antigens and does not induce chromosomal damage (Zhi-Jun *et al.*, 1997). Moreover, AlamarBlue does not have the other drawbacks of tritiated thymidine, such as the use of radioactive nucleotides and potential health and environmental hazard.

2.1.2. Methods: Exposure toxicity

To ensure that any inhibition of the quantified proliferative response was not due to non-specific cell toxicity or premature death, the cultures were tested for increased rates of lactate dehydrogenase leakage. Lactate dehydrogenase is an intracellular enzyme that leaks out into the culture media when the cell membrane is compromised, indicating the cell is no longer viable or has begun the process of cell death (Burcham *et al.*, 2002). Lactate dehydrogenase catalyses the oxidation of L-lactate to pyruvate, with nicotinamide adenine dinucleotide accepting the reducing equivalents. The fluorescence assay quantifies the formation of reduced nicotinamide adenine dinucleotide. The rate of formation is dependent on the amount of lactate dehydrogenase that has leaked into the media, representative of the degree of cell death.

2.1.3. Methods: *In vitro* bioavailability

In any experimental model where a known concentration of drug is added to the system and the quantified response is related to that concentration, it is vital to know the concentration of the drug that is available to act in the system. This will be referred to as the *in vitro* bioavailability of the drug. Factors in the experimental system such as serum, plastics, filters, degradative enzymes or the experimental environment including temperature and pH may reduce the amount of available drug and hence the *in vitro* bioavailability. These factors may alter the *in vitro* bioavailability by binding free drug or by degrading the drug to other compounds that may have altered activity (either increased or decreased). In the experimental system employed here, binding to the plastic wells of the 96 well plates or foetal calf serum and degradation of a few of the applied drugs were identified as factors which may reduce the

in vitro bioavailability. Therefore, steps were taken to quantify the affect each factor may have.

2.1.4. Methods: Diacetylmorphine and cell culture

Diacetylmorphine (heroin or 3,6-diacetylmorphine) is a highly addictive opioid agonist, whose use accounts for many drug-related illnesses, deaths and social problems (Ryan & White, 1996). Diacetylmorphine rapidly deacetylates (half-life of 3 minutes) *in vivo* to 6-monoacetylmorphine (Figure 2.1-1), which is further rapidly deacetylated to morphine (Inturrisi *et al.*, 1984; Rentsch *et al.*, 2001). *In vivo* these reactions are catalysed by serum butyrylcholinesterase (diacetylmorphine to 6-monoacetylmorphine) (Lockridge *et al.*, 1980; Salmon *et al.*, 1999), and erythrocyte acetylcholinesterase (diacetylmorphine to 6-monoacetylmorphine to morphine) (Salmon *et al.*, 1999). *In vitro*, the stability of diacetylmorphine is significantly affected by pH, temperature and light (Barrett *et al.*, 1992; Wijesekera *et al.*, 1994). Small changes in these conditions (pH or temperature) can cause increased spontaneous diacetylmorphine hydrolysis to 6-monoacetylmorphine, and 6-monoacetylmorphine to morphine.

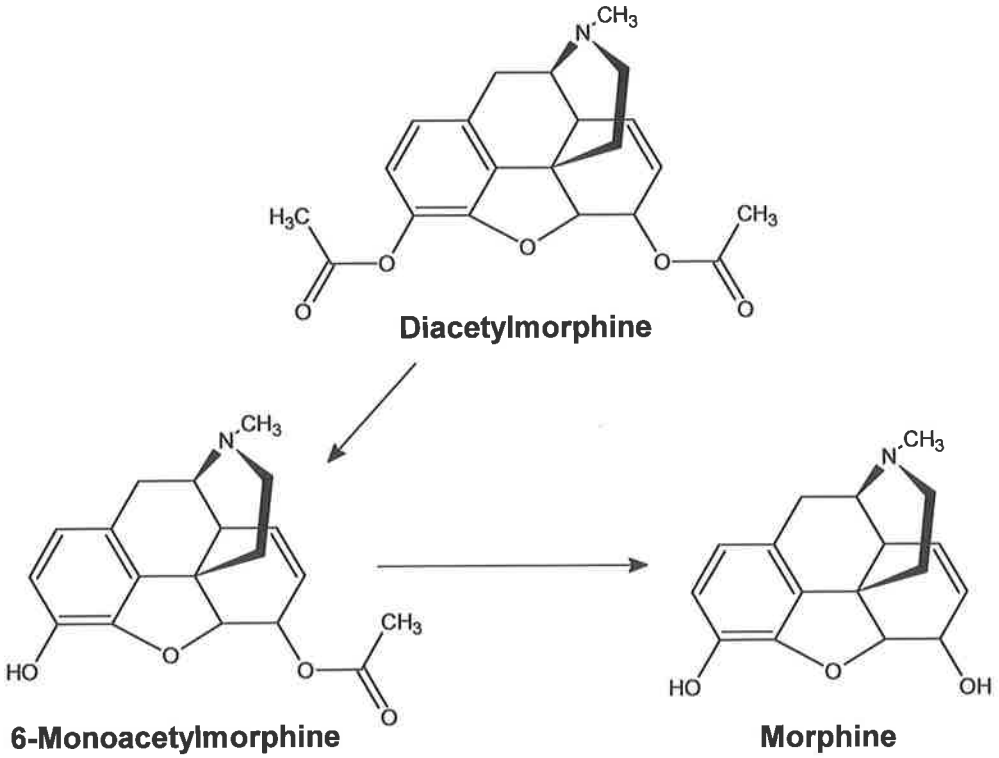


Figure 2.1-1 Diacetylmorphine degradation pathway.

The pharmacological activity of diacetylmorphine has been attributed to its metabolites 6-monoacetylmorphine, morphine, and morphine's metabolites all acting on opioid receptors (White & Irvine, 1999). Recently however, a specific diacetylmorphine receptor has been identified by which diacetylmorphine alone may produce analgesia (Brown *et al.*, 1997b; Rossi *et al.*, 1997). In light of the instability of diacetylmorphine, it is difficult to isolate its effects in *in vitro* systems and subsequently attribute these to diacetylmorphine and not its active metabolites. For example, a number of studies have investigated the immunomodulatory behaviour of diacetylmorphine *in vitro* (Perez-Castrillon *et al.*, 1992; Thomas *et al.*, 1995b). In the cell culture conditions employed by Thomas *et al.* (1995b), diacetylmorphine was incubated for greater than 72 hours at 37 °C in media which was subject to pH change, and possible exposure to various esterases, all of which are likely to cause increased diacetylmorphine degradation. 6-Monoacetylmorphine, morphine and morphine's metabolites (morphine-3-glucuronide, morphine-6-glucuronide) have significant

immunomodulatory effects (House *et al.*, 1997a; Pacifici *et al.*, 2000; Thomas *et al.*, 1995a). Hence, the effects of diacetylmorphine itself cannot be determined unless the extent of degradation and the primary catalyst(s) of the degradation processes are known and taken into consideration. There are no data on the degradation of diacetylmorphine in tissue culture. Therefore, the extent of diacetylmorphine degradation to 6-monoacetylmorphine and subsequently to morphine in cell culture (splenocyte mitogenesis assay) needed to be quantified, and determination of the mechanism by which diacetylmorphine hydrolyses under these conditions.

2.2. Aims

The aims of this study were to develop a method in which numerous 4,5-epoxymorphinans and non-4,5-epoxymorphinans could be examined for their immunomodulatory properties in a rapid and reproducible manner. Furthermore, the assay needed to be one in which the effects observed could be correlated with the concentration of drug applied to the system.

2.3. Methods and optimisation results

2.3.1. Chemicals

RPMI 1640 with HEPES and L-glutamine modification and Penicillin-streptomycin solution (10,000 units penicillin, 10 mg streptomycin.ml^{-1}) were purchased from Invitrogen (Mulgrave, Australia). Lipopolysaccharide from *Salmonella typhimurium*, phytohemagglutinin PHA-P, concanavalin A, sodium dodecyl sulphate phenylmethylsulfonyl fluoride, 1,5-bis 4 allydimethylammoniumphenyl pentane-3-one dibromide (BW284c51), oxycodone hydrochloride, morphine-3-glucuronide, morphine-6-glucuronide, thebaine hydrochloride, hydromorphone hydrochloride, naloxone methiodide, naloxone hydrochloride, naltrexone hydrochloride, 3-O-methylnaltrexone, naltrindole hydrochloride, naloxonazine dihydrochloride, nor-binaltorphimine dihydrochloride, DAMGO, U69593, DPDPE and *tert* butyl hydroperoxide were purchased from Sigma Chemical Co (St Louis, Missouri, USA). Oripavine and the plus inactive isomers of morphine and codeine were obtained from the National Institute of Drug Abuse (Bethesda, MD, USA). Levo-α-acetyl methadol hydrochloride, racemic methadone hydrochloride and (R)-(-)- and (S)-(+)-methadone HCl were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Levorphanol and nicotinamide adenine dinucleotide were purchased from Roche (Sydney, NSW, Australia). Fentanyl was supplied by David Bull Laboratories (Melbourne, Vic, Australia), remifentanil (as Ultiva®) was supplied by Glaxo Wellcome (Boronia, Vic, Australia) and alfentanil (as Rapifen®) was supplied by ICI Pharmaceuticals (Melbourne, Vic, Australia). Tramadol hydrochloride was purchased from Grünenthal GmbH (Stolberg, Germany). 6β-naltrexol was synthesised by the Department of Chemistry at the University of Adelaide (Porter *et al.*, 2000). Hydrocodone base, 6-hydroxyoxymorphone and norhydrocodone base were obtained from Dr S Hosztafi (ICN Alkaloida Company Ltd,

Tiszavasvári, Hungary). L-lactate was purchased from BDH Chemicals (Port Fairy, Vic, Australia). Buprenorphine and norbuprenorphine were obtained from Reckitt Benckiser Healthcare (Hull, UK). Foetal calf serum was obtained from Trace Scientific Ltd (Melbourne, Vic, Australia). Sterile clear plastic tissue culture 96 well plates were obtained from Sarstedt Inc. (Newton, NC, USA) and Nunc (Roskilde, Denmark). White plastic 96 well plates were supplied by BMG Labtechnologies (Offenburg, Germany). Eserine was obtained from BDH Australia (Smithfield, NSW, Australia), and diacetylmorphine base and 6-monoacetylmorphine from the National Analytical Reference Laboratory of the Australian Government Analytical Laboratories (Pymble, NSW, Australia). 6-Monoacetylmorphine was also a gift from Noel Sims (Forensic Science Centre, Adelaide, Australia). Noroxycodone and oxymorphone hydrochloride were obtained from Du Pont Pharmaceuticals (Wilmington, DE, USA). The active isomer of codeine phosphate was obtained from F.H. Faulding (Adelaide, SA, Australia) and morphine hydrochloride from McFarlane Smith (Edinburgh, UK). AlamarBlue was purchased from Astral Scientific (Gymea, NSW, Australia). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

2.3.2. Laboratory equipment maintenance

During the course of the laboratory work that is presented in this dissertation, several pieces of laboratory equipment were maintained and re-calibrated to reduce variability and to assure the quality of the research. Gilson pipetman pipettes (Middleton, WI, USA: P200, P1000 and P5000) were calibrated monthly, Beckman 50 pH meter was calibrated each day of use, whilst the Mettler AE 163 balance was validated every three months.

2.3.3. Cell isolation

2.3.3.1. Animals

Ethics approval to conduct the studies was obtained from the University of Adelaide Animal Ethics Committee (ethics numbers M/50/00 and M/64/01). Male Balb/c mice 6-8 weeks old were purchased from Central Animal Supplies (Waite Campus, University of Adelaide, SA, Australia) and used as donors of splenocytes for use in cell culture. Animals were provided with standard rodent feed and water *ad libitum*. Animals were housed in a standard 12 hour light dark cycle (starting at 7 am) under a constant room temperature of 22 ± 2 °C (mean ± range).

2.3.3.2. Lymphocyte preparation

Aseptic techniques were used during the preparation of the lymphocytes. Mice were sacrificed by cervical dislocation followed by prompt removal of the spleen. The spleen was prepared as a single-cell suspension by massaging and washing through a nylon mesh into a 15 ml tube (Sarstedt Inc, Nümbrecht, Germany) with up to 15 ml of RPMI 1640 (HEPES modification, 0.3 $mg.ml^{-1}$ L-glutamine, 5 ml penicillin-streptomycin solution.l^{-1}). The cells were centrifuged (Centurion Scientific, Sussex, England, Model K40 refrigerated centrifuge) at 4 °C for 5 minutes at 1000 rpm, the supernatant was discarded and the cells re-suspended in 1 ml of media followed by the addition of 10 ml of ice cold lysis buffer (1 ml of 20.56 $g.l^{-1}$ Tris base (pH 7.65), 9 ml 0.83% NH_4Cl in H_2O, mixed just prior to addition to cells). The suspension was placed on ice for 4 minutes, centrifuged (5 minutes at 1000 rpm), and the supernatant discarded. The suspension of cells were pooled (4 to 10 spleens depending on the size of the assay) and were re-suspended in 10 ml of media followed by centrifugation (5 minutes at 1000 rpm), removal of supernatant and re-suspension in 5 ml of RPMI 1640. The

number of viable lymphocytes in the suspension was counted using trypan blue and a haemocytometer.

2.3.4. Optimisation of the mitogenesis assay

2.3.4.1. AlamarBlue concentration

Fully reduced (indicative of proliferation and is a red colour) and unreduced AlamarBlue (new solution and is a deep shade of dark blue) were diluted in supplemented RPMI 1640 (10% foetal calf serum) and MilliQ water (14 concentrations 10% to 0.0006% vol/vol in 200 µl, n = 6) and plated into white 96 well plates (BMG Labtechnologies, Offenburg, Germany) to quantify the fluorescence of different concentrations of AlamarBlue in order to determine the optimal concentration of AlamarBlue for quantifying proliferation. Fluorescence was quantified (excitation 545 nm, emission 590 nm, gain 15) on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany).

The fluorescence of fully reduced AlamarBlue over a wide concentration range in both MilliQ water and supplemented RPMI 1640 was non-linear. Further dilution of AlamarBlue from 10% vol/vol caused an increase in the fluorescence signal that peaked at 1.25% vol/vol in supplemented RPMI 1640 and between 0.625% and 1.25% vol/vol in MilliQ water (Figure 2.3-1). AlamarBlue was therefore used at 2.5% vol/vol: each well received 5 µl AlamarBlue and 20 µl RPMI 1640 into a total volume of 200 µl. Of note is the quenching of the florescence signal by either foetal calf serum or RPMI 1640, or both. This degree of quenching is of no concern, however it should be investigated when developing such assays in the future if higher concentrations of foetal calf serum are used.

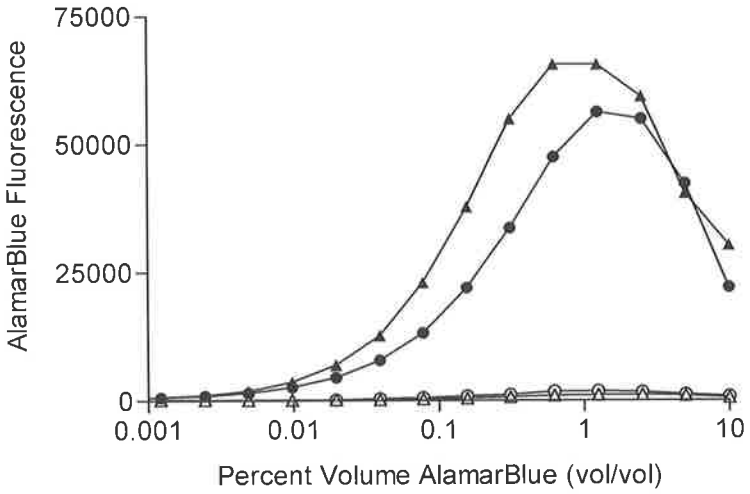


Figure 2.3-1 Optimisation of AlamarBlue concentration for quantification of fluorescence.

Different concentrations of AlamarBlue in MilliQ water (reduced ▲, unreduced O) and supplemented RPMI-1640 (reduced ●, unreduced △).

2.3.4.2. AlamarBlue incubation time

Subsequent to determining the optimal AlamarBlue concentration, the optimal AlamarBlue incubation time was established. Splenocytes (5×10^5 cells.well^{-1}, 10% foetal calf serum) isolated as previously described were incubated for 24 hours in 96 well plates at 37 °C, 5% CO_2 in a humidified incubator (Thermoline, Sydney, Australia) with concanavalin A (2.5 μg.ml^{-1}). AlamarBlue in RPMI 1640 (25 μl total: 5 μl AlamarBlue, 20 μl RPMI 1640 = 2.5% vol/vol) was then added to the wells and incubated for a further 0, 1, 3, 4, 6 or 8 hours. At the required times, 175 μl of the cell media was removed and pipetted into white 96 well plates for quantification of AlamarBlue fluorescence (as described previously). A two-tailed unpaired student t-test was performed at each time point compared with control with statistical significance set at $P < 0.05$. All data are presented as mean ± S.E.M. (n = 6).

AlamarBlue fluorescence increased with time in incubations containing concanavalin A (2.5 $\mu l.ml^{-1}$) reducing the AlamarBlue significantly (P = 0.04) more from three hours (Figure 2.3-2) compared to the control. An incubation time of four hours was chosen, since this time was short enough to demonstrate a significant difference in the proliferation by concanavalin A over control; proliferation rate was still in linear and the incubation time was short enough to be convenient.

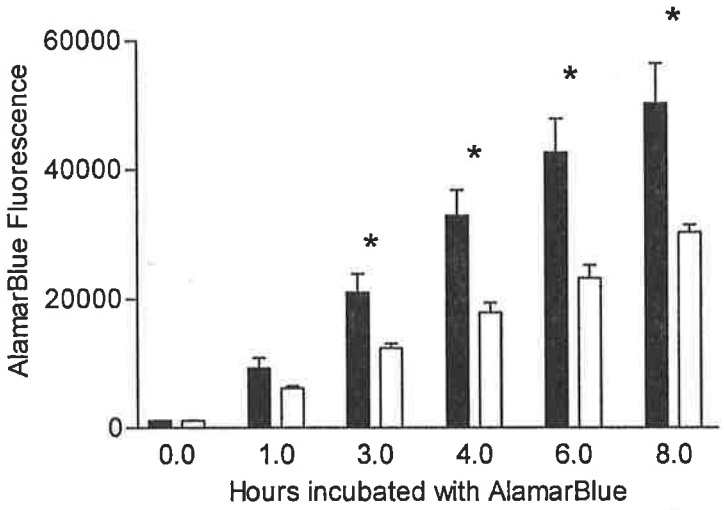


Figure 2.3-2 Time dependent increases in AlamarBlue fluorescence following addition to splenocytes.

Concanavalin A 2.5 $\mu g.ml^{-1}$ (■), control (□). Error bars are S.E.M. (n = 6) statistical significance is indicated by * = $P < 0.05$ compared with control at each time point.

2.3.4.3. Proliferation assay incubation time

Experiments were conducted to determine the incubation time required to achieve adequate concanavalin A proliferation. Splenocytes were isolated as before and plated into 96 well plates (5×10^5 cells.well^{-1}, 10% foetal calf serum) with concanavalin A (2.5 and 0.1 $\mu g.ml^{-1}$). The plates were incubated (5% CO_2 at 37 °C) for 0, 4, 8, 12, 16, 24, 32, 40 or 48 hours

followed by addition of AlamarBlue (2.5% vol/vol, 4 hour incubation) and fluorescence was quantified at 4, 8, 12, 16, 20, 28, 36, 44 and 52 hours as stated before (n = 6).

Proliferation of concanavalin A-induced splenocytes peaked initially at 12 hours (likely due to the handling and plating of the cells), but returned to control levels four hours later (Figure 2.3-3). The higher concentration of concanavalin A (2.5 $\mu g.ml^{-1}$) caused a time dependent linear increase in proliferation from 20 hours to 36 hours (significantly different from control at 28 hours, $P = 0.02$) and plateaued from 36 hours to 53 hours (Figure 2.3-3). Therefore, an incubation time of 28 hours (including the four hour AlamarBlue incubation) was chosen since significant mitogen-induced proliferation was observed and this time occurred in a linear portion of the proliferation response.

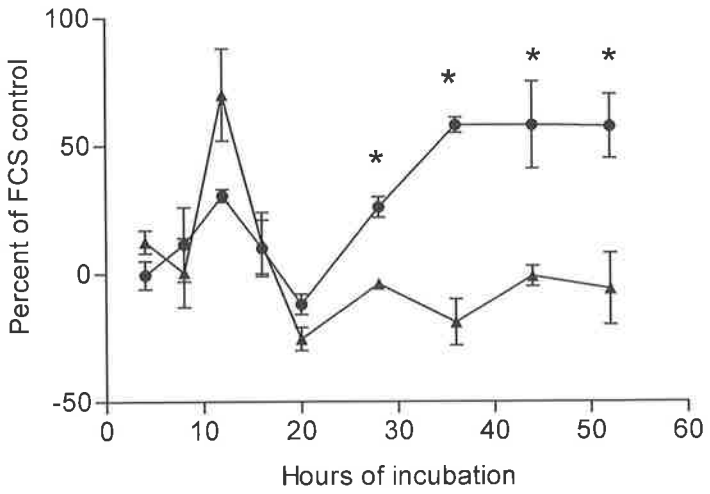


Figure 2.3-3 Time dependent concanavalin A-induced proliferation of splenocytes.

2.5 $\mu g.ml^{-1}$ (●) and 0.1 $\mu g.ml^{-1}$ (▲) Concanavalin A-induced proliferation expressed as the percent of the foetal calf serum control. Error bars are S.E.M. (n = 6) and statistical significance is indicated by * = $P < 0.05$.

2.3.4.4. Foetal calf serum concentration, cell number and concanavalin A concentration optimisation

Splenocytes were added to 96 well plates at 1×10^6, 1×10^5, 1×10^4 and 1×10^3 cells.well^{-1}, with either 5% or 10% foetal calf serum and 2.5 μg.ml^{-1} concanavalin A or RPMI 1640 control (n = 6). Plates were incubated for 24 hours followed by quantification of proliferation by AlamarBlue. The lower foetal calf serum concentration (5%) resulted in lower basal proliferation making the concanavalin A-induced proliferation easier to detect (Figure 2.3-4) and 1×10^5 cells.well^{-1} gave the best proliferation above control while remaining in the linear portion of the curve (Figure 2.3-4). Therefore, 1×10^5 cells.well^{-1} with 5% foetal calf serum were chosen as the optimal conditions.

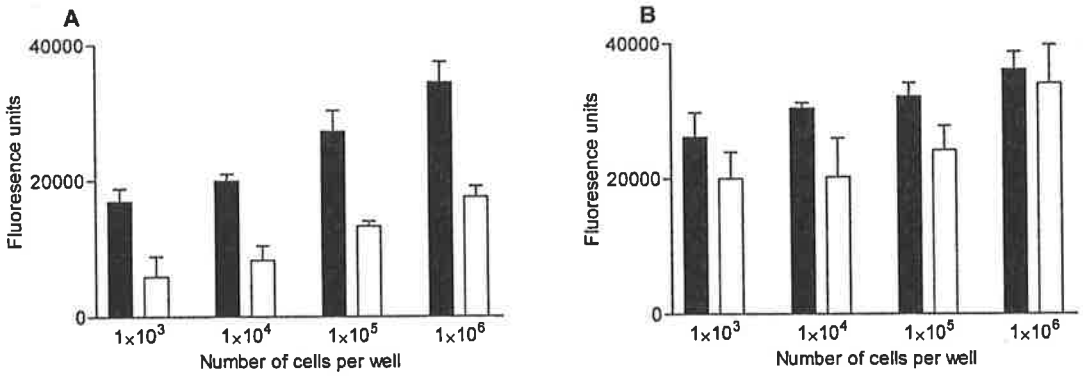


Figure 2.3-4 Optimisation of foetal calf serum concentration.

A: 5% and B: 10% foetal calf serum (2.5 μg.ml^{-1} concanavalin A ■ and control □) and cell number. Error bars are S.E.M. (n = 6).

Using splenocytes at 1×10^5 cells.well^{-1} in 5% foetal calf serum supplemented RPMI 1640, various concentrations of concanavalin A (5, 3.75, 2.5, 1, 0.1 μg.ml^{-1}) were examined (n = 6) to determine the best concentration to produce sub-maximal proliferation. Plates were incubated for 24 hours followed by quantification of proliferation by AlamarBlue (four hour

incubation). Proliferation caused by concanavalin A was biphasic with 3.75 $\mu g.ml^{-1}$ causing the greatest proliferation (Figure 2.3-5). A concentration at which sub-maximal proliferation occurred was required and therefore 2.5 $\mu g.ml^{-1}$ concanavalin A was chosen.

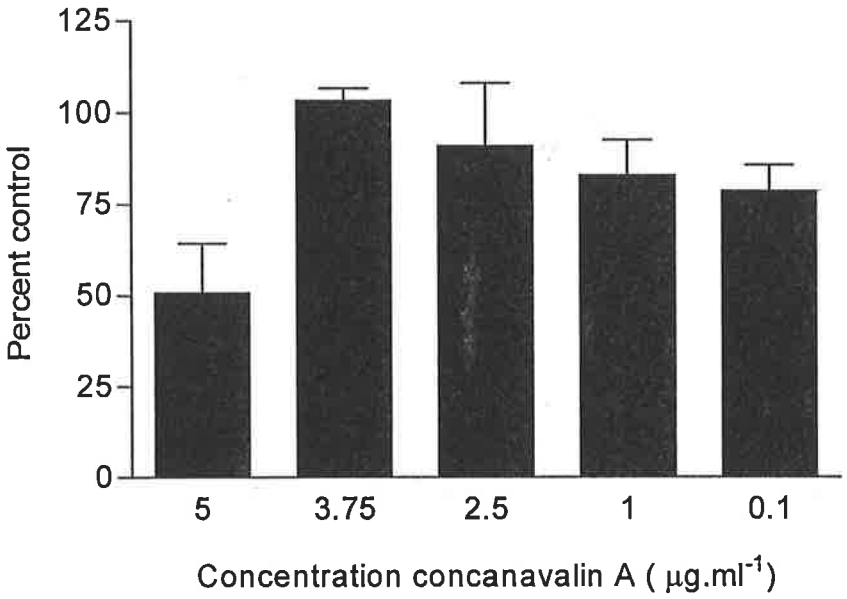


Figure 2.3-5 Optimisation of concanavalin A concentration to achieve sub maximal proliferation of mouse splenocytes.

Error bars are S.E.M. (n = 6).

2.3.5. Preparation of solutions of compounds used in studies

The compounds under investigation were supplied in various forms (e.g. salt or base) and therefore had varying degrees of aqueous solubility. Due to the concentration range (nM to mM), drugs that were supplied in their salt form were readily dissolved in MilliQ water. However, those compounds that were supplied as the base were not soluble in water and therefore were treated differently. Noroxycodone, norhydrocodone, the plus isomers of morphine and codeine, oripavine, norbuprenorphine and hydrocodone were all dissolved in hydrochloric acid such that the molarity of the acid and the base were the same.

Dissolving diacetylmorphine base directly into MilliQ water or an equimolar solution of hydrochloric acid caused spontaneous diacetylmorphine degradation of 10-15% as detected using HPLC (2.3.8.8). To prevent this degradation, diacetylmorphine base was dissolved in pH 5.0 phosphate buffer (monopotassium phosphate 65.9 mM and disodium phosphate 0.8 mM). Minimal diacetylmorphine degradation during storage was observed (< 1% over 3 months). Once in solution, diacetylmorphine and 6-monoacetylmorphine were stored at -80 °C until required. All other solutions were stored at 4 °C until used.

2.3.6. Incubation conditions

2.3.6.1. Characterisation experiments

In the experiments which characterised the affects of 4,5-epoxymorphinan and non-4,5-epoxymorphinans on concanavalin A proliferation the following methods were used. Splenocytes were isolated as described previously (2.3.3.2) and diluted in enriched media (RPMI 1640 with 10% foetal calf serum) to 1×10^6 cells.ml^{-1} (see 2.3.4.4 for optimisation data). One hundred microliters of this suspension (which is equal to 1×10^{-5} cells.well^{-1}) was added to each well of 96 well plates (Nunc, Roskilde, Denmark) and made up to 200 µl with 50 µl of concanavalin A (10 µg.ml^{-1}) and/or 50 µl of opioid (n = 12; 0 µM control, 0.4 nM, 4 nM, 0.04 µM, 0.4 µM, 4 µM, 40 µM and 400 µM). In some cases to further characterise the response curves, additional concentrations of opioids were included within the stated range or above the range. Unstimulated mitogen negative control wells were also prepared, using 50 µl of media instead of concanavalin A. The plates were incubated at 37 °C, 5% CO_2 in a humidified incubator for 24 hours following which 25 µl of a diluted AlamarBlue solution (5 µl AlamarBlue, 20 µl cell medium) was added to each well. The plates were incubated for a

further 4 hours following which 175 µl of media was transferred from the clear 96 well plates to white 96 well plates (BMG Labtechnologies, Offenburg, Germany) for fluorescence quantification (Excitation 545, Emission 590) on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany). Each experiment was repeated 6 times on separate days using different cell preparations.

2.3.6.2. Antagonist experiments

In experiments where opioid antagonists were also added to the culture media, the final volume remained unchanged. In each case, 25 µl of antagonist was added to the cells and left for 15 minutes; following this 50 µl of concanavalin A and 25 µl of the compound under investigation were added to the appropriate wells and subsequently treated as previously described.

2.3.7. Diacetylmorphine degradation

2.3.7.1. Incubation time-dependency study

Each well of the 96 well plates contained 100 µl of the cell suspension (5×10^6 cell.ml^{-1} diluted in enriched media), 50 µl of diacetylmorphine (final concentration 50 µM) and 50 µl of mitogen (final concentrations concanavalin A 2.5 µg.ml^{-1}, phytohemagglutinin 15 µg.ml^{-1} and lipopolysaccharide 25 µg.ml^{-1}) or cell media. Wells containing no cells were also prepared by adding 100 µl of enriched media. Plates were incubated at 37 °C, 5% CO_2 in a humidified incubator for 0, 1, 4, 6, 24, 30 and 53.5 hours at which time, samples were taken for analysis.

2.3.8. Esterase inhibitor study

Each well of the 96 well plates contained 100 µl of the cell suspension (5×10^6 cell.ml^{-1} diluted in enriched media), 50 µl of diacetylmorphine (final concentration 50 µM), 25 µl of mitogen (final concentrations concanavalin A 2.5 µg.ml^{-1}, phytohemagglutinin 15 µg.ml^{-1} and lipopolysaccharide 25 µg.ml^{-1}) and 25 µl of the esterase inhibitors; eserine (physostigmine) (200 µM), phenylmethylsulfonyl fluoride (500 µM) or BW284c51 (10 µM). Plates were incubated for 5 hours as previously described, following which samples were taken for analysis.

2.3.8.1. Diacetylmorphine concentration-dependency study

Each well of the 96 well plates contained 100 µl of the cell suspension (5×10^6 cell.ml^{-1} diluted in enriched media), 50 µl of diacetylmorphine (final concentrations 5, 10, 20, 40, 80 and 160 µM) and 50 µl of mitogen (final concentrations: concanavalin A 2.5 µg.ml^{-1}, phytohemagglutinin 15 µg.ml^{-1} and lipopolysaccharide 25 µg.ml^{-1}) or cell media. Plates were incubated for 5 hours as previously described following which samples were taken for analysis.

2.3.8.2. Mitogen concentration-dependency study

Each well of the 96 well plates contained 100 µl of the cell suspension (5×10^6 cell.ml^{-1} diluted in enriched media), 50 µl of diacetylmorphine (final concentration 50 µM) and 50 µl of mitogen (final concentrations concanavalin A 1, 2.5 and 10 µg.ml^{-1}; phytohemagglutinin 5, 15 and 50 µg.ml^{-1} and lipopolysaccharide 5, 25 and 50 µg.ml^{-1}) or cell media. Plates were

incubated for 5 hours as previously described following which samples were taken for analysis.

2.3.8.3. Erythrocyte lysis study

Cells were prepared as before except that preferential lysis of the erythrocytes was carried out after the first wash step. Cells were resuspended in 1 ml of media followed by the addition of 10 ml of ice-cold lysis buffer (1 ml of 20.56 $g.l^{-1}$ Tris base (pH 7.65), 9 ml 0.83% NH_4Cl in H_2O, mixed just prior to addition to cells). The suspension was placed on ice for 4 min, centrifuged, followed by a wash and cell count as before. Each well of the 96 well plates contained 100 µl of the cell suspension (5×10^6 $cell.ml^{-1}$ diluted in enriched media), 50 µl of diacetylmorphine (final concentration 50 µM) and 50 µl of mitogen (final concentrations concanavalin A 2.5 $\mu g.ml^{-1}$, phytohemagglutinin 15 $\mu g.ml^{-1}$ and lipopolysaccharide 25 $\mu g.ml^{-1}$) or cell media. Plates were incubated for 5 hours as previously mentioned following which samples were taken for analysis.

2.3.8.4. Cell number dependency study

Each well of the 96 well plates contained 100 µl of the cell suspension (5×10^4, 5×10^6 and 5×10^8 $cells.ml^{-1}$ cell suspensions diluted in enriched media), 50 µl of diacetylmorphine (final concentration 50 µM) and 50 µl of cell media. Plates were incubated for 5 hours as previously described following which samples were taken for analysis.

2.3.8.5. Effect of splenocytes on diacetylmorphine degradation study

To investigate the effect cellular constituents have on the rate of diacetylmorphine degradation, the whole splenocyte suspension was lysed after the cell count by adding 1 ml of 1 mM HCl (final volume 6 ml). The suspension was then centrifuged as before and diluted to a theoretical cell concentration of 5×10^6 cells.ml^{-1} using the previous cell count. One hundred microliters of the suspension was added to the 96 well plates as stated previously, along with 50 µl of diacetylmorphine (final concentration 50 µM) and 50 µl of cell media. Plates were incubated for 5 hours as previously described following which samples were taken for analysis.

2.3.8.6. Cell media evaporation study

The extent of evaporation during the period of incubation was assessed by incubating a plate for 53.5 hours, each well of which contained 50 µl of internal standard (codeine 100 µg.ml^{-1}) and 150 µl media. Following the incubation, 100 µl was taken from 12 wells, assayed for codeine and compared to freshly prepared controls. Statistical significance was assessed using a students t-test.

2.3.8.7. Sample preparation for HPLC quantification

Incubation samples were collected by adding 50 µl of internal standard (codeine 100 µg.ml^{-1}) to the wells, followed by removal of a 175 µl aliquot of the solution, which was then transferred to 10 ml polypropylene tubes containing 300 µl of 20% Na_2CO_3. Dichloromethane (4 ml) was added to the tubes followed by 10 minutes of rotary mixing and centrifugation at 1700g for 10 minutes. The aqueous layer was aspirated and the remaining organic phase was transferred to a new 10 ml tube containing 200 µl of 0.1 M HCl. The tubes

were rotary mixed for 10 minutes followed by centrifugation at 1700g for 10 minutes. Approximately 150 µl of the acid bubble was removed and prepared for auto injection onto the HPLC system.

2.3.8.8. Quantification for morphine and 6-monoacetylmorphine

The HPLC system comprised a SIL-9A autosampler (Shimadzu, Kyoto, Japan), LC-6A pump (Shimadzu), Jasco UVIDEC-100-V UV spectrophotometer (Japan Spectrocopic Co. Ltd., Tokyo, Japan) and a C-R6A integrator (Shimadzu). The stationary phase consisted of a Spherisorb 5µ C18 (15cm × 4mm) column and an in-line pre-column cartridge (10mm Almina C18 5µ, Alltech) was positioned ahead of the column. The mobile phase contained 30% acetonitrile, 0.01 M KH_2PO_4 and 230 $mg.l^{-1}$ sodium dodecyl sulphate adjusted to pH 2.3 with orthophosphoric acid. The mobile phase was filtered and subsequently sonicated for a minimum of 15 minutes. The flow rate through the column was 1.5 $ml.min^{-1}$ with absorbance measured at 210 nm (detector range 0.01 ABU). No interfering peaks were observed in the chromatography (Figure 2.3-6 A), and the peaks of interest were all well resolved (Figure 2.3-6 B). A calibration curve of ten standards was prepared consisting of morphine (1.4, 2.6, 5.3, 7.0, 8.8, 10.5, 17.5, 35.0, 52.6, 70.1 µM) and 6-monoacetylmorphine (7.5, 10, 15, 20, 30, 40, 50, 60, 80, 100 µM). The actual concentrations of the standards were four times greater, as they underwent a one in four dilution in the incubation (50 µl standard, 150 µl RPMI 1640). The samples were stored at -80 °C when not in use. The calibration curve samples were extracted at time zero with the time zero diacetylmorphine samples. Peak area ratios were calculated by dividing the peak area obtained from the chromatographic integrator by that of the internal standard, codeine. The equation to the line of best fit and coefficient of determination (r^2) for each calibration curve was calculated using linear regression analysis

(weighting $1/y^2$) of peak height ratios plotted against nominal concentrations (GraphPad Prism v3.02, GraphPad Software, San Diego, USA). Accuracy (as %) was determined as $\frac{\text{calculated concentration}}{\text{nominal concentration}} \times 100$, for each calibration and quality control sample and inaccuracy determined as $\text{accuracy} - 100$. The coefficient of variation, determined as $\frac{\text{standard deviation}}{\text{arithmetic mean}} \times 100$, was used as the index of precision.

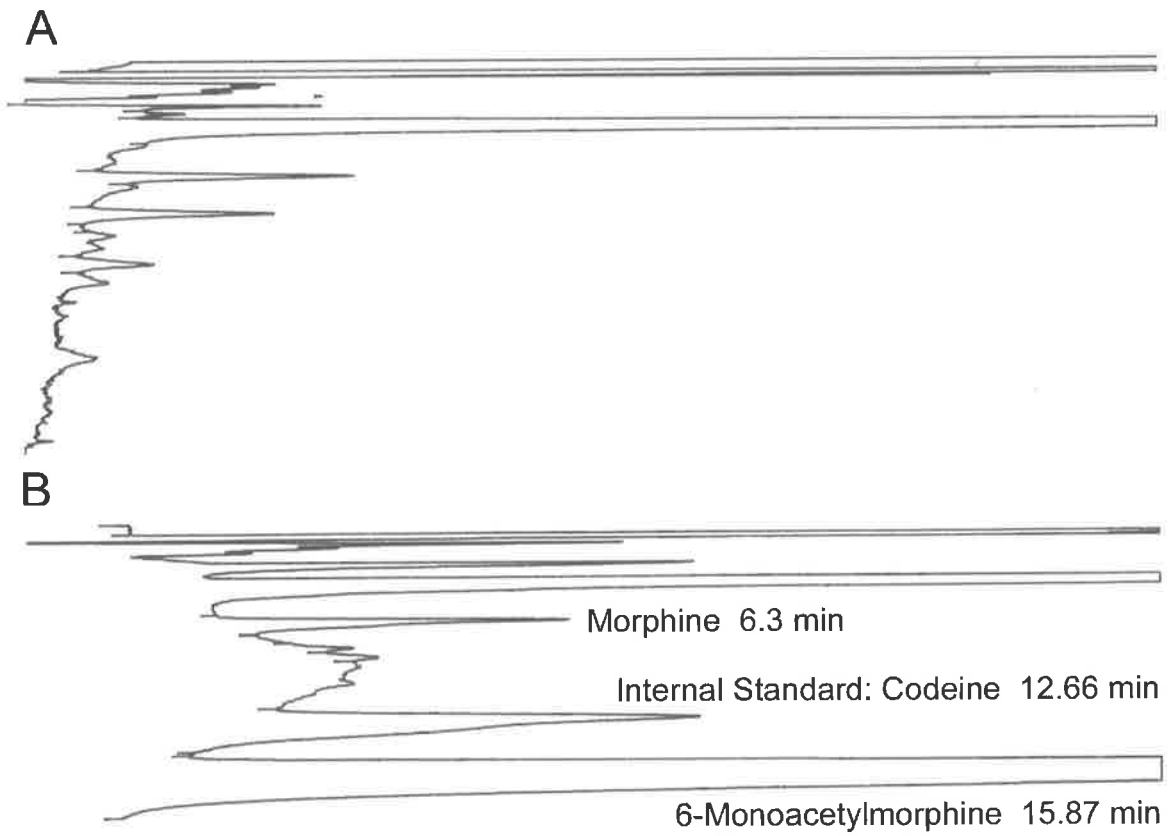


Figure 2.3-6 Example of HPLC chromatograms of diacetylmorphine degradative products.

A: Supplemented RPMI 1640 media blank extraction chromatogram. B: Cell incubation extraction chromatogram.

2.3.8.9. Data analysis – modelling of half-life

During the extraction process, diacetylmorphine spontaneously degraded (~15%) due to the alkaline conditions of the extraction buffer. Time zero extraction samples were prepared to determine the degradation due to this extraction procedure alone (n = 2). This degree of degradation was then subtracted from all subsequent data points resulting in the amount of morphine or 6-monoacetylmorphine formed due to the cell culture conditions alone. The concentration of diacetylmorphine was calculated by summing the molar concentrations of morphine and 6-monoacetylmorphine and subtracting this value from the initial concentration of diacetylmorphine (50 μM).

The rate of diacetylmorphine degradation to 6-monoacetylmorphine (k_1) and subsequently to morphine (k_2) were determined using the following equations:

$$\frac{dA}{dt} = -k_1 A + k_1 x$$

$$\frac{dB}{dt} = k_1 A - k_1 x - k_2 B + k_2 y$$

$$\frac{dC}{dt} = k_2 B - k_2 y$$

where A, B and C are diacetylmorphine, 6-monoacetylmorphine and morphine concentrations, respectively and, x and y are constants which allow optimal modelling since the degradations of diacetylmorphine and 6-monoacetylmorphine were not complete. The equations were fitted to the data to calculate k_1 and k_2 using MicroMath Scientist for Windows version 2.0 (MicroMath Inc., MI, USA).

Half-life was calculated by dividing ln(2) by the respective rate constants. Area under the curve and statistical calculations were performed using Prism 3.02 (GraphPad, San Diego,

USA). Statistical significance was assessed using a two-way analysis of variance with Bonferroni post hoc test. Significance was set at $P < 0.05$.

2.3.9. Binding of opioids to 96 well microplates

To ascertain if methadone had reduced *in vitro* bioavailability due to its potential ability to bind to the plastic 96 well plates used for cell culture, quantification of free and unbound racemic methadone was conducted following contact with the plates. Duplicate samples of 10 μM racemic methadone (final volume 200 μl) were prepared in Nunc 96 well plates in supplemented and un-supplemented RPMI 1640 (with or without 5% foetal calf serum) with concanavalin A (2.5 $\mu g.ml^{-1}$) and matching samples were prepared in 1.5 ml micro-centrifuge tubes. The samples were incubated for 45 minutes at 37 °C 5% CO_2 and 150 μl of the media was then taken from each sample and prepared for racemic methadone quantification using a previously described method (Foster *et al.*, 2000) by Dr DJR Foster of the Drug Metabolism and Pharmacokinetics Laboratory, Department of Clinical and Experimental Pharmacology, University of Adelaide. Data were expressed as a ratio of the internal standard peak area, and converted to a percent of the negative control (sample with no foetal calf serum prepared in a 1.5 ml micro-centrifuge tube). Statistical significance was assessed using a two-way analysis of variance with Bonferroni post-hoc test.

The degree of methadone binding to foetal calf serum was then assessed using an MPS micropartition kit (Amicon, Beverly, MA, USA) with YMT filtration membranes (10000 kDa molecular weight cut off, Amicon). The devices were used according to the manufacturer's directions using a Beckman J2-21 centrifuge with both the chamber and the fixed angle rotor (JA 20.1, Beckman) pre-warmed to 37 °C. Duplicate samples of 10 μM racemic methadone

were prepared in the devices (final volume 400 µl) in supplemented or un-supplemented RPMI 1640 with concanavalin A (2.5 $\mu g.ml^{-1}$) and incubated for 45 minutes at 37 °C 5% CO_2 followed by a 2 to 5 minutes centrifuge at 1000 g. The resultant filtrate was then injected directly onto the HPLC system for quantification as previously described. The data were expressed as percent of control containing no foetal calf serum minus on hundred percent, which had not been in contact with the filtration device.

2.3.10. Toxicology studies

The cellular toxicity resulting from incubations with methadone, morphine and buprenorphine were assessed using the leakage of lactate dehydrogenase as a marker of toxicity. The lactate dehydrogenase method was based on that of Richards *et al.* (1975) and adapted to 96 well plates by Dr Frank Fontaine of the Toxicology Laboratory in the Department of Clinical and Experimental Pharmacology, University of Adelaide. Isolated splenocytes were incubated for 48 hours with racemic methadone (100 µM and 1 µM), morphine (0.1 µM) or buprenorphine (10 µM or 0.01 µM). An untreated control was also included in the experiment. Lactate dehydrogenase activity in 10 µl aliquots of the culture media were measured by quantifying nicotinamide adenine dinucleotide reduction (excitation 340 nm and emission 460 nm) in 200 µl of 0.25 M Tris-HCl (pH 8.9) containing 50 mM L-lactate and 14 mM nicotinamide adenine dinucleotide in white BMG 96 well plates (final volume per well 210 µl). Fluorescence readings were taken at 37 °C every 8 seconds for 184 seconds (25 cycles) using a BMG Polarstar microplate reader. Data were expressed as $slope.second^{-1}$ and then converted to percent of the negative control. *Tert* butyl hydroperoxide (5% $vol.vol^{-1}$ incubation for 45 minutes) was used as a positive control of cellular toxicity. A one-way analysis of variance

with Bonferroni post-hoc test comparing data to negative control was used to assess statistical significance.

2.4. Results

2.4.1. Mitogenesis assay optimisation

As described previously (2.3.4), the assay conditions for the mouse splenocyte assay were optimised as best as possible. The optimised conditions were 5% foetal calf serum supplemented RMPI 1640, 2.5 $\mu g.ml^{-1}$ concanavalin A, 1×10^5 $cells.well^{-1}$ incubated for 24 hours at 37 °C 5% CO_2 in a humidified environment, addition 25 µl of AlamarBlue (5 µl AlamarBlue and 20 µl RPMI 1640) followed by a final 4 hour incubation.

2.4.2. Diacetylmorphine degradation

Morphine and 6-monoacetylmorphine formations from diacetylmorphine increased significantly ($P < 0.0001$) in the presence of cells (mitogen treated or untreated) from 4 hours compared to RPMI 1640 alone (Figure 2.4-1). Morphine and 6-monoacetylmorphine formation peaked at 6 hours thereafter, minimal increase in degradation occurred (Figure 2.4-1). Phytohemagglutinin was the only mitogen that caused a significant ($P = 0.036$) increase in morphine formation compared to cells alone (Figure 2.4-1), whilst there was no significant difference ($P > 0.36$) between cell treatments for 6-monoacetylmorphine degradations. These differences were also observed in the area under the curve data for morphine concentrations (no cells 488 $\mu M.hour^{-1}$, cells 924 $\mu M.hour^{-1}$, lipopolysaccharide 942 $\mu M.hour^{-1}$, concanavalin A 966 $\mu M.hour^{-1}$ and phytohemagglutinin 1049 $\mu M.hour^{-1}$) and

6-monoacetylmorphine (no cells 327 $\mu M.hour^{-1}$, cells 494 $\mu M.hour^{-1}$, lipopolysaccharide 676 $\mu M.hour^{-1}$, concanavalin A 716 $\mu M.hour^{-1}$ and phytohemagglutinin 727 $\mu M.hour^{-1}$).

Total diacetylmorphine degradation, calculated from morphine and 6-monoacetylmorphine concentrations, peaked at 6 hours with 73% of the initial diacetylmorphine (50 μM) converted to morphine (~65%) and 6-monoacetylmorphine (~35%) (Figure 2.4-1). The half-lives of diacetylmorphine and 6-monoacetylmorphine degradations were 1.4 hours and 3.1 hours, respectively in cell media alone. Coefficient of determination (r^2) values ranged from 0.97 for phytohemagglutinin to 0.99 for cells. The half-life of diacetylmorphine and 6-monoacetylmorphine degradation were similar in the incubations containing cells, ranging from 1.2 hours (cells alone) to 2.2 hours (concanavalin A) for diacetylmorphine and 0.99 hours (lipopolysaccharide) to 1.2 hours (cells alone) for 6-monoacetylmorphine.

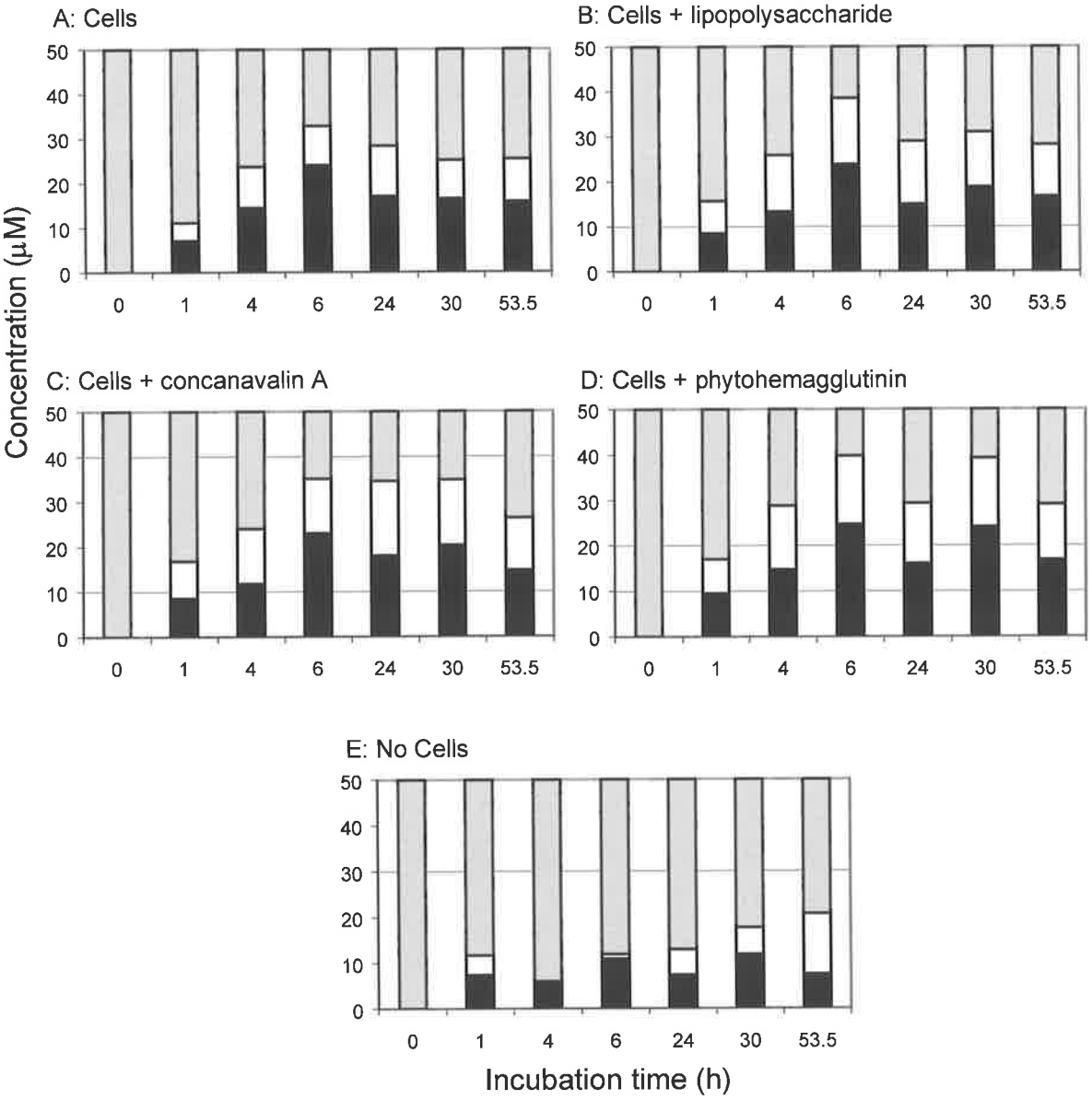


Figure 2.4-1 Time dependent degradation of diacetylmorphine in cell culture under different conditions.

Diacetylmorphine ( ), morphine (■) and 6-monoacetylmorphine (□). A: Cells, B: Cells + lipopolysaccharide (25 mg.ml^{-1}), C: Cells + concanavalin A (2.5 mg.ml^{-1}), D: Cells + phytohemagglutinin (15 mg.ml^{-1}), E: No Cells. Initial diacetylmorphine concentration = 50 μM. Data represent 4 independent samples, taken at each time point under each condition.

There was no significant evaporation ($P > 0.74$) of the cell culture media during the incubation period (codeine peak areas mean ± SD: control 229915 ± 21036 vs 53.5 hour

incubation 230883 ± 23424). Mitogens alone (no cells) or the presence of splenocyte lysate did not increase the rate of diacetylmorphine degradation in RPMI 1640.

As the original diacetylmorphine concentration increased in the cell culture media, formation of morphine and 6-monoacetylmorphine increased linearly ($r^2 > 0.94$) over a diacetylmorphine concentration range of 5 μM to 160 μM after 5 hours incubation. Morphine formation was related to increasing cell number after a 5 hours incubation, with 5×10^7 cells causing the greatest degradation, whilst 6-monoacetylmorphine formation was not related to cell number (Figure 2.4-2). The use of esterase inhibitors or the removal of erythrocytes had no significant effect ($P > 0.18$ and $P > 0.22$, respectively) on diacetylmorphine degradation.

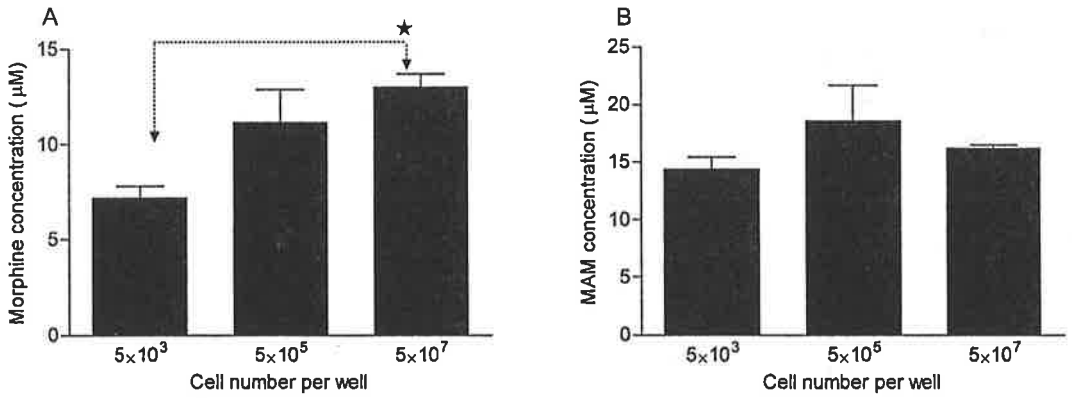


Figure 2.4-2 Cell number dependent diacetylmorphine degradation.

Diacetylmorphine degradation to morphine (A) and 6-monoacetylmorphine (MAM) (B). Error bars are S.E.M. and statistical significance is indicated by ★ = $P < 0.05$

2.4.3. *In vitro* bioavailability of methadone

Methadone did not significantly bind to the 96 well plates ($P > 0.05$), with no difference between the isomers in any of the treatments (Figure 2.4-3). There was a slight reduction in recovered methadone from incubations with the plate alone, however this was not significant

and returned to 100% when foetal calf serum was included in the media (Figure 2.4-3). If this reduced recovery is due to binding to the 96 well plates, it appears that foetal calf serum blocks this binding, ensuring adherence to plastic does not reduce the bioavailability of methadone.

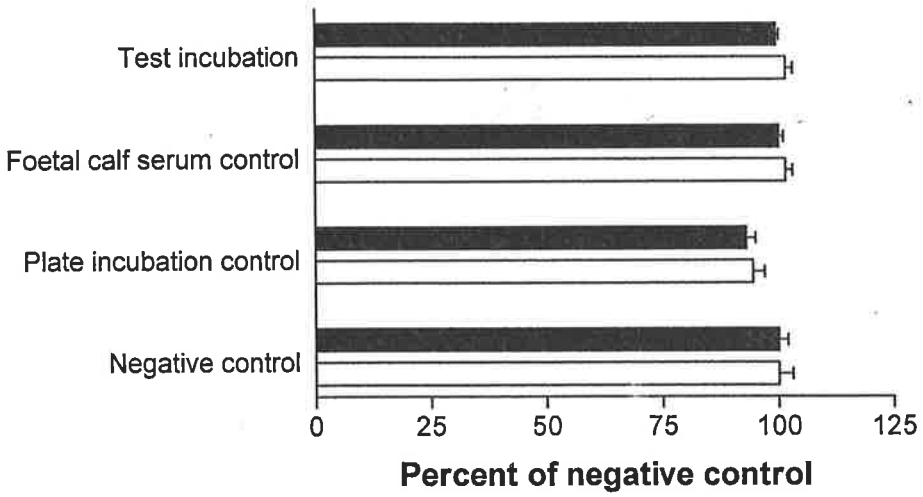


Figure 2.4-3 Quantification of methadone not bound to 96 well plates.

(R)-(-)- (□) and (S)-(+)-methadone (■) recovery expressed as percent of negative control. Sample names: negative control – no foetal calf serum prepared in a microcentrifuge tube; plate incubation control - no foetal calf serum prepared in 96 well plate; foetal calf serum control - foetal calf serum prepared in a micro-centrifuge tube; and test incubation - foetal calf serum prepared in 96 well plate. Error bars are S.E.M.

Methadone bound slightly, but not significantly ($P > 0.05$), to the filtration device (likely the membrane), but did not bind to the low concentration of foetal calf serum in the supplemented media (Figure 2.4-4). Therefore, we can assume that the concentration of drug applied to the system is likely to be fully available to act at its cellular target.

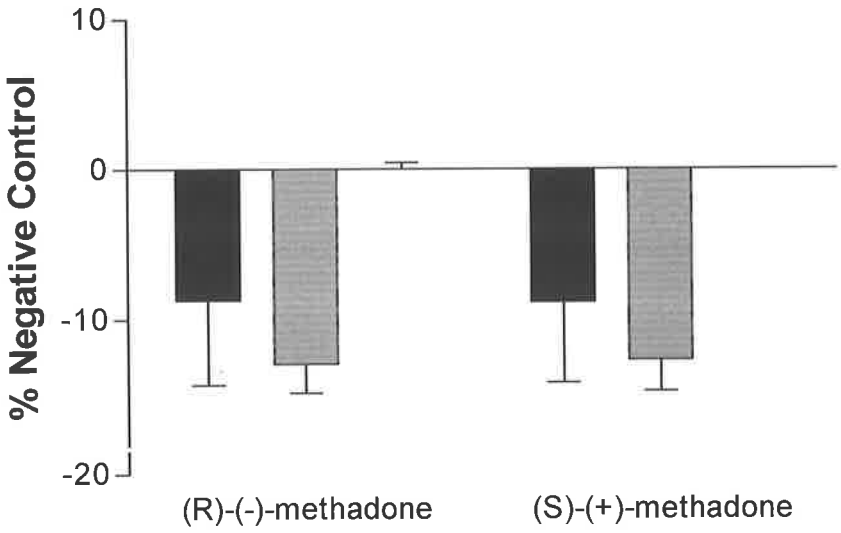


Figure 2.4-4 (R)-(-)- and (S)-(+)-methadone binding to foetal calf serum.

Foetal calf serum in device (■), no foetal calf serum in device (◩) and no foetal calf serum which had no contact with a device (□). Error bars are S.E.M.

2.4.4. Toxicology data

Buprenorphine, morphine or methadone did not cause increased lactate dehydrogenase leakage (Figure 2.4-5) at any of the concentrations used, whilst the positive control, *tert* butyl hydroperoxide caused significant cellular toxicity ($P < 0.001$).

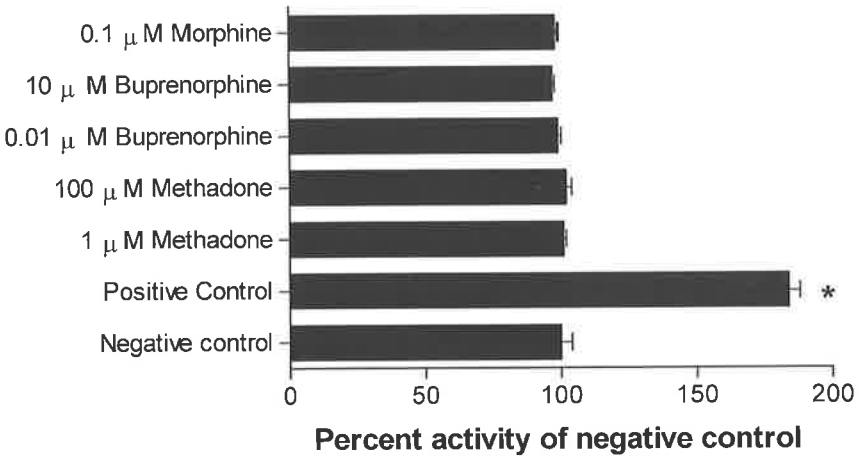


Figure 2.4-5 Lactate dehydrogenase activity of culture media following 48 hour incubation with methadone, morphine and buprenorphine.

Positive control toxicity was induced by tert butyl hydroperoxide (5% 45 minute incubation). Error bars are S.E.M. and statistical significance is indicated by * = $P < 0.001$

2.5. Discussion

The assay developed and optimised is similar to those mitogenesis assays previously reported in the literature. However, there are a few significant and important differences, including the use of AlamarBlue to quantify proliferation and the use of the shortened incubation time. It can be assumed that the concentration of drug applied to the system causes the affect, since the *in vitro* bioavailability of the drug is not affected by binding to plastic or serum, although unstable compounds such as diacetylmorphine and possibly other compounds with labile functional groups, such as remifentanil, will degrade in the culture media (discussed below).

2.5.1. Implications of diacetylmorphine degradation

This part of the study was undertaken to quantify diacetylmorphine degradation in cell culture and to attempt to determine the mechanism by which this occurs, thus enabling future investigations of the specific effects of diacetylmorphine *in vitro* to be firmly established. Morphine and 6-monoacetylmorphine were detected in the cell culture media after just 1 hour when incubated at 37 °C. Up to 80% of diacetylmorphine had been hydrolysed after 6 hours in some of the conditions used in this study. The degradation of diacetylmorphine was significantly affected by the presence of cells as determined from the area under the curve data. Interestingly, the half-life of diacetylmorphine was similar in incubations with and without cells, whilst the half-life of 6-monoacetylmorphine was reduced by the presence of cells. Therefore, diacetylmorphine degradation is dependent on the conditions of the cell culture and not the presence of cells. This is supported by the findings that esterase inhibitors, mitogen concentration, removal of erythrocytes and reduction of the lymphocyte concentration in the cell culture had no effect on 6-monoacetylmorphine formation.

Morphine formation is dependent largely on the presence of cells, since the half-life of 6-monoacetylmorphine without cells was greater than with cells and morphine formation could be reduced with lower lymphocyte concentrations. However, this reaction does not appear to be mediated by esterases since the non-selective esterase inhibitors eserine and phenylmethylsulfonyl fluoride (Zhang *et al.*, 1999), and selective erythrocyte acetylcholinesterase inhibitor BW284c51 (Salmon *et al.*, 1999) had no effect on morphine formation. Taken together, these results suggest that the major cause of 6-monoacetylmorphine degradation was non-enzymatic and possibly due to reactions or bi-products associated with lymphocyte activity.

Barrett *et al.* (1992) estimated the half-life of diacetylmorphine to be 32.9 hours at 37 °C, pH 7.4 (phosphate buffer). The half-life of diacetylmorphine obtained in the current study in RPMI-1640 cell media alone (37 °C, assumed pH of 7.4) was 1.4 hours, significantly shorter than that previously found for similar pH and temperatures (Barrett *et al.*, 1992; Poochikian & Cradock, 1979). However, Barrett *et al.* (1992) found that small increases in pH resulted in a significant decrease in the half-life of diacetylmorphine (pH 8.0, half-life 8.3 hours). A small increase in the pH of the cell culture media and the differences in the complexity of the media compared to the phosphate buffer may account for the difference found in diacetylmorphine's half-life.

The conclusions drawn from previous *in vitro* tissue culture studies investigating the effects of diacetylmorphine *per se* (Perez-Castrillon *et al.*, 1992; Thomas *et al.*, 1995b), have not taken into account the presence of morphine and 6-monoacetylmorphine in their experimental systems. Even though diacetylmorphine is rapidly metabolised to its active metabolites *in vivo*, the pharmacodynamic effects of diacetylmorphine alone must be elucidated, in order to better understand the properties of this compound over those of morphine and 6-

monoacetylmorphine. This study has highlighted the difficulty of using unstable compounds such as diacetylmorphine in cell culture. Unless precautions are taken to limit the degradation of diacetylmorphine, future studies which use diacetylmorphine in cell culture, must acknowledge that significant quantities of morphine and 6-monoacetylmorphine are formed and must not assume the responses observed are due entirely to diacetylmorphine alone. At present, there appears to be no straightforward method to circumvent diacetylmorphine degradation. Therefore, shorter exposure times may need to be used in combination with media replenishment to avoid accumulation of diacetylmorphine's active degradation products in the media.

2.5.2. Cellular toxicity

As discussed previously, non-specific cellular toxicity resulting from *in vitro* exposure to opioids has been disproved (e.g. Jessop *et al.* (1991a)). However, apoptosis has been observed (Singhal *et al.*, 1998). It is believed that the lactate dehydrogenase activity assay, utilised here to detect non-specific toxicity, may also detect large amounts of apoptosis due to the inevitable leakage of small quantities of lactate dehydrogenase from the apoptotic bodies owning to the sensitivity of the assay. Methadone, buprenorphine and morphine caused pronounced inhibition of proliferation at 100 μM, 10 μM and 0.1 μM, respectively (Chapter 4). However, there were no differences in lactate dehydrogenase leakage, and therefore cellular toxicity, between control incubations and concentrations of agonist that caused reduced proliferation (100 μM methadone, 0.1 μM morphine or 10 μM buprenorphine) or those that did not (1 μM methadone or 0.01 μM buprenorphine). These data indicate that there is no enhanced cellular toxicity despite significant reductions in proliferation demonstrating no non-specific cellular toxicity. Whether apoptosis is induced by exposure to

these opioid agonists is not clear, as it was not assessed specifically. However, the extent to which proliferation was reduced by methadone and buprenorphine would have indicted wide spread apoptosis and it would be expected that apoptosis to this degree would be detected by the lactate dehydrogenase leakage assay. More sensitive apoptosis assays should be used in the future to investigate the potential influence these agonists have on triggering apoptosis.

2.5.3. Conclusion

The assay that was developed, optimised and subsequently utilised to investigate opioid immunomodulation, is a reproducible, reliable and a robust assay, and it can be assumed that the concentration of drug applied to the system is related to the response obtained (with exception of the compounds discussed). The use of AlamarBlue has made the assay rapid, sensitive and safe. This assay has been employed to investigate the immunomodulation of numerous 4,5 epoxymorphinans and non-4,5 epoxymorphinans which are presented in the following chapters.

Chapter 3. *In vitro* 4,5-epoxymorphinan effects on immunocompetent cells

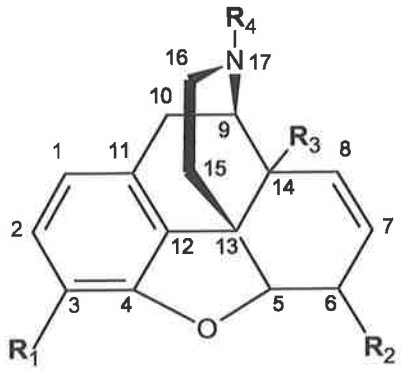
3.1. Introduction

Morphine, the prototypical opioid agonist belongs to the 4,5-epoxymorphinan class of compounds (Table 3.1-1). Other common either clinically prescribed or abused 4,5-epoxymorphinans include codeine, oxycodone and diacetylmorphine (heroin). Apart from their analgesic properties, 4,5-epoxymorphinans and endogenous opioids, such as β-endorphin, can directly influence immune cell function. Wybran *et al.* (1979) demonstrated that morphine was able to inhibit T cell rosetting in a naloxone-reversible manner. Subsequently, numerous research groups have used a plethora of both *in vitro* and *in vivo* infection and immunity models to investigate the extent and mechanism underlying opioid-induced alterations in immune function, as reviewed by Eisenstein *et al.* (1998), Glasel (2000) and Carr *et al.* (1996b), and discussed previously in Chapter 1 (1.4). However, few studies have departed from using morphine as the prototypic 4,5-epoxymorphinan.

Centrally, endogenous opioid peptides, 4,5-epoxymorphinans and other synthetic opioids act on the "classical" μ, κ and δ opioid receptors and their subclasses (Dhawan *et al.*, 1996). When administered *in vivo*, opioids have centrally- and peripherally-mediated actions (King *et al.*, 2001). Therefore, it is possible for these compounds to alter immune function indirectly via neuronal innervation of lymphoid organs (Felten *et al.*, 1985) or via endocrine pathways, such as opioid-induced activation of the HPA axis, which subsequently results in the release of immunosuppressive glucocorticoids (Bryant *et al.*, 1991). A concerted effort has been made to identify the specific receptors that mediate the direct opioid effects on immune cells using biological activity, ligand and fluorescent antibody binding and mRNA detection by reverse transcription-polymerase chain reaction (Madden *et al.*, 1998; Sharp *et*

al., 1998b). Low expression levels of neuronal-like opioid receptors on immune cells has made detection difficult. However, the current evidence indicates the presence of neuronal like (classical) opioid receptors on some immunocompetent cell types (specifically μ: Sedqi *et al.* (1995) and Chuang *et al.* (1995b); κ: Bidlack *et al.* (1995) and Belkowski *et al.* (1995c); δ: Gaveriaux *et al.* (1995) and Li *et al.* (1999); and reviewed by Sharp *et al.* (1998b)). In addition, non-opioid (non-classical) receptors that do not behave in the same manner as their neuronal counterparts have been identified on immune cells. The differences in their pharmacological properties include non-stereoselectivity (Roy *et al.*, 1991), naloxone insensitivity (Jessop & Taplits, 1991a; Roy *et al.*, 1998b), decreased affinity for opioid agonists (Roy *et al.*, 1991) and altered optimal ion concentrations for agonist binding (Madden *et al.*, 2001). A receptor which binds opioid alkaloids, but not opioid peptides has also been characterised on immune cells and has been named the μ_3 opioid receptor (Makman *et al.*, 1995b). Specific β-endorphin receptors which are naloxone-insensitive and which display other non-classical opioid characteristics are also expressed on immune cells (Hazum *et al.*, 1979; Shahabi *et al.*, 1992; Shahabi *et al.*, 1990a; Woods *et al.*, 1997). Despite the low expression levels and differing pharmacological properties, activation of these receptors is able to alter biological function (Gaveriaux-Ruff *et al.*, 1998).

Table 3.1-1 Comparative structures of 4,5-epoxymorphinans used in this study.



Compound	R_1	R_2	R_3	R_4	Other changes	Degrees of separation
Morphine	OH	OH	H	CH_3		0
6-Hydroxyoxymorphone	OH	OH	OH	CH_3	1	1
Hydromorphone	OH	=O	H	CH_3	1	1
Oripavine	OH	OCH_3	H	CH_3	2	1
Morphine-6-glucuronide	OH	$OC_6H_9O_5$	H	CH_3		1
6-Monoacetylmorphine	OH	$OCOCH_3$	H	CH_3		1
Codeine	OCH_3	OH	H	CH_3		1
Morphine-3-glucuronide	$OC_6H_9O_5$	OH	H	CH_3		1
6β-Naltrexol	OH	OH	OH	C_4H_7	1	2
Oxymorphone	OH	=O	OH	CH_3	1	2
Hydrocodone	OCH_3	=O	H	CH_3	1	2
Thebaine	OCH_3	OCH_3	H	CH_3	2	2
Diacetylmorphine	$OCOCH_3$	$OCOCH_3$	H	CH_3		2
Naloxone	OH	=O	OH	C_3H_5	1	3
Naltrexone	OH	=O	OH	C_4H_7	1	3
Oxycodone	OCH_3	=O	OH	CH_3	1	3
Norhydrocodone	OCH_3	=O	H	H	1	3
Naloxone Methiodide	OH	=O	OH	C_4H_8I	1	4
3-O-Methylnaltrexone	OCH_3	=O	OH	C_4H_7	1	4
Noroxycodone	OCH_3	=O	OH	H	1	4
Buprenorphine	OH	OCH_3	H	C_4H_7	1, 3, 4	
Norbuprenorphine	OH	OCH_3	H	H	1, 3, 4	

1: single bond between C_7 and C_8; 2: double bond between C_6 and C_7 and C_8 and C_{14} and single bond between C_7 and C_8; 3: $C_6H_{13}O$ at position 7; 4: 2 carbon bridge between C_6 and C_{14}.

The direct pharmacodynamic effects of 4,5-epoxymorphinans on immune cells, via both classical and non-classical opioid receptors, depend on the model used and the concentration range of agonist investigated. Morphine caused a naloxone-insensitive concentration-dependent decrease in thymocyte (Roy *et al.*, 1997) and splenocyte proliferation (Jessop & Taplits, 1991a), whilst others were unable to alter splenic or peripheral blood lymphocyte proliferation except at high (100 μM) concentrations (Fecho *et al.*, 1996a). Conversely, Bidlack *et al.* (1990) demonstrated a naloxone-insensitive increase in proliferation of lymph node T cells by morphine. Inter-animal strain differences in response to opioids has also been observed (Eisenstein *et al.*, 1995). These conflicting data have led to confusion regarding the biological and physiological relevance of opioid receptors expressed on immune cells; however, the expression and release of endogenous opioid agonist peptides by activated immune cells during inflammation (Cabot *et al.*, 1997a) and stress (Rittner *et al.*, 2001) indicates the potential for a regulatory role. Furthermore, since immune-derived cytokines are

integral to the development of opioid tolerance (Raghavendra *et al.*, 2002), and immune status alters the analgesic efficacy of morphine (Kamei *et al.*, 1992) and the severity of opioid withdrawal (Dougherty *et al.*, 1990), suggesting a substantial role for the immune system in opioid pharmacodynamic effects and highlighting the need to understand the effects of a wider range of 4,5-epoxymorphinans on immune function.

4,5-Epoxymorphinans have varying affinities for different classes of the classical μ, κ and δ opioid receptors. Chen *et al.* (1991) demonstrated a structure-binding affinity relationship using a large number of structurally-related 4,5-epoxymorphinans and displacement of tritiated DAMGO from rat brain homogenate μ opioid receptors. Therefore, if a similar model is applied *in vitro*, as suggested by Sibinga *et al.* (1988), and utilised *in vivo* by Sacerdote *et al.* (1997b), to investigate immunomodulatory effects of 4,5-epoxymorphinans, a better understanding of the receptors which mediate these responses may be gained, and a greater appreciation of how opioid immunomodulation influences opioid pharmacodynamics.

3.2. Aims

The aim of this study was to investigate the *in vitro* immunomodulatory effects of a large number of structurally related 4,5-epoxymorphinans and to ascertain if any structure-effect relationship is apparent. This also enabled a comparison of the rank order of effect with currently established μ opioid receptor binding affinity and binding efficacy rank order data from previous studies. Selective and non-selective opioid antagonists were also used to determine the opioid receptor subtypes that may be involved. A splenocyte mitogenesis assay was used as the experimental model. However, in contrast to many previously applied models, a rapid non-toxic and sensitive proliferation detection method was used (Ahmed *et*

al., 1994), combined with incubation conditions optimised for the detection of altered immune response by *in vitro* exposure to 4,5-epoxymorphinans (see Chapter 2).

3.3. Materials and Methods

3.3.1. Chemicals

RPMI 1640 with HEPES and L-glutamine modification was purchased from Invitrogen (Mulgrave, Australia). Penicillin-streptomycin solution (10,000 units penicillin, 10 mg streptomycin per ml), concanavalin A, oxycodone hydrochloride, morphine-3-glucuronide, morphine-6-glucuronide, thebaine hydrochloride, hydromorphone hydrochloride, naloxone methiodide, naloxone hydrochloride, naltrexone hydrochloride, 3-O-methylnaltrexone, naltrindole hydrochloride, naloxonazine dihydrochloride and nor-binaltorphimine dihydrochloride were purchased from Sigma (St. Louis, MO, USA). Oripavine and the plus inactive isomers of morphine and codeine were obtained from the National Institute of Drug Abuse (Bethesda, MD, USA). 6β-naltrexol was synthesised by the Department of Chemistry at the University of Adelaide (Porter *et al.*, 2000). Hydrocodone base, 6-hydroxyoxymorphone and norhydrocodone base were obtained from Dr S Hosztafi (ICN Alkaloida Company Ltd, Tiszavasvári, Hungary). Buprenorphine and norbuprenorphine were obtained from Reckitt Benckiser Healthcare (Hull, UK). Foetal calf serum was obtained from Trace Scientific Ltd (Melbourne, Australia). Diacetylmorphine base and 6-monoacetylmorphine were purchased from the National Analytical Reference Laboratory of the Australian Government Analytical Laboratories (Pymble, Australia). Noroxycodone and oxymorphone hydrochloride were obtained from Du Pont Pharmaceuticals (Wilmington, DE, USA). Codeine phosphate was obtained from F.H. Faulding (Adelaide, Australia) and morphine hydrochloride from McFarlane Smith (Edinburgh, UK). AlamarBlue was

purchased from Astral Scientific (Gymea, Australia). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

3.3.2. Preparation of Solutions

As discussed previously in Chapter 2 (2.3.5) dissolving diacetylmorphine base directly into Milli Q water or an equimolar solution of hydrochloric acid caused spontaneous diacetylmorphine degradation of 10-15%. To prevent this, diacetylmorphine base was dissolved in pH 5.0 phosphate buffer (monopotassium phosphate 65.9 mM and disodium phosphate 0.8 mM). Once in solution, diacetylmorphine was stored at -80 °C until required. This process was also used for 6-monoacetylmorphine base. Noroxycodone, norhydrocodone, the plus isomers of morphine and codeine, oripavine, norbuprenorphine and hydrocodone were all dissolved in dilute hydrochloric acid such that the molarity of the acid and base were the same, while the remaining compounds were dissolved in Milli Q water. All solutions were store at 4 °C until used.

3.3.3. Animals

Ethics approval to conduct the studies was obtained from the University of Adelaide Animal Ethics Committee (ethics numbers M/50/00 and M/64/01). Male Balb/c mice 6-8 weeks old were purchased from Central Animal Supplies (Waite Campus, University of Adelaide, SA, Australia) and used as donors of splenocytes for use in cell culture. Animals were provided with standard rodent feed and water *ad libitum*. Animals were housed in a standard 12 hour light dark cycle (starting at 7 am) under a constant room temperature of 22 ± 2 °C (mean ± range).

3.3.4. Lymphocyte preparation

The splenocyte cultures were prepared in the same fashion as discussed in Chapter 2 (2.3.3.2), using the optimised conditions presented previously. Each 4,5-epoxymorphinan was assessed on six separate occasions using six separate splenocyte preparations.

3.3.5. Inhibitor studies

Incubation conditions were only slightly modified from those described in 2.3.3.2. The cells were incubated with 25 µl of antagonist for 15 minutes prior to the addition of 50 µl concanavalin A and 25 µl agonist (concentrations doubled due to altered volume added). Naloxone was used at four concentrations 0.0001 µM, 0.01 µM, 1 µM and 100 µM. Specific opioid receptor subtype antagonists naltrindole (δ), naloxonazine (µ) and nor-binaltorphimine (κ) were initially incubated at concentrations from 0.0001 µM to 100 µM, to determine a concentration at which no response from the antagonist alone was observed, as at least naltrindole has been shown to alter cell proliferation (Gaveriaux-Ruff *et al.*, 2001). The concentrations used were 1µM for naltrindole and naloxonazine, and 0.1µM for nor-binaltorphimine. All antagonists were used in combination with (-)-morphine (10 µM and 0.1 µM), (+)-morphine (100 µM and 0.1 µM), oxycodone (0.01 µM), morphine-6-glucuronide (0.01 µM) and buprenorphine (10 µM).

3.3.6. Time course studies

The time dependent inhibition of splenocyte proliferation by morphine subsequent to mitogenic stimulation was also investigated. Cultures were prepared as before with mitogen.

However, the final volume was 190 µl with no opioid included in the media. The plates were then incubated as in other studies. At 0, 4, 8, 12, 16, 20 and 24 hours, 10 µl of morphine (0.1 and 10 µM) was added to the media and proliferation was quantified after 24 hours incubation.

3.3.7. Data analysis

The data comprised baseline proliferation (unstimulated proliferation) that was subtracted from all other proliferation data and then expressed as the percent of proliferation of the mitogen control minus one hundred percent. Equations relating modulation of proliferation to drug concentrations were fitted to the data using Prism 4.0 (GraphPad, CA, USA). Two types of equations were used for these calculations: A basic Hill equation $y = \left(\frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - x) \times \text{Slope}}} \right)$, where Bottom is the minimum and Top is the maximum response (proliferation) and LogEC_{50} the concentration required to achieve 50% of the response, and Slope is the slope of the relationship. Secondly, the sum of two Hill equations were fitted to the data as an equation that fitted both the upward and downward parts of a bell-shaped curve $y = \left(\frac{\text{Bottom}_{DT} + (\text{Top}_{DT} - \text{Bottom}_{DT})}{1 + 10^{(\text{LogEC}_{50DT} - x) \times \text{Slope}_{DT}}} \right) + \left(\frac{\text{Bottom}_{UT} + (\text{Top}_{UT} - \text{Bottom}_{UT})}{1 + 10^{(\text{LogEC}_{50UT} - x) \times \text{Slope}_{UT}}} \right)$.

Parameters were obtained for the downward (DT) and upward (UT) parts of the concentration response relationship. Where possible the Top_{DT}, Bottom_{UT}, Bottom (on a positive slope) and Top (on a negative slope) were set at zero to simulate a physiological situation where if no drug was present there would be no effect. However, in some cases the data did not allow this, because the responses obtained at low ligand concentrations did not tend to zero resulting in an inappropriate fit; instead these points were constrained as close to zero as the model would allow (upper or lower limits were set in Prism 4.0). Using the best-fit parameters

generated by Prism, the maximum proliferative responses and the concentration at which these responses occurred were calculated using Excel 2000 and the Solver analysis pack. Statistical significance was assessed using a one-way analysis of variance with Bonferroni post hoc test comparing the mitogen control with all other data points. All data are presented as mean ± S.E.M. Significance was set at $P < 0.05$.

3.4. Results

In vitro exposure of mitogen-stimulated splenocytes to 4,5-epoxymorphinans caused varied responses, however these were divided into 5 major groups.

1. *Inverted bell-shaped curve:* Inhibition of the proliferative response and subsequent return to baseline response was found for oxycodone (Figure 3.4-1 A), morphine-6-glucuronide (Figure 3.4-1 B), diacetylmorphine (Figure 3.4-1 C), 6-monoacetylmorphine (Figure 3.4-1 D), (-)-codeine (Figure 3.4-1 E) and (+)-codeine (Figure 3.4-1 F). These data are also summarised in Table 3.4-1. This response was characterised by minimal alteration in response from control at low concentrations (0.0001 μM), significant inhibition (range -22% to -51 %, $P < 0.026$) at mid-range concentrations (0.01 μM to 1 μM), followed by a return to control proliferation at high concentrations (10 μM to 100 μM). The sum of two Hill equations adequately fitted to these data (Figure 3.4-1). The nadir of proliferation and the concentration at which this occurred varied. The nadir for oxycodone was the greatest (-51%) whilst the lowest concentration at which a nadir occurred was 0.003 μM for (-)-codeine (-27%).

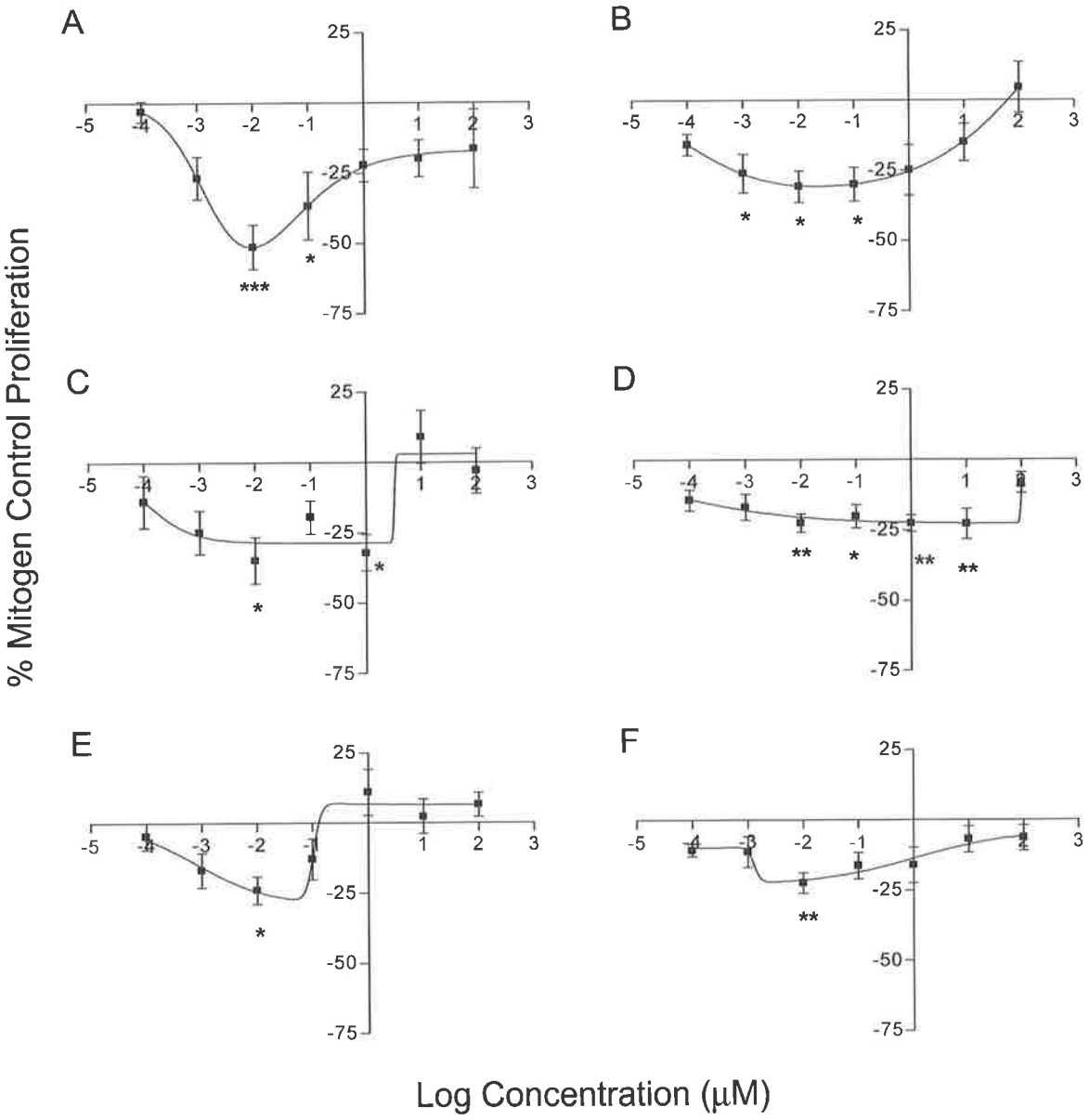


Figure 3.4-1 Group 1: 4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

Oxycodone (A), morphine-6-glucuronide (B), diacetylmorphine (C), 6-monoacetymorphine (D), (-)-codeine (E) and (+)-codeine (F). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2. *Inhibitory concentration response curve:* Significant inhibition ($P < 0.02$) at high concentrations (> 1 μM) was found for oripavine (Figure 3.4-2 A), buprenorphine (Figure 3.4-2 B), norbuprenorphine (Figure 3.4-2 C), thebaine (Figure 3.4-2 D) and 3-O-methylnaltrexone (Figure 3.4-2 E). These data are also summarised in Table 3.4-1. A Hill equation with a negative slope was modelled to these data. The responses were characterised by minimal response over the low and mid-range concentrations (0.0001 μM to 1 μM), followed by significant inhibition at high concentrations (100 μM). The concentration at which the nadir occurred was 100 μM for all compounds, however the nadir ranged from -28% to -219%. The inhibitory response had not plateaued at the highest concentration tested indicating even greater inhibition may be possible with higher concentrations.

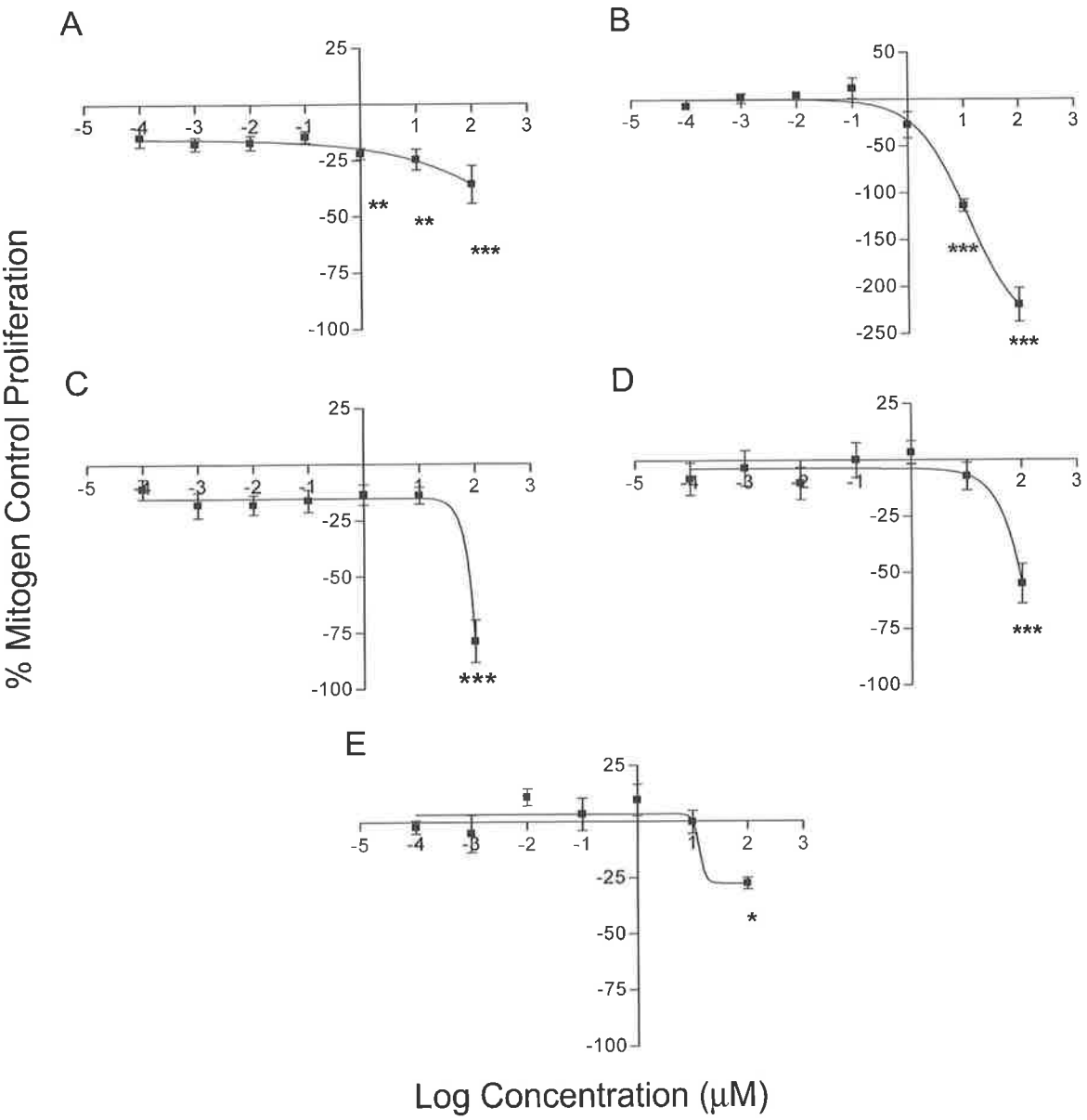


Figure 3.4-2 Group 2: 4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

Oripavine (A), buprenorphine (B), norbuprenorphine (C), thebaine (D) and 3-O-methylnaltrexone (E). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

3. *Inverted bell-shaped curve with induction:* The effect of (-)-morphine (Figure 3.4-3 A), 6-hydroxyoxymorphone (Figure 3.4-3 B) and (+)-morphine (Figure 3.4-3 C) at the low concentrations was similar to that of oxycodone and the first group of compounds. The lowest concentrations did not affect the proliferative response but significant inhibition ($P <$

0.022) was found at the mid-range concentrations (0.01 μM to 1 μM) (Table 3.4-1). In contrast, with oxycodone and the first group of 4,5-epoxymorphinans, at the high concentrations there was a significant induction of proliferation ($P < 0.026$). The nadir of the responses was greatest for (-)-morphine (-32%), as was the zenith (41%) (Figure 3.4-3 A). The lowest concentration at which the nadir and zenith occurred was 0.04 μM for (+)-morphine (-11%) and 1.7 μM (-)-morphine (41%), respectively.

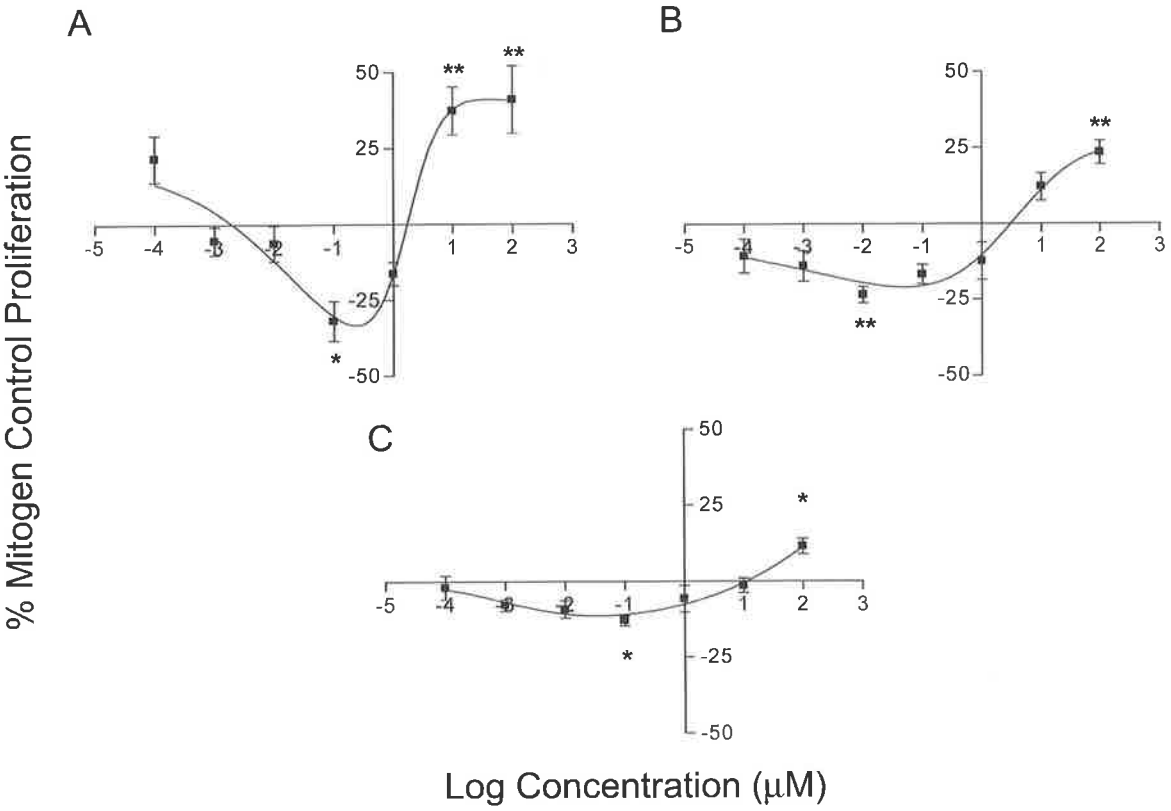


Figure 3.4-3 Group 3: 4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

(-)-morphine (A), 6-hydroxyoxymorphone (B) and (+)-morphine (C). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

4. *Induction concentration response curve:* Significant induction ($P < 0.023$) of the proliferative response was observed when cells were incubated with oxymorphone (Figure 3.4-4 A), morphine-3-glucuronide (Figure 3.4-4 B), naltrexone (Figure 3.4-4 C), 6β-naltrexol

(Figure 3.4-4 D) and hydromorphone (Figure 3.4-4 E). These data are also summarised in Table 3.4-1. A Hill equation with a positive slope was modelled to these data. Oxymorphone had the greatest maximum response (48.9%), at 100 μM.

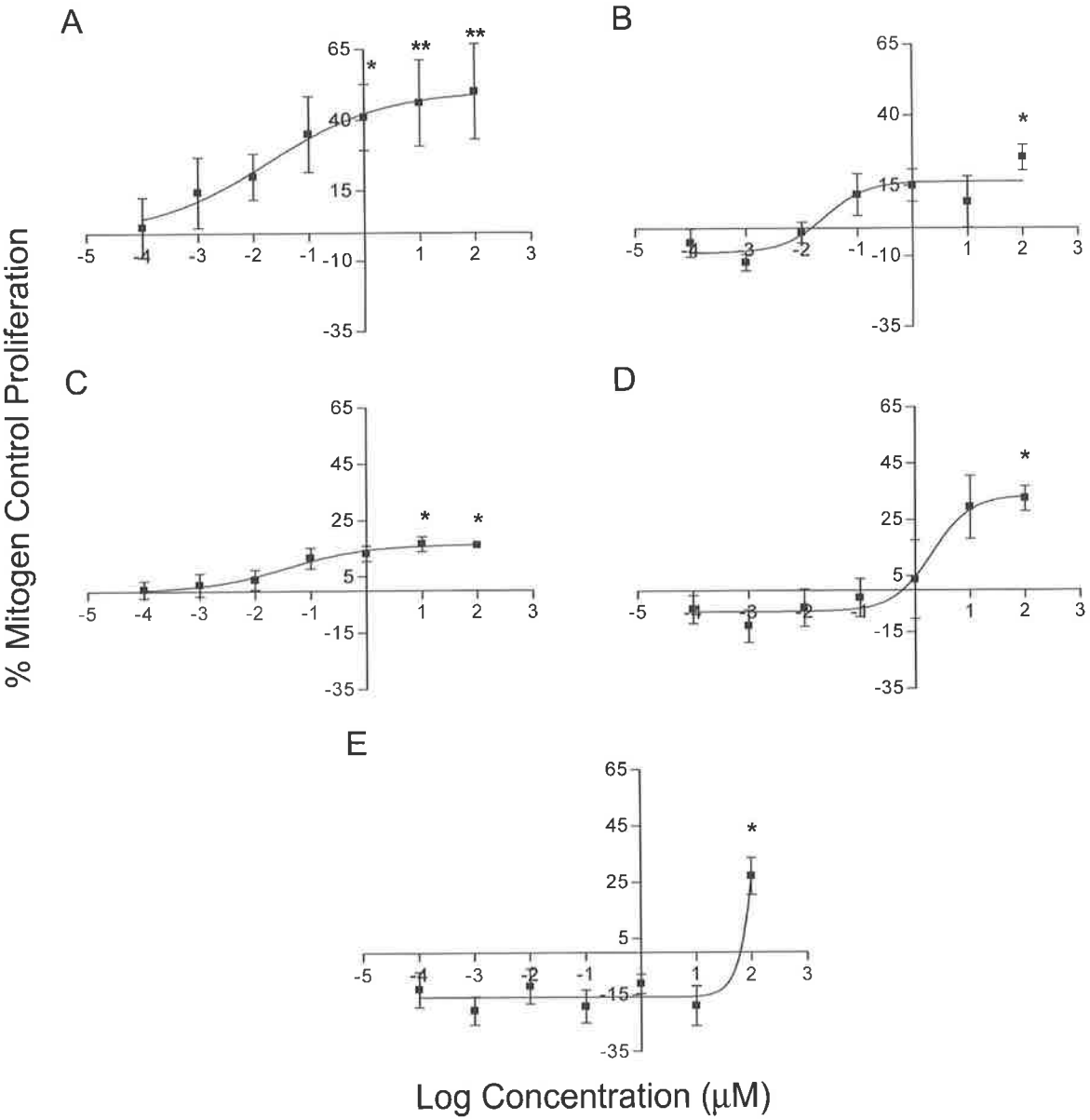


Figure 3.4-4 Group 4: 4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

Oxymorphone (A), morphine-3-glucuronide (B), naltrexone (C), 6β-naltrexol (D) and hydromorphone (E). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

5. Hydrocodone (Figure 3.4-5 A), naloxone (Figure 3.4-5 B), norhydrocodone (Figure 3.4-5 C), noroxycodone (Figure 3.4-5 D) and naloxone methiodide (Figure 3.4-5 E) had no significant effect on the proliferative response.

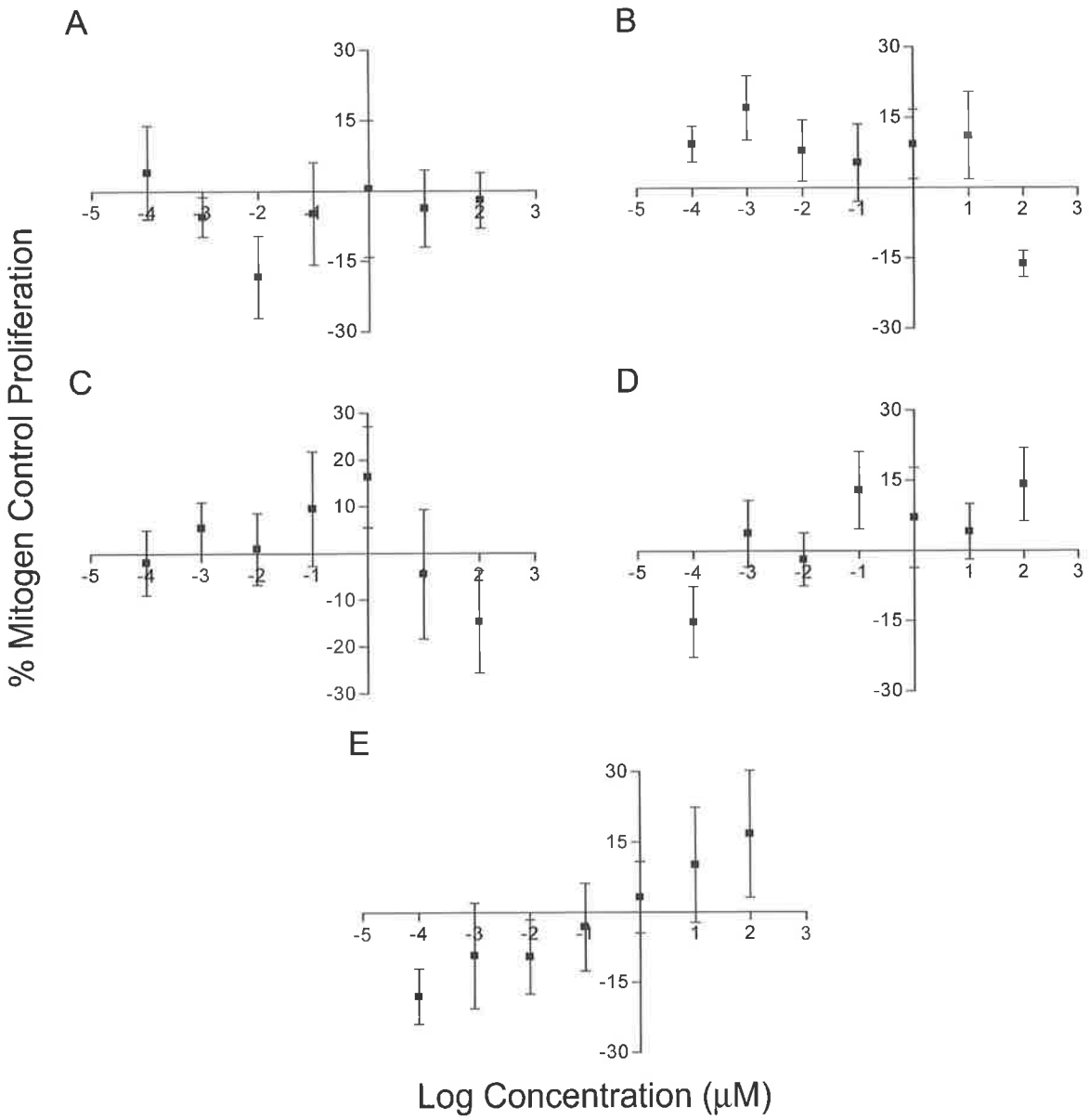


Figure 3.4-5 Group 5: 4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

Hydrocodone (A), naloxone (B), norhydrocodone (C), noroxycodone (D) and naloxone methiodide (E). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Comparison of rank orders:

1. *Inhibition (downward):* The rank order of maximal inhibitory effect was buprenorphine > norbuprenorphine > thebaine > oxycodone > oripavine > (-)-morphine > morphine-6-glucuronide > diacetylmorphine > 3-O-methylnaltrexone > (-)-codeine > 6-monoacetylmorphine > (+)-codeine > 6-hydroxyoxymorphone > (+)-morphine (Table 3.4-1). However, the rank order of the EC_{50} values for this response was 6-monoacetylmorphine < diacetylmorphine < morphine-6-glucuronide < (-)-codeine < (+)-morphine < (+)-codeine < oxycodone < (-)-morphine < 6-hydroxyoxymorphone < buprenorphine < 3-O-methylnaltrexone < norbuprenorphine < thebaine < oripavine (Table 3.4-1).

2. *Induction (upward):* The rank order for the maximal stimulatory effect was oxymorphone > (-)-morphine > 6β-naltrexol > hydromorphone > 6-hydroxyoxymorphone > morphine-3-glucuronide > naltrexone > (+)-morphine (Table 3.4-1). The maximal responses achieved in group one were not included as this groups' maximal response was not above baseline. The rank order of the EC_{50} values of the stimulant response including the group one data was oxymorphone < morphine-3-glucuronide < oxycodone < naltrexone < (-)-codeine < (+)-codeine < (-)-morphine < 6β-naltrexol < diacetylmorphine < 6-hydroxyoxymorphone < 6-monoacetylmorphine < hydromorphone < morphine-6-glucuronide < (+)-morphine (Table 3.4-1).

Table 3.4-1 Data for all 4,5-epoxymorphinan compounds analysed.

	Compound	Downward Response			Upward Response		
		EC_{50}	Max effect	Conc	EC_{50}	Max effect	Conc
1	Oxycodone	1.6 nM	-51%	10 nM	0.03 μM		
	Morphine-6-glucuronide	0.1 nM	-31%	20 nM	> 100 μM		
	Diacetylmorphine	0.1 nM	-29%	2.0 μM	3.2 μM		
	(-)-Codeine	0.8 nM	-27%	50 nM	0.1 μM		
	6-Monoacetylmorphine	0.03 nM	-23%	76 μM	100 μM		
	(+)-Codeine	1.3 nM	-22%	2.5 nM	1.1 μM		
2	Buprenorphine	12.6 μM	-219%	100 μM			
	Norbuprenorphine	> 100 μM	-79%	100 μM			
	Thebaine	> 100 μM	-55%	100 μM			
	Oripavine	> 100 μM	-36%	100 μM			
	3-O-Methylnaltrexone	20 μM	-28%	100 μM			
3	(-)-Morphine	25 nM	-33%	234 nM	1.7 μM	41%	40 μM
	6-Hydroxyoxymorphone	316 nM	-21%	50 nM	3.5 μM	24%	100 μM
	(+)-Morphine	1 nM	-11%	40 nM	> 100 μM	11%	100 μM
4	Oxymorphone				20 nM	49%	100 μM
	6β-naltrexol				2 μM	33%	100 μM
	Hydromorphone				> 100 μM	27%	100 μM
	Morphine-3-glucuronide				25 nM	17%	100 μM
	Naltrexone				40 nM	16%	100 μM
5	Hydrocodone Naloxone Norhydrocodone Noroxycodone Naloxone Methiodide	NO RESPONSE					

Maximum effect responses and EC_{50} concentrations reported were obtained from the modelled Hill equations.

Other response characteristics: The responses were not stereoselective since both codeine and to a lesser degree morphine stereoisomers affected proliferation to a similar degree.

Antagonists

Naloxone significantly antagonised the inhibition caused by (+)- and (-)-morphine (0.1 μM, $P < 0.01$), oxycodone (0.01 μM, $P < 0.05$) and morphine-6-glucuronide (0.01 μM, $P < 0.05$). However, it potentiated inhibition at 100 μM (Table 3.4-2). Naloxone was only able to partially reverse buprenorphine inhibition, with greater antagonistic effect from 0.0001 μM to 1 μM naloxone. Conversely, naloxone had no effect on the induction of proliferation caused by (-)-morphine (10 μM). However, naloxone at 1 μM and 10 μM antagonised (+)-morphine (100 μM) induction of proliferation.

Table 3.4-2 Effect of naloxone on immunomodulation by different 4,5-epoxymorphinans.

Naloxone	Control	0.0001 μM	0.01 μM	1 μM	100 μM
(+)-Morphine 0.1 μM	-17.3 ##	-6.9	**1.5 ****	**4.5 *****	-12.2
(-)-Morphine 0.1 μM	-32.8 ###	**16.3 *****	**19.2 *****	**19.6 *****	-30.2
Oxycodone 0.01 μM	-36.2 #	**30.2***	**31.5 ***	3.7	-70.3
M6G 0.01 μM	-32.7 #	**22.7 ****	**18.8 ***	**16.4 ***	-37.2
(+)-Morphine 100 μM	51.7 ###	42.8	26.7	**16.4 ***	**9.2 ****
(-)-Morphine 10 μM	35.7 ###	32.8	39.0	43.2	29.7
Buprenorphine 10 μM	-137.5 ###	**-44.7 *****	**-56.0 *****	**-47.3 *****	**-82.2 ****

Data are expressed as the percent of the mitogen control minus one hundred percent. Statistical differences in the control column are from comparison with the mitogen control and statistical significance is signified as # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$. All other statistical analysis are compared to the control column using a one-way ANOVA with Bonferroni post hoc. Statistical significance are signified as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. M6G is morphine-6-glucuronide.

Naloxonazine antagonised the inhibition caused by (+)- and (-)-morphine (0.1 μM, $P < 0.01$), oxycodone (0.01 μM, $P < 0.001$) and morphine-6-glucuronide (0.01 μM, $P < 0.001$) and partially antagonised the inhibition of buprenorphine (10 μM, $P < 0.05$), whilst nor-binaltorphimine and naltrindole had no significant effects (Table 3.4-3). None of the selective antagonists were able to inhibit the induction of proliferation caused by (-)-morphine (10 μM) or (+)-morphine (100 μM).

Table 3.4-3 Effect of the selective opioid receptor subtype antagonists on the immunomodulation by different 4,5-epoxymorphinans.

	Control	Naltrindole (δ) 1 μM	Naloxonazine (μ) 1μM	Nor-BNI (κ) 0.1 μM
(-)-Morphine 0.1 μM	-27.9 ###	-25.7	**4.0** ***	-36.5
(+)-Morphine 0.1 μM	-18.8 #	-18.2	**8.3** **	-19.3
Oxycodone 0.01 μM	-30.5 ###	-25.0	**1.8** ***	-28.5
M6G 0.01 μM	-21.6 #	-16.3	**8.5** ***	-31.3
Buprenorphine 10 μM	-120.1 ###	-109.0	**-87.3** *	-131.5
(-)-Morphine 10 μM	22.4 ##	5.7	7.3	11.3
(+)-Morphine 100 μM	20.7 #	8.8	13.0	15.3

Data are expressed as the percent of the mitogen control minus one hundred percent. Statistical differences in the control column are from comparison with the mitogen control and statistical significance is signified as # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$. All other statistical analysis compare are compared to the control column using a one-way ANOVA with Bonferroni post hoc. Statistical significance are signified as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Immunomodulation time course

Addition of 0.1 μM morphine into the culture media after mitogenic stimulation of proliferation caused suppression following 16 hours exposure, which reached statistical significance ($P < 0.01$) after 24 hours exposure (Figure 3.4-6 A). Increasing the morphine concentration in the incubation media to 10 μM caused statistically significant ($P < 0.001$) induction of proliferation following 12 hours exposure, which plateaued from this time (Figure 3.4-6 B).

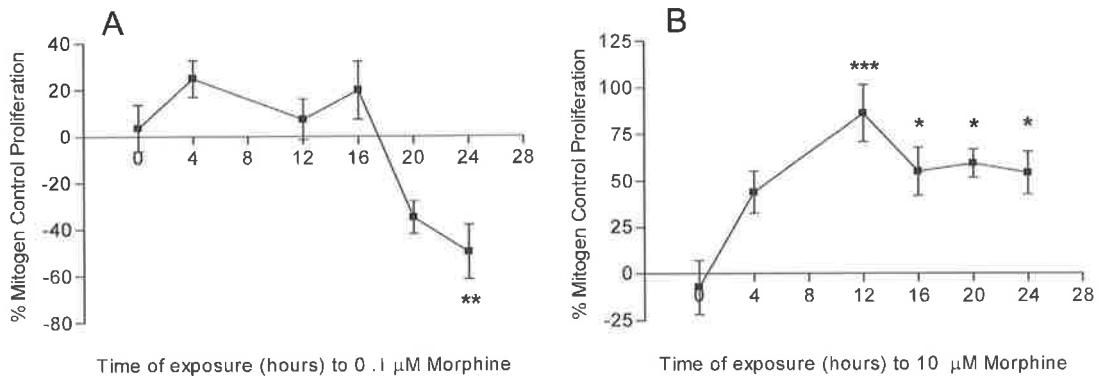


Figure 3.4-6 Influence of exposure time on the effects of morphine on splenocyte proliferation.

Error bars are S.E.M. and statistical significance are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.5. Discussion

The aim of this study was to characterise the *in vitro* immunomodulatory effects of a large number of 4,5-epoxymorphinans. The use of AlamarBlue in this investigation allowed a short exposure time (24 hours), rapid detection of proliferation (4 hours) and the removal of toxic influences from the experimental model (Ahmed *et al.*, 1994). Although previous studies have produced conflicting results with the data presented here (Fecho *et al.*, 1996a; Jessop & Taplits, 1991a), we have tested a wider range of compounds using the same experimental model. Hence, we are in a position to allow more meaningful comparisons to be made between the effects of different 4,5-epoxymorphinans, thereby allowing elucidation of a structure-effect relationship.

In contrast to other biological systems for evaluating the effects of structurally similar 4,5-epoxymorphinans, five different types of responses were obtained: an inverted bell-shaped curve (e.g. oxycodone), a concentration-dependent inhibitory response curve (e.g. buprenorphine), an inverted bell-shaped curve with induction (e.g. morphine), a concentration-dependent induction response curve (e.g. oxymorphone), and lack of response (e.g. hydrocodone). The diverse characteristics of these responses brings into question the classical opioid nature of the receptor(s) through which these responses were mediated. If the 4,5-epoxymorphinans were acting via a single classical opioid receptor, the directions of the response would be similar and potency easily assigned. Furthermore, the rank order of the maximal effect and EC_{50} values would be similar to currently available neuronal opioid binding data. However, this was not the case. Table 3.5-1 compares the rank order of μ opioid receptor binding affinities for the 24 4,5-epoxymorphinans used in this study and previously reported by others with the rank order of immunomodulatory effects found in this

study. It is clearly apparent that the comparative rank orders are dissimilar, implying therefore that the immunomodulatory effects observed here are not mediated by the neuronal μ opioid receptor.

Other classical opioid characteristics such as stereoselectivity and antagonist sensitivity were also inconsistent among the 4,5-epoxymorphinans used. For example, the opioid-active (-) and opioid-inactive (+) stereoisomers of codeine and morphine caused similar immunomodulation, thereby displaying non-stereoselectivity. Furthermore, (-)-morphine caused both naloxone- and naloxonazine-sensitive inhibition, and naloxone- and naloxonazine-insensitive induction of the proliferative response, which has been reported previously for naloxone (Roy *et al.*, 1998b), whilst nor-binaltorphimine and naltrindole were without antagonistic effects. These antagonist data would normally suggest the presence of classical μ opioid receptor(s) causing the inhibitory response and non-classical opioid receptor(s) mediating the induction of proliferation. However, the rank order data in Table 3.5-1 and the lack of stereoselectivity indicate that the opioid inhibition of proliferation was a non-classical opioid response, although elements of opioid antagonist sensitivity remain. Hence, if only one opioid agonist and antagonist was used in the assay, an incorrect conclusion would have been made, since the available data would have supported the involvement of a classical opioid receptor.

For example, (-)-morphine caused both naloxone-sensitive inhibition and naloxone-insensitive induction of the proliferative response. Naloxone-sensitivity and -insensitivity within an experimental model has been reported previously for cytokine production by lipopolysaccharide-stimulated peritoneal macrophages (Roy *et al.*, 1998b), where the response to 50 nM (-)-morphine was naloxone-sensitive, whilst the 50 μM response was

naloxone-insensitive. Interestingly, the selective μ opioid antagonist naloxonazine, had the same effect as naloxone, whereby it antagonised (-)-morphine inhibition but had no effect on the induction of proliferation. Both nor-binaltorphimine and naltrindole were unable to antagonise any responses. These (-)-morphine antagonist data would normally suggest the presence of both a classical opioid receptor causing the inhibitory response and a non-classical opioid receptor mediating the induction of proliferation. However, the morphine response was non-stereoselective, and when all the data are viewed, there is significant evidence suggesting the inhibitory responses are not classically opioid mediated based on the lack of rank order of μ opioid receptor binding. The naloxone- and naloxonazine-sensitivity results were consistent within the response groups. For example, the inhibitory response resulting from exposure to 0.1 μM (+)-morphine and (-)-morphine, and 0.01 μM oxycodone and morphine-6-glucuronide were all responsive to both naloxone and naloxonazine antagonism, whereby the inhibitory response caused by 10 μM buprenorphine was only partially reduced by these antagonists. The increased inhibition by 100 μM naloxone is likely due to it acting as an agonist at this concentration. However, the induction of proliferation by 10 μM (+)-morphine was sensitive to naloxone but not naloxonazine and this divergence is difficult to explain. This group 1 antagonist data indicate that despite these responses being unlikely classically opioid, they still display opioid antagonist-sensitivity, which must be taken into account in future studies.

Table 3.5-1 Rank order of available μ opioid receptor binding affinities.

	Rank Orders				
Compound	**Binding affinity**	**Downward maximal effect**	**Downward EC_{50}**	**Upward maximal effect**	**Upward EC_{50}**
Buprenorphine	1	1	10	NR	
Norbuprenorphine	2	2	12	NR	
Naltrexone	3	NR		7	4
Naloxone	4	NR		NR	
Morphine-6-glucuronide	5	7	3		13
Hydromorphone	6	NR		4	12
Oxymorphone	7	NR		1	1
(-)-Morphine	8	6	8	2	7
Oxycodone	9	4	7		3
Diacetylmorphine	10	8	2		9
6-Monoacetylmorphine	11	11	1		11
Hydrocodone	12	NR		NR	
Naloxone Methiodide	13	NR		NR	
Morphine-3-glucuronide	14	NR		6	2
(-)-Codeine	15	10	4		5
Thebaine	16	3	13	NR	
(+)-Morphine	17	14	5	8	14

Gathered from Chen *et al.* (1991), Codd *et al.* (1995), Lewanowitsch *et al.* (2003), Metzger *et al.* (2001), Spetea *et al.* (1998), Selley *et al.* (2001), Magnan *et al.* (1982), Law and Loh (1999) and Huang *et al.* (2001) and rank orders of responses obtained from the current study. NR represents that no deviations from baseline response in that direction was observed.

The time course of inhibitory and enhancing effects of morphine on splenocyte proliferation also offers an interesting insight into the mechanisms of action of these different concentrations. The low concentration of morphine (0.1 μM) did not start to inhibit proliferation until it had been in contact with the cells for greater than 16 hours. In contrast, 10 μM morphine caused increased proliferation after just 4 hours exposure and remained elevated for the rest of the treatment. These data suggest that the mechanisms that mediated these response are different, thereby implying different receptors.

The non-classical opioid hypothesis and proliferation response data found in this study conflict with previously published results. Fecho *et al.* (1996a) found no change in morphine-induced splenocyte proliferation except for significant suppression at high concentrations (100 μM), whilst Jessop and Taplits (1991a) only found inhibition of concanavalin A-induced splenocyte proliferation. Thomas *et al.* (1995a) were unable to demonstrate any clear dose dependent relationship in the proliferation response following exposure to morphine, morphine-6-glucuronide and morphine-3-glucuronide and found the inactive metabolite normorphine possessed more activity than the others. In contrast, our data agree with Bidlack and Hemmick (1990) who reported induction of proliferation at 10 μM morphine but did not report the effect at lower concentrations. The reason for these discrepancies is likely to be experimental differences between the studies, including animal strain and other experimental parameters such as exposure and proliferation time. We considered that proliferation should be quantified before the maximum effect is achieved and well before media depletion. Furthermore, *in vitro* immune responses to opioids are reduced following longer incubation times (Shahabi & Sharp, 1995a) possibly caused by receptor down regulation (Sharp *et al.*, 1997), emphasising the need for a shorter incubation period. The use of AlamarBlue allowed short exposure (24 hours) and rapid detection of proliferation (4 hours) due to assay sensitivity, thereby avoiding some of these experimental limitations.

Due to the apparent non-classical opioid nature of these responses and the need to further characterise these responses, an hypothesis for a structure-effect relationship for the effect these 4,5-epoxymorphinans have on concanavalin A-induced splenocyte proliferation was formulated.

There are four different functional group modifications at the R_1 position of the 4,5-epoxymorphinans tested: hydroxyl, glucuronide, acetyl and methoxyl (Table 3.1-1). Changing from the polar hydroxyl or glucuronide functional group to the relatively non-polar methoxyl group (morphine-3-glucuronide or morphine to codeine; oxymorphone to oxycodone; naltrexone to 3-O-methylnaltrexone; hydromorphone to hydrocodone) caused loss of induction at high concentrations. The change in response from an hydroxyl to glucuronide exchange (morphine to morphine-3-glucuronide) resulted in loss of inhibition, whilst the induction at high concentrations remained, suggesting the more polar the R_1 functional group, the more likely induction of proliferation would occur. The substitution of an hydroxyl to methoxyl at R_1 (oripavine to thebaine) was more difficult to explain, as it appeared that the methoxyl at position R_2 caused significant inhibition at high concentrations. As highlighted previously (Chapter 2), it is difficult to draw any conclusions from the use of diacetylmorphine in cell culture as it is rapidly degraded to 6-monoaceylmorphine and morphine. However, the diacetylmorphine response is in keeping with the presence of both morphine and 6-monoacetylmorphine in the media.

At the R_2 position, there were five different functional groups: hydroxyl, methoxyl, glucuronide, acetyl and ketone. Modifications of the functional group from the polar hydroxyl or glucuronide to the non-polar ketone caused loss of response; however, in this case the response was inhibition at low concentrations. For example, morphine or morphine-6-glucuronide to hydromorphone; 6-hydroxyoxymorphone to oxymorphone; and codeine to hydrocodone all showed these characteristics. The scenario is not supported by the changes from 6β-naltrexol to naltrexone, since 6β-naltrexol did not inhibit proliferation, possibly due to the presence of the cyclopropylmethyl group at the R_4 position. Changes at R_2 from a methoxyl to a ketone (oripavine to hydromorphone and hydrocodone to thebaine), methoxyl to hydroxyl (thebaine to codeine or oripavine to morphine) or methoxyl to acetyl (oripavine to

6-monoacetylmorphine) caused loss of significant inhibition at high concentrations. It appears therefore that a methoxyl functional group at R_2 causes significant inhibition at high concentrations. Replacement of an acetyl by a glucuronide, hydroxyl or ketone group (6-monoacetylmorphine to morphine-6-glucuronide, morphine or hydromorphone, respectively) produced no change in function possibly because of the further degradation of 6-monoacetylmorphine to morphine (2.4.2). In the case of morphine to morphine-6-glucuronide, the change of a hydroxyl to the more polar glucuronide caused more pronounced inhibition suggesting that increasing the polarity of the group at the R_2 position results in greater inhibition.

Alterations of the functional group at R_3 from an hydroxyl to an hydrogen caused a reduction in response (oxycodone to hydrocodone, 6-hydroxyoxymorphone to morphine and oxymorphone to hydromorphone). Removal of the methyl group at R_4 resulted in loss of responses over the concentration range used (hydrocodone to norhydrocodone; and oxycodone to noroxycodone) and removal of the cyclopropylmethyl at R_4 caused a shift to the left of the response for norbuprenorphine (compared to buprenorphine) and loss of response for noroxycodone (compared to 3-O-methylnaltrexone). Substitution of a methyl with a cyclopropylmethyl (oxycodone to 3-O-methylnaltrexone and 6-hydroxyoxymorphone to 6β-naltrexol) caused loss of inhibition at low concentrations or reduced induction of proliferation (oxymorphone to naltrexone). Finally, the change from a methyl or cyclopropylmethyl to an alkene (oxymorphone or naltrexone to naloxone) caused loss of effect. The substitutions at R_4 indicate that more than a hydrogen atom needs to be present for activity to remain, although there is no clear explanation for the change in the responses associated with other functional groups at this position.

Overall, these structure-effect data indicate that a relatively polar R_1 functional group is responsible for the induction of proliferation, whilst increasing the polarity of the R_2 and R_3 functional groups appear to be responsible for inhibition of proliferation. A clear role for the R_4 functional group is not apparent, although substitution with a hydrogen substantially reduces activity. This structure-effect hypothesis does not correlate well with previous structure affinity data for opioid agonist binding to μ opioid receptors. Chen *et al.* (1991) demonstrated that for 4,5-epoxymorphinan binding to rat brain homogenates, the functional group at the R_1 position was the most important determinant of binding affinity, whilst the group at the R_2 position had little effect on binding affinity (Table 3.5-1). This is not the case for the responses observed for these compounds' effect on the stimulated splenocyte culture. Indeed, the opposite occurred, with changes at the R_2 position resulting in changes in response at significantly lower concentrations, indicating that this part of the molecule is more important for this pharmacodynamic effect (Table 3.5-1).

Several parent drugs and their major metabolites were evaluated in this system, enabling a comparison of parent to metabolite relative activities. For example, morphine-6-glucuronide was a more potent inhibitor of proliferation than morphine and morphine-3-glucuronide but a less potent inducer of proliferation than morphine and morphine-3-glucuronide at higher concentrations, indicating that activation of different immunomodulatory potentials may occur *in vivo* due to metabolism of the parent drug. Similarly, oxycodone was a more potent inhibitor at low concentrations but less potent inducer at high concentrations than its O-demethylated metabolite oxymorphone. Conversely, codeine was a more potent inhibitor at low concentrations than its O-demethylated metabolite morphine. These results highlight the immunological significance of the functional groups at R_1 and R_2, which are metabolically labile, leading to potential changes in immuno-pharmacodynamics across a dosing interval due to the different time courses of concentrations of immunologically active metabolites.

The differences between immune opioid effects and central opioid effects for the 4,5-epoxymorphinans examined may be found in the microenvironments in which immune cells operate. Peripheral immune cells can potentially experience the whole gamete of physiological conditions that exist within the human body due to their ability to move with relative ease from the circulation into peripheral tissues and back into circulation. Therefore, the cell membrane receptors must operate in these varying conditions. The optimal sodium ion concentrations for ligand binding for one of the non-opioid receptors characterised is different to that of neuronal opioid receptors (0.15 mM versus 0.05 mM) and is more closely related to the ionic conditions found peripherally (Madden *et al.*, 2001). It is possible that the varied extracellular conditions that immune cells experience cause the opioid receptors to lose their specificity due to altered tertiary structure or receptor co-localisation. Alternatively, proteins released by immune cells may interact with opioid receptors and alter their affinity, as has been reported for IL-1 increasing the specific binding of endogenous opioids to rat brain slices (Wiedermann, 1989) or increased binding affinity of immune opioid receptors after proliferation (Roy *et al.*, 1991). If the latter is the case, then inter-laboratory variability in results could be expected due to the likely differences in availability of effector molecules because of binding to different plastics and blocking of potential binding sites by serum supplemented media (McLure *et al.*, 2000; Obach *et al.*, 1997). Furthermore, the use of a mixed culture such as splenocytes is likely to produce different effects, since different co-stimulation signals will be available, compared to single cell type or cell line cultures (Nguyen & Miller, 2002). Finally, the presence of splice variants of opioid receptors on immune cells (McCarthy *et al.*, 2001b) may also explain the diversity of responses that have been observed, as these types of receptors display altered ligand selectivity and sensitivity (Pasternak, 2001). It is important that the characteristics of peripheral opioid receptors are

further investigated and characterised, and the effect they have on opioid pharmacodynamics is elucidated.

In conclusion, the structure-effect relationships formulated here indicate that the R_2 functional group at position 6 of the 4,5-epoxymorphinan structure determines inhibition of proliferation, whilst the R_1 at position 3 predicts induction of the proliferative response. Despite the inhibition of the response at low concentrations being naloxone- and naloxonazine-sensitive, the classical neuronal opioid nature of the response is not supported by the non-stereospecificity and lack of normal rank order of effect to μ opioid receptor binding. The induction of the response at high concentrations was not a classical opioid response due to the naloxone insensitivity. These conflicting rank order, antagonists and stereoselectivity data emphasise the need to conduct multiple analyses to determine the opioid nature of a response, especially when dealing with opioid modulation of these immune responses. This model could be used to evaluate the immunomodulatory effects of non-4,5-epoxymorphinan opioids (e.g. fentanyl, methadone and peptide opioids). Furthermore, the use of these structurally diverse compounds may further clarify the nature of the receptors mediating opioid immunomodulation. As our understanding of the direct effects of opioids on the various cells in the immune system increases and how these responses affect *in vivo* pharmacodynamics of opioids, an appreciation of the balance between central effects of opioids such as analgesia and dependence and immunomodulation may allow better selection of medication in some clinical settings.

Chapter 4. *In vitro* non-4,5 epoxymorphinan effect on immunocompetent cells

4.1. Introduction

Exogenous opioids, such as morphine (a 4,5-epoxymorphinan) and peptide endogenous opioids alter immune function *in vitro* (Wybran *et al.*, 1979) and *in vivo* (Sacerdote *et al.*, 1997b). We have challenged whether the effects of 4,5-epoxymorphinan on immune function are via the neuronal or "classical" opioid receptor (4,5-epoxymorphinan immunomodulation) and suggested that this widely prescribed and abused class of drugs may cause direct cellular immunomodulation via a non-classical opioid receptor (Chapter 3).

By using a structure-activity relationship as suggested by Sibinga *et al.* (1988), and utilised *in vivo* by Sacerdote *et al.* (1997b), the *in vitro* immunomodulatory effects of 24 4,5-epoxymorphinans were characterised (Chapter 3). Five different types of concentration-response relationships were observed: an inverted bell-shaped curve (e.g. oxycodone), an inhibitory concentration-response curve (e.g. buprenorphine), an inverted bell-shaped curve with induction (e.g. morphine), an induction concentration-response curve (e.g. oxymorphone), and no response (e.g. noroxycodone). Non-stereoselectivity, naloxone-sensitivity and -insensitivity, and importantly, a disparity between reported receptor binding affinity data and rank order of immunomodulatory potency were also found. The structure-effect relationship produced substantial evidence for non-classical opioid receptor involvement in the quantified immune proliferation (Chapter 3). These immunomodulation data were gathered using a single structural class of opioids, the 4,5-epoxymorphinans (Chapter 3). However, opioid agonists and antagonists of "classical" neuronal opioid receptors are structurally diverse. This characteristic was exploited by medicinal chemists who synthesised opioid compounds that were more potent, longer acting and which had fewer side effects (Casy & Parfitt, 1986). This gave rise to numerous new and structurally-diverse

chemical classes such as morphinans (e.g. levorphanol), 4-anilinopiperidines (e.g. fentanyl) and 3,3-diphenylpropylamines (e.g. methadone) (Casy & Parfitt, 1986). Derivatives of endogenous opioid peptides, such as DAMGO and DPDPE, have also been developed which have differing pharmacological properties from their parent peptide, such as increased binding affinity and opioid receptor selectivity (Casy & Parfitt, 1986). The immunomodulation caused by some of these non-4,5-epoxymorphinan compounds has been investigated. However, in most cases few compounds have been compared using the same experimental model and experimental conditions. In one collection of studies House, Thomas and colleagues (1995a; 1996; 1997a; 1997b; 1995b; 1995a; 1995b) undertook the characterisation of several μ, κ and δ opioid receptor ligands using a series of immunological models; although, comparisons of structure-activity relationships were not discussed in detail. However, some non-classical opioid characteristics were observed (e.g. non-classical rank order of effect). Others have also reported non-classical opioid immunomodulation (Jessop & Taplits, 1991a; Madden *et al.*, 2001; Roy *et al.*, 1998b; Roy *et al.*, 1991), although, the presence of "classical" opioid receptors has also been demonstrated (Bidlack *et al.*, 1995; Ovadia *et al.*, 1989).

4.2. Aims

Characterisation of non-4,5-epoxymorphinan immunomodulation using the same experimental model and subsequent comparative analysis has not been conducted. Therefore, the aim of this study was to investigate the *in vitro* immunomodulatory effects of 17 structurally unrelated non-4,5-epoxymorphinans to complement the previous work of this thesis using 4,5-epoxymorphinans (Chapter 3). This enabled comparison of the immunomodulatory potencies with available neuronal μ opioid receptor binding data,

including rank order of effect, stereospecificity, antagonist selectivity and grouping of similar ligand effects. Selective and non-selective opioid antagonists were also used to clarify the role of classical opioid receptors. A splenocyte mitogenesis assay was used as the experimental model, however in contrast to many previously applied models, a rapid non-toxic and sensitive proliferation detection method was used (Ahmed *et al.*, 1994), combined with incubation conditions optimised for the detection of altered immune response by *in vitro* exposure to opioids.

4.3. Materials and Methods

4.3.1. Chemicals

RPMI 1640 with HEPES modification and L-glutamine were purchased from Invitrogen (Mulgrave, Vic, Australia). Penicillin-streptomycin solution (10,000 units penicillin, 10 mg streptomycin.ml^{-1}), concanavalin A, naltrindole hydrochloride, naloxonazine dihydrochloride, nor-binaltorphimine dihydrochloride, naloxone hydrochloride, DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin), U69593 ((+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide) and DPDPE ([D-Pen^{2,5}]-Enkephalin) were purchased from Sigma (St. Louis, MO, USA). Levorphanol was purchased from Roche (Sydney, NSW, Australia), dextromethorphan and dextrorphan from Parke-Davis (Ann Arbor, MI, USA), fentanyl from David Bull Laboratories (Melbourne, Vic, Australia), remifentanil from Glaxo Wellcome (Boronia, Vic, Australia) and alfentanil from ICI Pharmaceuticals (Melbourne, Vic, Australia). Tramadol hydrochloride was purchased from Grünenthal (Stolberg, Germany). Levo-α-acetyl methadol hydrochloride, racemic methadone hydrochloride and (R)-(-)- and (S)-(+)-methadone bases were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Foetal calf serum was obtained from Trace

Scientific Ltd (Melbourne, Vic, Australia). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality. Compounds that were not supplied as a soluble salt were dissolved in an equi-molar solution of hydrochloric acid, while the remaining compounds were dissolved in Milli Q water. All solutions were store at 4 °C until used.

4.3.2. Animals

Ethics approval to conduct the studies was obtained from the University of Adelaide Animal Ethics Committee (ethics numbers M/50/00 and M/64/01). Male Balb/c mice 6-8 weeks old were purchased from Central Animal Supplies (Waite Campus, University of Adelaide, SA, Australia) and used as donors of splenocytes for use in cell culture. Animals were provided with standard rodent feed and water *ad libitum*. Animals were housed in a standard 12 hour light dark cycle (starting at 7 am) under a constant room temperature of 22 ± 2 °C (mean ± range).

4.3.3. Lymphocyte preparation

The splenocyte cultures were prepared in the same fashion as discussed in Chapter 2 (2.3.3.2), using the optimised conditions presented previously.

4.3.4. Inhibitor studies

Incubation conditions were only slightly modified from those described in 2.3.3.2. The cells were incubated with 25 µl of antagonist for 15 minutes prior to the addition of 50 µl

concanavalin A and 25 µl agonist (concentrations doubled due to altered volume added). Naloxone was used at four concentrations 100 µM, 1 µM, 0.01 µM and 0.0001 µM (final concentration). Methadone and its' stereoisomers were used at 100 µM in the naloxone antagonist experiment. Specific opioid receptor subtype antagonists naltrindole (δ), naloxonazine (µ) and nor-binaltorphimine (κ) were initialling incubated at concentrations from 0.0001 µM to 100 µM to determine a concentration at which no response from the antagonist alone was observed, since naltrindole has been shown to alter cell proliferation (Gaveriaux-Ruff *et al.*, 2001). The concentrations used were 1µM for naltrindole and naloxonazine, and 0.1µM for nor-binaltorphimine. All selective opioid receptor antagonists were used in combination with levorphanol (100 µM), fentanyl (0.1 µM) and DAMGO (0.1 µM).

4.3.5. Time course studies

The time dependent inhibition of splenocyte proliferation by methadone subsequent to mitogenic stimulation was also investigated. Cultures were prepared as before with mitogen, however the final volume was 190 µl with no opioid included in the media. The plates were then incubated as in other studies. At 0, 4, 8, 12, 16, 20 and 24 hours 10 µl of methadone (100 µM) was added to the media and proliferation was quantified after 24 hours incubation.

Chronic *in vitro* exposure to various concentrations of methadone was also conducted. Splenocytes were isolated as described previously and 6×10^7 cells.flask^{-1} were placed into sterile 175 cm^2 flasks (Nunc, Roskilde, Denmark) with in 50 ml RMPI 1640 with 2.5% foetal calf serum and methadone (final concentration 0, 0.1, 1, 10, 50 and 100 µM). The flasks were incubated 37 °C, 5% CO_2 in a humidified incubator for 48 hours. The cells were then

harvested from the flasks by gently rocking each flask for 5 minutes followed by two washes with fresh RPMI 1640 (total volume used < 50 ml) and collected into two 50 ml tubes. The suspension were then centrifuged for 10 min at 2000 rpm and the pellets were re-suspended and combined in a 15 ml tube. This suspension was then centrifuged for 5 minutes at 1000 rpm. Following this the cell pellet was gently re-suspended and the cells were plated into 96 well plates and treated as before.

4.3.6. Data analysis

The data comprised baseline proliferation (unstimulated proliferation) that was subtracted from all other proliferation data and then expressed as the percent of proliferation of the mitogen control minus one hundred percent. Equations relating modulation of proliferation to drug concentrations were fitted to the data using Prism 4.0 (GraphPad, CA, USA). Two types of equations were used for these calculations: A basic Hill equation $y = \left(\frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - x) \times \text{Slope}}} \right)$, where Bottom is the minimum and Top is the maximum response (proliferation) and LogEC_{50} the concentration required to achieve 50% of the response, and Slope is the slope of the relationship. Secondly, the sum of two Hill equations were fitted to the data as an equation that fitted both the upward and downward parts of a bell-shaped curve $y = \left(\frac{\text{Bottom}_{DT} + (\text{Top}_{DT} - \text{Bottom}_{DT})}{1 + 10^{(\text{LogEC}_{50DT} - x) \times \text{Slope}_{DT}}} \right) + \left(\frac{\text{Bottom}_{UT} + (\text{Top}_{UT} - \text{Bottom}_{UT})}{1 + 10^{(\text{LogEC}_{50UT} - x) \times \text{Slope}_{UT}}} \right)$.

Parameters were obtained for the downward (DT) and upward (UT) parts of the concentration response relationship. Where possible the Top_{DT}, Bottom_{UT}, Bottom (on a positive slope) and Top (on a negative slope) were set at zero to simulate a physiological situation where if no drug was present there would be no effect. However, in some cases the data did not allow this, because the responses obtained at low ligand concentrations did not tend to zero resulting

in an inappropriate fit; instead these points were constrained as close to zero as the model would allow (upper or lower limits were set in Prism 4.0). Using the best-fit parameters generated by Prism, the maximum proliferative responses and the concentration at which these responses occurred were calculated using Excel 2000 and the Solver analysis pack. Statistical significance was assessed using a one-way analysis of variance with Bonferroni post hoc test comparing the mitogen control with all other data points. All data are presented as mean ± S.E.M. Significance was set at $P < 0.05$.

4.4. Results

The proliferative responses resulting from exposure to the various non-4,5-expoymorphinans were divided into four groups dependent on the response observed: inhibition dose-response curve, inverted bell-shaped curve, induction and inhibition response and an induction response curve.

1. *Inhibition dose response curve:* Significant inhibition of proliferation from 10 μM to 100 μM was observed for levo-alpha-acetyl-methadol (LAAM) (Figure 4.4-1 A), racemic methadone (Figure 4.4-1 B) and it's two stereoisomers ((R)-(-)-: Figure 4.4-1 C, (S)-(+)-: Figure 4.4-1 D), dextromethorphan (Figure 4.4-1 E), naloxonazine (Figure 4.4-1 F) and naltrindole (Figure 4.4-1 G) ($P < 0.0001$, Table 4.4-1). The maximal inhibition was at the highest concentrations used of 100 μM and was greatest for naltrindole (-511%, $P < 0.0001$). A Hill equation with a negative slope was fitted to these data.

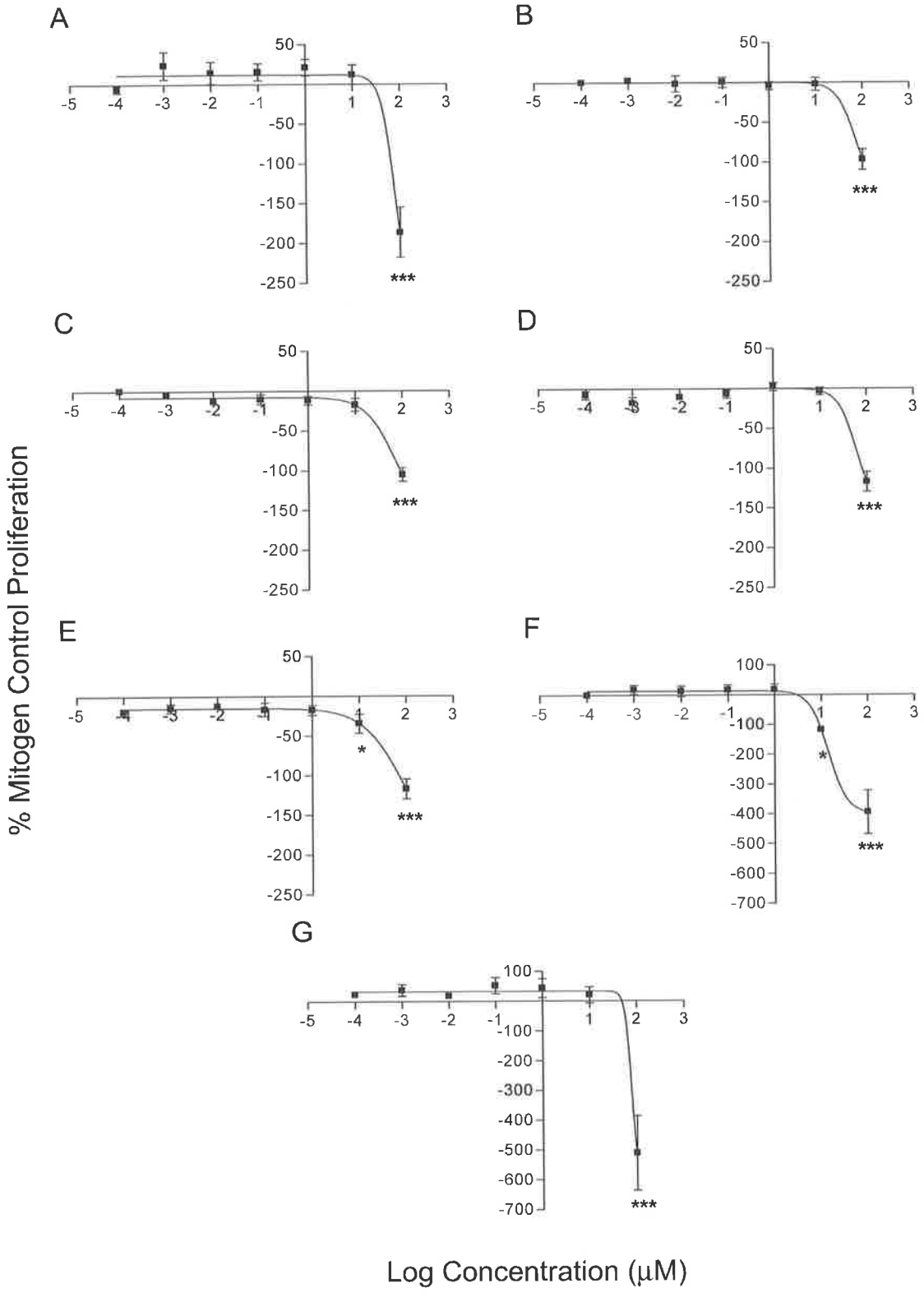


Figure 4.4-1 Group 1: non-4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

LAAM (A), racemic methadone (B), (R)-(-)-methadone (C), (S)-(+)-methadone (D), dextromethorphan (E), naloxonazine (F) and naltrindole (G). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2. *Inverted bell-shaped curve:* DAMGO (Figure 4.4-2 A) and the 4-anilinopiperidines fentanyl (Figure 4.4-2 B), alfentanil (Figure 4.4-2 C) and remifentanil (Figure 4.4-2 D) caused an inverted bell-shaped type profile with significant inhibition of proliferation between 0.0001 μM to 10 μM ($P < 0.01$, Table 4.4-1), with minimal difference in proliferation at 100 μM compared to control. The sum of two Hill equations was fitted to these data. The nadir of proliferation was greatest for fentanyl (-48%, $P = 0.002$) and occurred at the lowest concentration (0.35 μM) compared to the other compounds in this group.

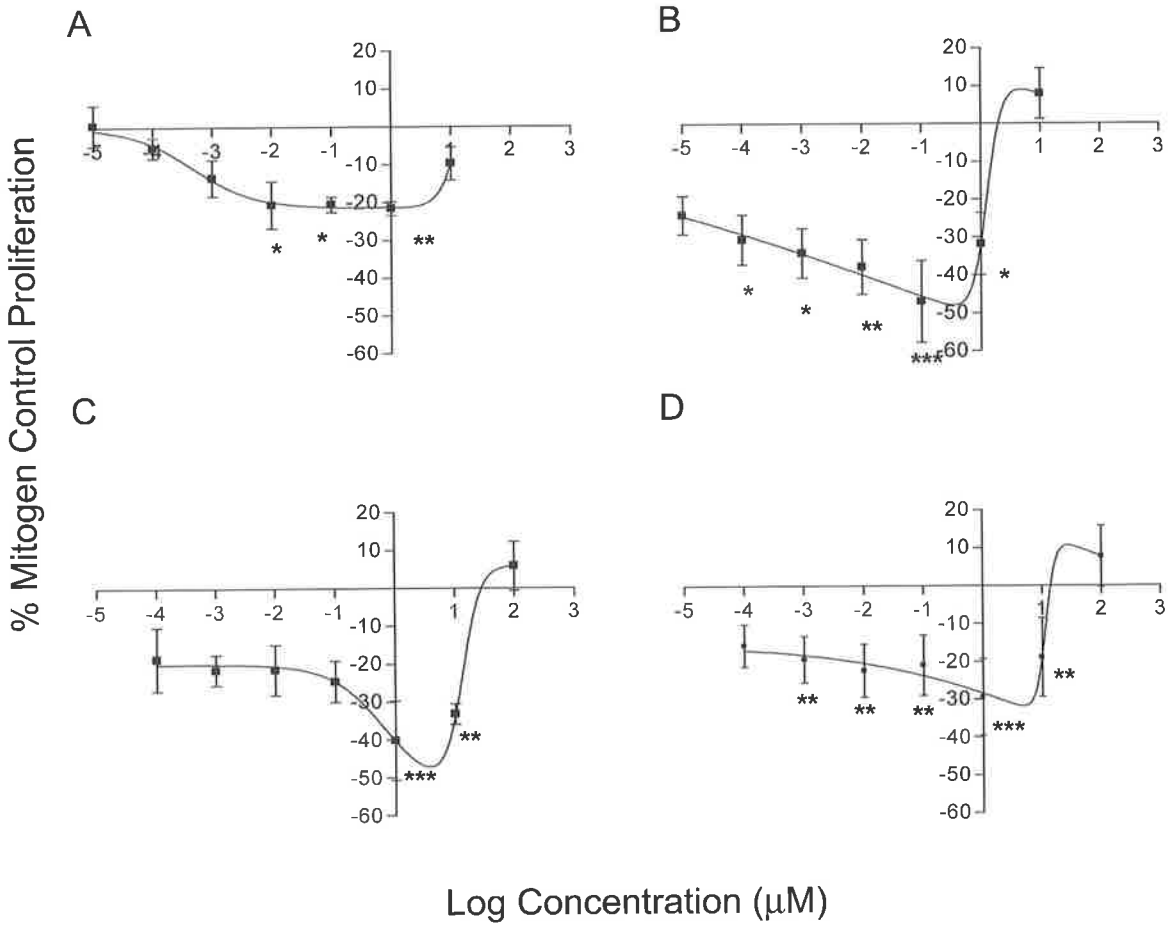


Figure 4.4-2 Group 2: non-4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

DAMGO (A), fentanyl (B), alfentanil (C) and remifentanil (D). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

3. *Induction and inhibition response:* Exposure to tramadol only (Figure 4.4-3) caused significant inhibition of proliferation at the lowest (0.0001 μM, $P = 0.003$) and induction of proliferation at the highest concentration (100 μM, $P = 0.02$) used (Table 4.4-1). The sum of two Hill equations was fitted to these data.

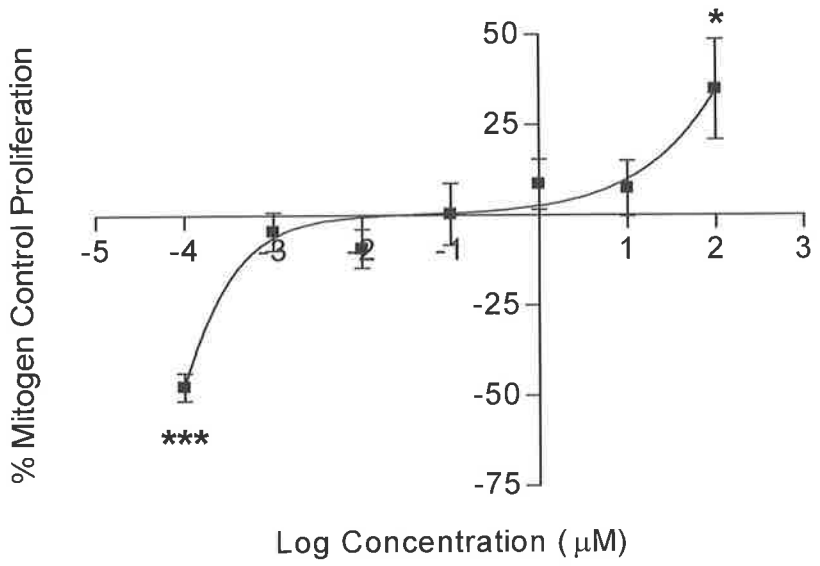


Figure 4.4-3 Tramadol modulation of splenocyte proliferation following 24 hour incubation.

Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

4. *Induction dose response curve:* U69593 (Figure 4.4-4 A), levorphanol (Figure 4.4-4 B), nor-binaltorphimine (Figure 4.4-4 C) and DPDPE (Figure 4.4-4 D) caused significant induction of proliferation at the higher concentrations ($P < 0.0151$) with U69595 and levorphanol having the maximal effect at 100 μM (51%, $P = 0.0008$). A Hill equation with a positive slope was fitted to these data. Dextrorphan caused significant ($P = 0.02$, 33%) induction of proliferation at 0.05 μM and returned to non-significant levels of proliferation at 100 μM. Dextrorphan required the sum of two Hill equations to fit the data.

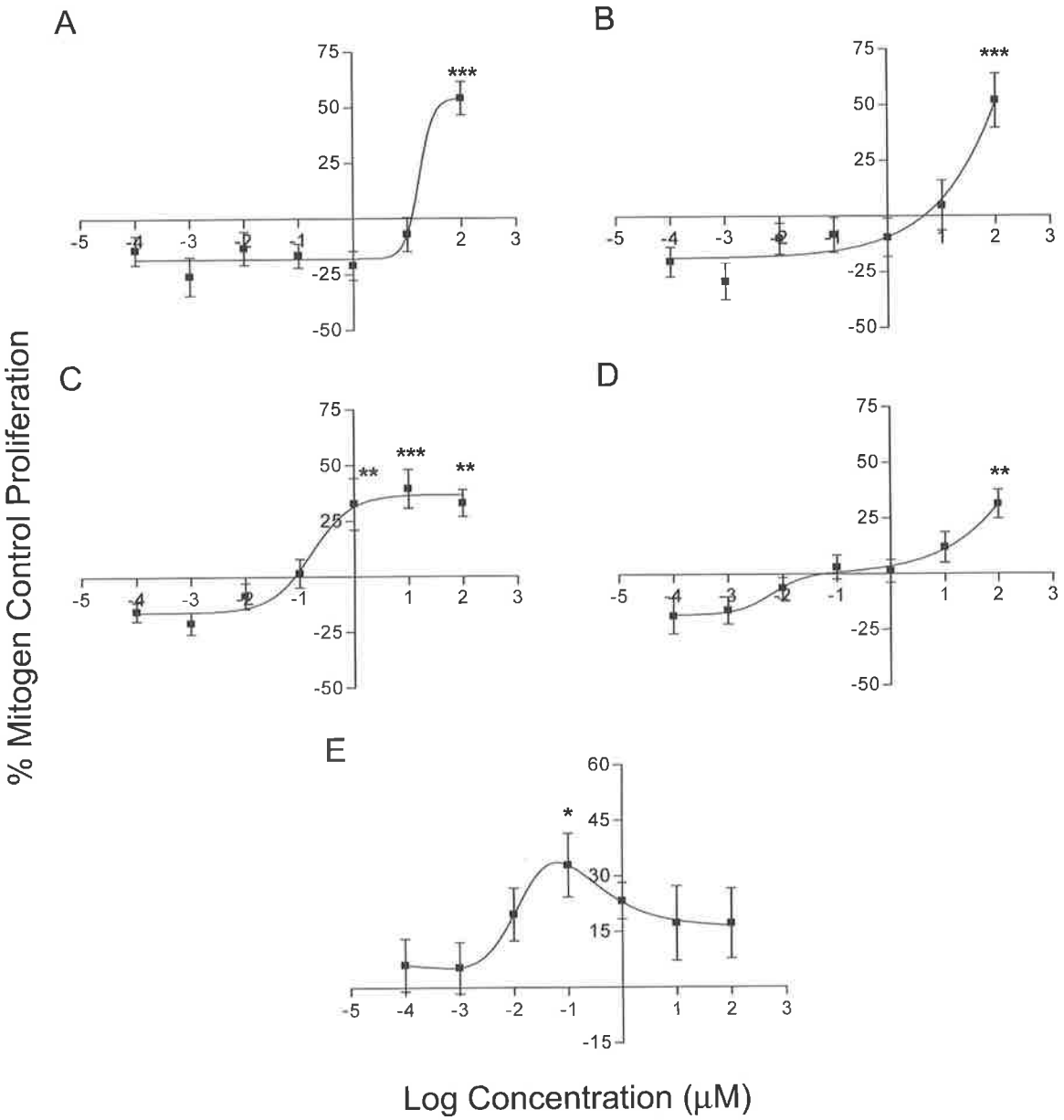


Figure 4.4-4 Group 4: non-4,5-epoxymorphinan modulation of splenocyte proliferation following 24 hour incubation.

U69593 (A), levorphanol (B), nor-BNI (C), DPDPE (D) and dextrorphan (E). Error bars are S.E.M. of 6 independent experiments and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Comparison of rank orders:

1. *Inhibition (downward):* The rank order of mean maximal inhibitory effect was naltrindole > naloxonazine > LAAM > dextromethorphan = (S)-(+)-methadone > (R)-(-)-methadone > racemic methadone > fentanyl > alfentanil > remifentanil > DAMGO.

However, the rank order of the EC_{50} values of the response was DAMGO < fentanyl < alfentanil < LAAM < naloxonazine < remifentanil < (S)-(+)-methadone = (R)-(-)-methadone < racemic methadone = naltrindole = dextromethorphan (Table 4.4-2 and Table 4.5-1).

2. *Induction (upward):* The rank order for the maximal stimulatory effect was U69593 = levorphanol > nor-binaltorphimine > dextrorphan > DPDPE. However, the rank order of EC_{50} values of the response was dextrorphan < nor-binaltorphimine < fentanyl < remifentanil < alfentanil < U69593 = DAMGO < DPDPE < levorphanol (Table 4.4-2 and Table 4.5-1).

Table 4.4-1 Characterisation of the immunomodulatory effects of synthetic opioid compounds.

	Compound	Downward Response			Upward Response		
		Down EC_{50}	Max effect	Conc	Up EC_{50}	Max effect	Conc
1	Naltrindole	79.4 μM	-511%	100 μM			
	Naloxonazine	15.8 μM	-394%	100 μM			
	LAAM	12.6 μM	-187%	100 μM			
	Dextromethorphan	79.4 μM	-117%	100 μM			
	(S)-(+)-Methadone	63.1 μM	-117%	100 μM			
	(R)-(-)-Methadone	63.1 μM	-105%	100 μM			
	Racemic Methadone	79.4 μM	-97.1%	100 μM			
2	Fentanyl #	0.06 μM	-47.9%	0.35 μM	1.26 μM		
	Alfentanil	0.66 μM	-47.2%	3.8 μM	13.5 μM		
	Remifentanil	31.6 μM	-31.8%	4.7 μM	12.6 μM		
	DAMGO #	0.5 nM	-21.3%	0.72 μM	15.8 μM		
3	Tramadol	0.04 nM	-47.3%	0.01 μM	1.6 mM	34.5%	100 μM
4	U69593				15.9 μM	51.1%	100 μM
	Levorphanol				5 mM	51.1%	100 μM
	Nor-BNI				0.16 μM	36.9%	100 μM
	DPDPE				158 μM	31.3%	100 μM
	Dextrorphan	0.85 μM	5.7%	0.1 nM	0.01 μM	33.0%	0.05 μM

Signifies that the concentration range used was 10 μM to 0.00001 μM due to concentrations of stock supplied. Maximum effect responses and EC_{50} concentrations reported were obtained from the modelled Hill equations.

Antagonists

Induction of proliferation by levorphanol (100 μM) was partially antagonised only by nor-binaltorphimine ($P < 0.05$), whilst fentanyl (0.1 μM) and DAMGO (0.1 μM) inhibition was completely antagonised by naloxonazine ($P < 0.05$) (). Naloxone was unable to antagonise the inhibition caused by 100 μM (R)-(-)- or (S)-(+)-methadone and significantly ($P = 0.0291$) augmented the inhibition of (R)-(-)-methadone at 100 μM (Figure 4.4-5).

Table 4.4-2 Selective opioid receptor subtype antagonist effects on the immunomodulation by different opioids.

	Control	Naltrindole (δ) 1 μM	Naloxonazine (μ) 1μM	Nor-BNI (κ) 0.1 μM
Levorphanol (100 μM)	45.2 #	40.5	40.3	20.5 *
Fentanyl (0.1 μM)	-26.3 #	-22.1	2.8 *	-19.8
DAMGO (0.1 μM)	-28.1 #	-21.0	-0.1 *	-20.1

Data are expressed as the percent of the mitogen control minus one hundred percent. Statistical differences expressed as # $P < 0.05$ in the control column were obtained from comparisons with the mitogen control. All other statistical analyses were compared to the control column using a one-way ANOVA with Bonferroni post hoc. Statistical significance are signified as * $P < 0.05$

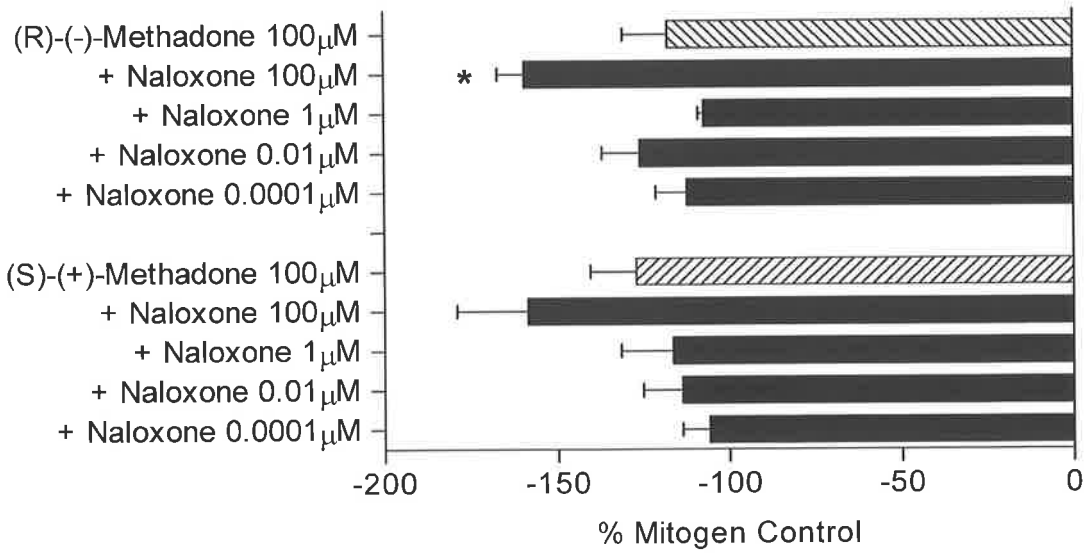


Figure 4.4-5 Lack of effect of naloxone on (R)-(-)- and (S)-(+)-methadone inhibition of concanavalin A-induced splenocyte proliferation.

Error bars are S.E.M. of 6 independent experiments and statistical significance from (R)-(-)- or (S)-(+)-methadone alone is represented as * = $P < 0.05$.

Addition of methadone into the culture media after initiation of proliferation caused significant ($P < 0.001$) suppression of proliferation even when added at the same time as AlamarBlue (time 0) and incubated with the cells while the proliferation was quantified (Figure 4.4-6). Methadone induced inhibition of proliferation increased as the exposure time increased.

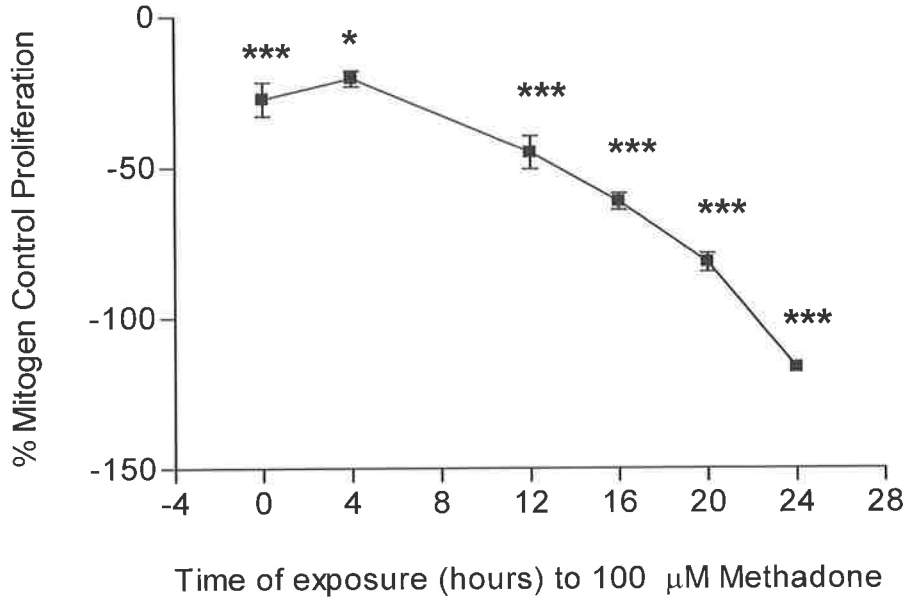


Figure 4.4-6 Influence of exposure time on the suppressive effects of methadone on splenocyte proliferation.

Error bars are S.E.M. and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Chronic *in vitro* methadone exposure caused concentration dependent alterations in sensitivity to mitogen-induced proliferation. Following 48 hours exposure to 0.1 and 1 μM methadone, splenocytes proliferated significantly more ($P < 0.01$) when stimulated with concanavalin A for 24 hours, compared to the untreated controls (Figure 4.4-7 A). However, the response to 100 μM methadone under mitogenic conditions (bottom point in each cluster in Figure 4.4-7 A) was significantly greater ($P < 0.05$) in the cells exposed to 0.1 and 1 μM methadone, when compared to their matched controls (Figure 4.4-7 B).

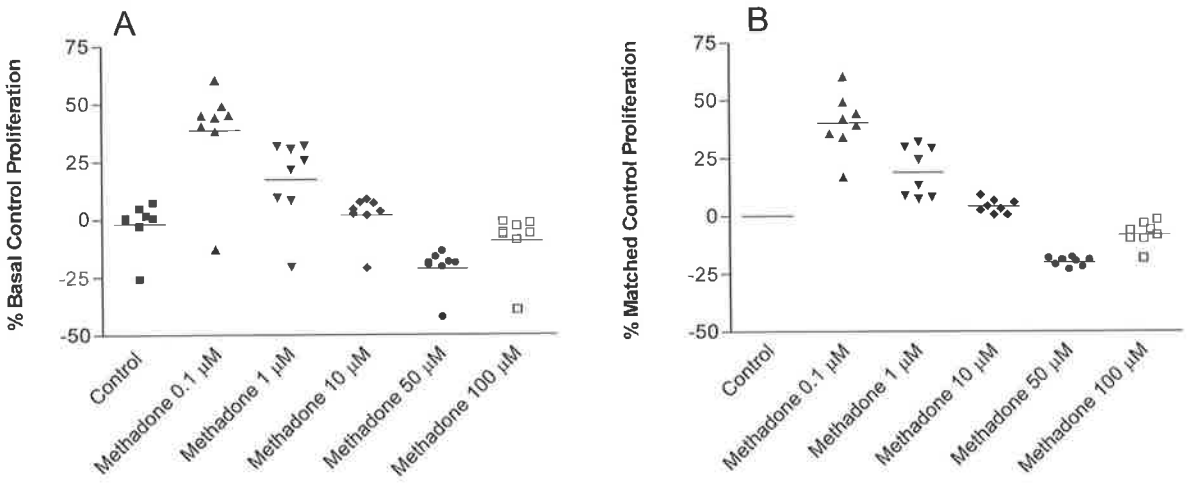


Figure 4.4-7 The influence of chronic *in vitro* methadone exposure on mitogen-stimulated splenocyte proliferation.

Proliferation of splenocytes chronically exposed to various concentrations of methadone (x-axis), followed by mitogen stimulation and treatment with 0.0001 µM to 100 µM methadone. A: Expressed as percent of basal control mitogen-stimulated proliferation minus one hundred percent. B: Expressed as percent of matched methadone concentration control.

4.5. Discussion

The aim of this study was to characterise the *in vitro* immunomodulatory effects of 17 structurally diverse synthetic non-4,5-epoxymorphinan opioids. The use of AlamarBlue to quantify the immunomodulatory effects of non-4,5-epoxymorphinan opioids allowed a short exposure time (24 hour), rapid detection of proliferation (4 hour) and the removal of toxic influences from the experimental model (i.e. the use of AlamarBlue instead of other reagents used in quantification of proliferation)(Ahmed *et al.*, 1994).

The *in vitro* proliferative responses following exposure to opioids were divided into separate groups due to the different concentration-response curves observed, as was also found for the 4,5-epoxymorphinans in Chapter 3. Four types of responses were identified: an inhibition dose response curve (e.g. methadone), an inverted bell-shaped curve (e.g. fentanyl), an

induction and inhibition response (e.g. tramadol) and an induction response curve (e.g. nor-binaltorphimine). The disparity of these responses once again is the first indication here of a non-classical opioid response as observed in Chapter 3. Moreover, the rank order of maximal effect and EC_{50} values differ substantially from available μ opioid receptor binding affinity data (Table 4.5-1). In addition, other classical opioid characteristics were not observed. For example, the μ opioid receptor inactive isomer (S)-(+)-methadone was as potent as the active (R)-(-)-methadone thereby demonstrating non-stereoselectivity (Figure 4.4-1). Furthermore, classical opioid antagonist sensitivities were absent in methadone's response (Figure 4.4-5).

The time course and chronic methadone exposure data provide a number of interesting comparisons. Firstly, the time course of inhibition caused by methadone following mitogenic stimulation is different to the inhibition time course profile that morphine displayed (3.4), in that inhibition occurred immediately following exposure to methadone, whilst morphine inhibition was only evident after 16 hours. This may suggest a different mechanism of action, which is also highlighted by the different response shape and concentration sensitivity. Chronic exposure to methadone, followed by mitogenic stimulation and further methadone exposure, demonstrated that exposure to low methadone doses, achievable *in vivo* caused increased splenocyte proliferation. However, simultaneous mitogenic stimulation and exposure to high methadone concentrations resulted in the same inhibitory response as the controls, irrespective of prior methadone exposure. Therefore, no tolerance developed to the 100 μM methadone response, although perhaps the chronic exposure to low concentrations of methadone caused a dis-inhibition of pro-proliferative mechanism. This result would require further work to tease out a potential mechanism.

Some data are available for the immunomodulatory capacity of the compounds analysed. For example, racemic methadone has previously been shown to inhibit numerous immune cell types and functions in a similar fashion to that reported here, despite the use of a different immunological assay and endpoint quantification methods (Ochshorn *et al.*, 1990; Singh, 1980; Thomas *et al.*, 1995b). The characteristic inhibition curve observed for methadone was also found for naltrindole, naloxonazine, LAAM and dextromethorphan. This type of immunomodulation has been reported previously for naltrindole (Carr *et al.*, 1990; Gaveriaux-Ruff *et al.*, 2001; House *et al.*, 1995b), but not for the other compounds in this group. It would appear that the responses caused by this group of compounds are not classically opioid due to the high concentration at which responses are elicited, the non-stereoselectivity and methadone's opioid antagonist insensitivity. This is substantiated by the report of Gaveriaux-Ruff *et al.* (2001) who used a combined μ, κ and δ opioid receptor-deficient mouse strain and found the characteristic inhibition of the mixed lymphocyte reaction at high naltrindole concentrations, thereby implicating the presence and action via a non-classical opioid receptor(s). Interestingly, we previously reported a similar profile of suppression of proliferation by oripavine, buprenorphine, norbuprenorphine, thebaine and to a lesser extent 3-O-methynaltrexone (Chapter 3). Despite their structural dissimilarities, all these compounds appear to be acting in a similar manner possibly by the same mechanism.

The inverted bell-shaped responses of DAMGO, fentanyl, alfentanil and remifentanil (Figure 4.4-2, Table 4.4-1) are very similar to the responses observed previously for oxycodone, morphine-6-glucuronide, diacetylmorphine, 6-monoactylmorphine, and both stereoisomers of codeine (Chapter 3). Similarly, naloxonazine was able to antagonise the DAMGO and fentanyl response as previously reported in Chapter 3 for morphine, oxycodone and morphine-6-glucuronide. Therefore, this suggests that these compounds act in a very similar manner to the previously described 4,5-epoxymorphinans, despite their structural disparity.

Interestingly, the action of DAMGO and fentanyl eliminates the possible role of the opioid alkaloid selective receptor μ_3 described by Makman *et al.* (1996). It must be noted that despite the opioid antagonist sensitivity of these responses, the rank order of effect of the responses was not comparable to that of the classical opioid response based on the available binding affinity data (Table 4.5-1). Therefore, these data indicate that the inverted bell-shaped responses to these compounds are not mediated via a classical opioid receptor.

Table 4.5-1 Rank order of available μ opioid receptor binding affinities

	Rank orders				
Compound	**Binding affinity**	**Downward maximal effect**	**Downward EC_{50}**	**Upward maximal effect**	**Upward EC_{50}**
DAMGO	1	10	1		6
Levorphanol	2	NR		1	8
(R)-(-)-Methadone	3	5	7	NR	
Racemic Methadone	4	6	8	NR	
Fentanyl	5	7	2		3
Naloxonazine	6	2	5	NR	
LAAM	7	3	4	NR	
Naltrindole	8	1	8	NR	
Alfentanil	9	8	3		5
NorBNI	10	NR		2	2
Remifentanil	11	9	6		4
(S)-(+)-Methadone	12	4	7	NR	
DPDPE	13	NR		4	7
Dextrorphan	14	NR		3	1
U69593	15	NR		1	6
Dextromethorphan	16	4	8	NR	

Gathered from Codd *et al.* (1995), Chen *et al.* (1991), Yeadon and Kitchen (1988), Bonner *et al.* (2000), Metzger *et al.* (2001) and Cox *et al.* (1999) and the rank orders of responses obtained from this study.

The nor-binaltorphimine antagonism of induction of proliferation caused by levorphanol was unexpected as levorphanol is a potent μ opioid receptor ligand, whilst nor-binaltorphimine is a κ opioid receptor antagonist. Our data indicate nor-binaltorphimine and dextrorphan were very effective enhancers of concanavalin A-induced proliferation. If nor-binaltorphimine was

acting as a classical κ opioid receptor antagonist and the immune system was under an inhibitory or an excitatory tone, it would be expected that U69593 would have the opposite effect to nor-binaltorphimine due to its potent κ opioid agonist activity; however this was not the case. Meanwhile, the stereoisomer of levorphanol, dextrorphan which is devoid of opioid activity, caused induction of proliferation at lower concentrations than levorphanol (0.05 μM vs 100 μM, Table 4.4-1). These data confirm the non-opioid nature of these responses and support the non-stereoselective responses of methadone, morphine and codeine (Chapter 3).

In comparison to the group 1 data which agreed with previous literature, earlier reports for compounds in group 2 and 4 are inconsistent, even before the current data were obtained. For example, studies have found DAMGO to cause naloxone sensitive increases (Kowalski, 1998b; Pignatti *et al.*, 1998; Radulescu *et al.*, 1991) and decreases (Eisenstein *et al.*, 1991) in immune responses, whilst others have found no effect (Hucklebridge *et al.*, 1990; Morgan, 1996). Similar disparity in the literature can also be found for fentanyl (Hole, 1984; House *et al.*, 1995a), DPDPE (House *et al.*, 1996; Hucklebridge *et al.*, 1990; Kowalski, 1998b; Morgan, 1996; Shahabi & Sharp, 1995b) and U69593 (Pignatti *et al.*, 1998; Rogers *et al.*, 1991). The differences in these reports could be due to differences in experimental methods and models, thereby making inter-study comparisons difficult. However, by investigating a wide range of opioids of structurally diverse origins using the one experimental model, we have continued and expanded on the findings from our previous study (Chapter 3).

The expression of classical opioid receptor mRNA in immunocompetent cells may also provide an explanation of the different opioid responses achieved. The expression of μ opioid receptor mRNA has not been found in mouse splenocytes (Gaveriaux *et al.*, 1995; Sedqi *et al.*, 1996), whilst there is no consensus for κ opioid receptor mRNA expression (Miller, 1996;

Pampusch *et al.*, 1998a). In contrast, several groups have found δ opioid receptor expression (Gaveriaux *et al.*, 1995; Li *et al.*, 1999; Sharp *et al.*, 1997) although some only found it following mitogen stimulation (Miller, 1996). Therefore, in considering these opioid receptor mRNA expression data, it is not surprising that classical opioid receptor responses were not observed due to the potential absence of expressed μ and κ opioid receptors. Moreover, the potential δ opioid receptor responses did not display robust opioid characteristics, casting doubt on the role of classical opioid receptors in the direct effect of opioids on splenocytes.

In the light of this work and our previous study (Chapter 3) it would appear that the immunomodulation observed following *in vitro* exposure to the vast array of opioids, of both 4,5-epoxymorphinan and non-4,5-epoxymorphinan structure, are not mediated by a classical neuronal opioid receptor. This does not eliminate the possibility that the receptor(s) mediating these responses occur is opioid-like or splice variants of the traditional opioid receptor (McCarthy *et al.*, 2001b) or opioid receptors with significantly altered receptor co-localisation, or opioid receptors altered by their environment (e.g. peripheral ion concentrations, Madden *et al.* (1998)). Significant questions remain unanswered regarding these responses. For instance, it is unclear what receptor(s) is mediating the responses. Secondly, the physiological significance of this uncharacterised receptor(s) and the responses mediated by it are unknown. Finally, the reason why these agonists act in this fashion at this receptor and other similar opioid ligands has yet to be elucidated. Determination of the mRNA sequence and protein structure of these elusive receptors would be critical to our understanding of their site of action and function and regulation. Furthermore, despite naloxone and naloxonazine sensitivity of DAMGO, fentanyl, morphine, oxycodone and morphine-6-glucuronide responses (Chapter 3), these immunomodulations may not be mediated via a classical opioid receptor, due to the lack of a classical opioid rank order of

effect, non-stereoselectivity of the responses and obscure grouping of responses. Therefore, to circumvent these experimental issues of determining opioid involvement, the method suggested by Sibinga *et al.* (1988), utilised *in vivo* by Sacerdote *et al.* (1997b) and *in vitro* in the current study and previously (Chapter 3), should be employed. This method engages the use of various opioid ligands to allow comparison of dose-response curves in combination with the use of non-selective and selective opioid antagonists.

Chapter 5. In vivo animal study

5.1. Introduction

Methadone is a widely used synthetic 3,3-diphenylpropylamine opioid which primarily acts at the μ opioid receptor (Codd *et al.*, 1995; Kristensen *et al.*, 1995). Its most common use is in substitution therapy for opioid dependence (Farrell *et al.*, 1994) but is also being increasingly used in the management of chronic pain (Ayonrinde & Bridge, 2000). Methadone is administered as a racemic mixture of equal amounts of (R)-(-)- and (S)-(+)-enantiomers. Only (R)-(-)-methadone possesses significant μ opioid receptor agonist activity with 10-20 fold higher binding affinity than the (S)-(+)-enantiomer (Codd *et al.*, 1995; Kristensen *et al.*, 1995). It is this stereoselectivity which is characteristic of an opioid response (Sibinga & Goldstein, 1988).

As has been discussed in great detail, opioids can modulate the immune system at a cellular level *in vitro* and indirectly *in vivo* (Carrigan & Lysle, 2001; Fecho *et al.*, 1996b). Considerable debate surrounds the identity and "classical" opioid nature (stereoselectivity, agonist sensitivity and rank order of potency of effect) of the receptor(s) that are responsible for these responses and this has been recently highlighted *in vitro* (Chapters 3 and 4). Several factors must be consistent for a response to be deemed classically opioid, including ligand stereoselectivity, antagonist-, ion-, and toxin-sensitivity (Sibinga & Goldstein, 1988). Previous studies have shown non-stereoselectivity and naloxone insensitivity for methadone's *in vitro* immunomodulatory behaviour using various *in vitro* immunological assays and immune cell sources (Ochshorn *et al.*, 1990; Singh, 1980; Thomas *et al.*, 1995b). However, these responses have only occurred at very high ligand concentrations (10 to 100μM) above the range of concentrations achieved clinically and so have been dismissed as physiologically

insignificant (Ochshorn *et al.*, 1990). In animal models, methadone has also been found to be less inhibitory than morphine in several immune functional assays (de Waal *et al.*, 1998; LeVier *et al.*, 1995; Pacifici *et al.*, 1994). However, the stereoselectivity of the affect of methadone on these responses has not been investigated.

5.2. Aims

The aim of this study was to determine if *in vivo* administration of methadone acts stereoselectively to inhibit concanavalin A-induced splenocyte proliferation *ex vivo*. Also, does *in vivo* administration of methadone act stereoselectively to inhibit concanavalin A-induced splenocyte proliferation *ex vivo*. In contrast to many previously applied models, a rapid non-toxic (Ahmed *et al.*, 1994), sensitive proliferation detection method was used, combined with incubation conditions optimised for the detection of altered immune response by *in vitro* exposure to opioids.

5.3. Materials and Methods

5.3.1. Animals

Ethics approval to conduct the studies was obtained from the University of Adelaide Animal Ethics Committee (ethics number M/54/02). Male Balb/c mice 6-8 weeks old (22 ± 0.5 g) were purchased from Central Animal Supplies (Waite Campus, University of Adelaide, Adelaide, Australia) and used as donors of splenocytes for use in cell culture. Animals were provided with standard rodent feed and water *ad libitum*. Animals were housed in a standard 12 hour light dark cycle (starting at 7am) under a constant room temperature of 22 ± 2 °C (mean ± range). The observer was blinded to all treatments.

5.3.2. Chemicals

RPMI 1640 with HEPES modification and L-glutamine were purchased from Invitrogen (Mulgrave, Vic, Australia). Penicillin-streptomycin solution (10,000 units penicillin, 10 mg streptomycin.ml^{-1}) and concanavalin A were purchased from Sigma (St. Louis, MO, USA). Racemic methadone hydrochloride was obtained from McFarlane Smith (Edinburgh, UK), whilst (R)-(-)- and (S)-(+)-methadone bases were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Foetal calf serum was obtained from Trace Scientific Ltd (Melbourne, Vic, Australia). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality. Compounds that were not supplied as a soluble salt were dissolved in an equimolar solution of hydrochloric acid (0.1 M), while the remaining compounds were dissolved in Milli Q water. The vehicle used for all *in vivo* drug delivery was saline (0.9% NaCl) and all injections were administered intraperitoneally (i.p.) in a volume of 10 ml.kg^{-1}. All solutions were stored at 4 °C until used.

5.3.3. Chronic *in vivo* treatment

Mice (n = 6 for methadone and saline treatments; n = 5 for morphine treatment due to the death of one animal after the first day of treatment) received morphine (20 mg.kg^{-1}), racemic methadone (20 mg.kg^{-1}) or saline twice a day in a 6 day regimen (Table 5.3-1). The analgesic responses to these treatments were then tested 20 min after the afternoon dose by placing the animals on a 50 °C hot plate and recording the time taken (seconds) until any jumping, paw shaking or paw licking was observed. Animals that did not respond in this fashion after 60 seconds were removed from the plate.

Table 5.3-1 Chronic opioid treatment regimen.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Morning		Dose	Dose	Dose	Dose	Dose
Afternoon	Dose & Hot plate	Dose & Hot plate	Dose & Hot plate	Dose & Hot plate	Dose & Hot plate	Splenocyte isolation

5.3.4. Acute *In vivo* treatment

Mice (n = 3 for each treatment) received a single i.p. injection of (R)-(-)- (1.5 $mg.kg^{-1}$), (S)-(+)- (1.5 $mg.kg^{-1}$), racemic methadone (3 $mg.kg^{-1}$) or saline. The analgesic responses to these treatments were then tested 20 min later by placing the animals on a 50 °C hot plate and recording the time taken (seconds) until any jumping, paw shaking or paw licking was observed. Animals that did not respond in this fashion after 60 seconds were removed from the plate.

5.3.5. Lymphocyte preparation

Animals were sacrificed 120 minutes after the final drug administration by cervical dislocation followed by prompt removal of the spleen. The spleen was prepared as a single cell suspension as presented in previous Chapters. In the chronic treatment study the splenocyte preparations were treated with methadone (n = 6; 0 μM control, 0.01 μM, 0.1 μM, 1 μM, 10 μM, 25 μM, 50 μM and 100 μM) and morphine (n = 6; 0 μM control, 0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM). In the acute treatment study splenocyte preparations were treated with (R)-(-)-, (S)-(+)- or racemic methadone (n = 6; 0 μM control, 0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM). Unstimulated mitogen negative control wells were also prepared, using 50 μl of media instead of concanavalin A. The plates were incubated at 37 °C, 5% CO_2 in a humidified incubator

(Thermoline, Sydney, Australia) for 24 hours following which 25 µl of a diluted AlamarBlue solution (5 µl AlamarBlue, 20 µl cell medium) was added to each well. The plates were incubated for a further 4 hours following which 175 µl of media was transferred from the clear 96 multiwell plates to white 96 multiwell plates (BMG Labtechnologies, Offenburg, Germany) for fluorescence quantification on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) (Excitation 545, Emission 590).

5.3.6. Data analysis

The analgesic response was calculated as the percent of maximum possible effect (%MPE) using the following equation: $\%MPE = \frac{\text{test latency - control latency}}{60 - \text{control latency}} \times 100$ (Carmody, 1995).

The control latency (20 ± 3 seconds (n = 6)) was obtained from untreated controls.

The *in vitro* mitogenesis data comprised baseline proliferation from saline treated animals (unstimulated proliferation) subtracted from all data from all treatment groups; the data were then expressed as the percent of the saline treated animals mitogen control minus one hundred percent. All data are expressed as mean ± S.E.M. Statistical significance was assessed using either a one- or two-way analysis of variance with Bonferroni post hoc test. Significance was set at $P < 0.05$.

5.4. Results

High multi-dose regimen

Morphine and methadone administration caused substantially increased latency of hot plate withdrawals. Following 5 days treatment partial tolerance developed to the analgesic effects

of morphine and methadone (Figure 5.4-1). The analgesic effects of methadone and morphine were the same across all days, including the development of tolerance ($P < 0.05$).

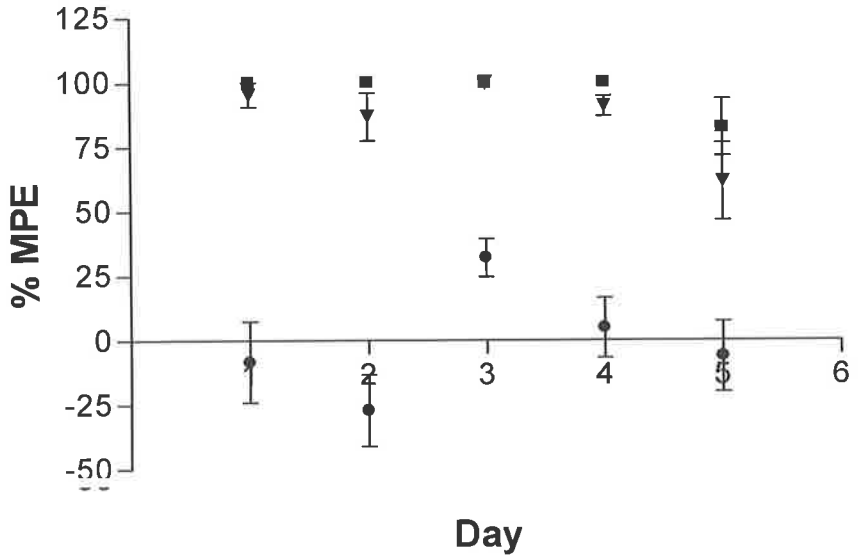


Figure 5.4-1 Hot plate latencies over 6 day treatment regimen of morphine and methadone.

Mice were treated with saline (●), morphine (▼) (20 mg.kg^{-1}) or methadone (■)(20 mg.kg^{-1}). Error bars are S.E.M.

Splenocyte proliferation was significantly ($P < 0.001$) suppressed by *in vivo* morphine and methadone treatment (Figure 5.4-2). The suppressive effects of the opioid treatment were so great that a lower dose and shorter exposure regimen were also conducted.

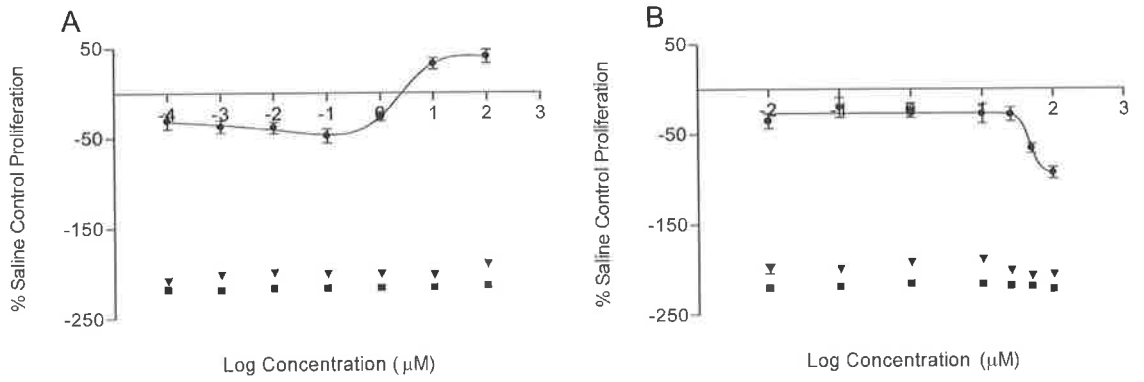


Figure 5.4-2 Splenocyte proliferation following chronic *in vivo* opioid treatment.

In vivo mice were administered saline (●), morphine (▼) (20 mg.kg^{-1}) or methadone (■)(20 mg.kg^{-1}). The proliferative responses of isolated splenocytes from the *in vivo* treated animals to *in vitro* exposure to morphine (A) or methadone (B). Error bars are S.E.M.

Single low dose regimen

Significant increases in hot plate latencies were observed in animals that received a single dose of 3 mg.kg^{-1} racemic methadone (P = 0.0012) and 1.5 mg.kg^{-1} (R)-(-)-methadone (P = 0.0002) when compared to saline treated controls (Figure 5.4-3 A). However, a single dose of (S)-(+)-methadone caused no increase in hot plate latency compared to saline control (P = 0.79), and therefore caused significantly less changes in hot plate latencies than racemic methadone (P = 0.0008) or (R)-(-)-methadone (P = 0.0001) (Figure 5.4-3 A).

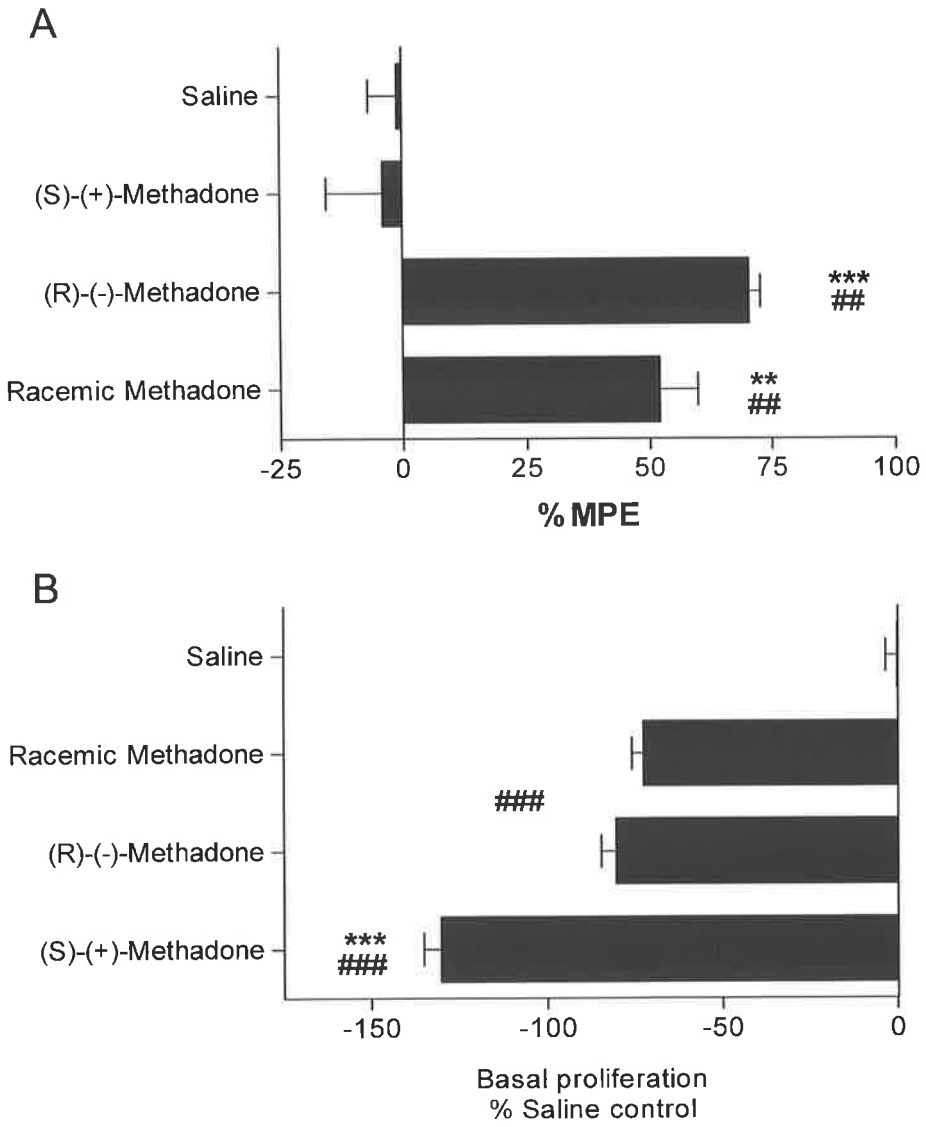


Figure 5.4-3 Analgesic and immunomodulatory effects of acute *in vivo* exposure to racemic methadone and its' enantiomers

A: Hot plate latencies 20 minutes after single injection of saline, (S)-(+)- (1.5 $mg.kg^{-1}$) , (R)-(-)- (1.5 $mg.kg^{-1}$) or racemic methadone (3 $mg.kg^{-1}$). Data represent mean ± S.E.M. ** $P < 0.01$, *** $P < 0.001$ vs (S)-(+)-methadone; ## $P < 0.01$ vs saline. No significant difference between (R)-(-)-methadone and racemic methadone was observed ($P = 0.12$). B: Effect of (S)-(+)- (1.5 $mg.kg^{-1}$), (R)-(-)- (1.5 $mg.kg^{-1}$) and racemic methadone (3 $mg.kg^{-1}$) on *ex vivo* concanavalin A-induced proliferation of splenocytes. Error bars are S.E.M. and ### represents $P < 0.001$ compared to saline control, whilst *** signifies $P < 0.001$ compared to (R)-(-)-methadone and racemic methadone.

The proliferative response of splenocytes isolated from each of the single dose treatment groups were quantified. Basal splenocyte proliferation was significantly suppressed in (R)-(-)-methadone (1.5 $mg.kg^{-1}$) and racemic methadone (3 $mg.kg^{-1}$) treated animals compared to saline controls ($P < 0.001$, Figure 5.4-3 B). However, unlike the hot plate latency data, (S)-

(+)-methadone (1.5 $mg.kg^{-1}$) caused significantly ($P < 0.001$) greater inhibition than (R)-(-)-methadone, racemic methadone and saline treated animals (Figure 5.4-3 B).

Ex vivo methadone treatment resulted in significant ($P > 0.001$) non-stereoselective inhibition of proliferation at 100 μM. The single data point below each cluster in Figure 5.4-4 is the 100 μM response. This *ex vivo* response was independent of *in vivo* treatment (Figure 5.4-4). There were no significant differences between *ex vivo* responses to (R)-(-)-, (S)-(+)- or racemic methadone in animals treated with racemic methadone ($P = 0.22$) or saline ($P = 0.07$) *in vivo* accounting for 0.38% and 0.73% of the variability, respectively as assessed using a two-way ANOVA with Bonferroni post hoc. However, this statistical analysis did detect significant differences between *ex vivo* treatments in (R)-(-)-methadone and (S)-(+)-methadone treated animals, although post hoc analysis showed that these differences were only singular events and therefore were discounted.

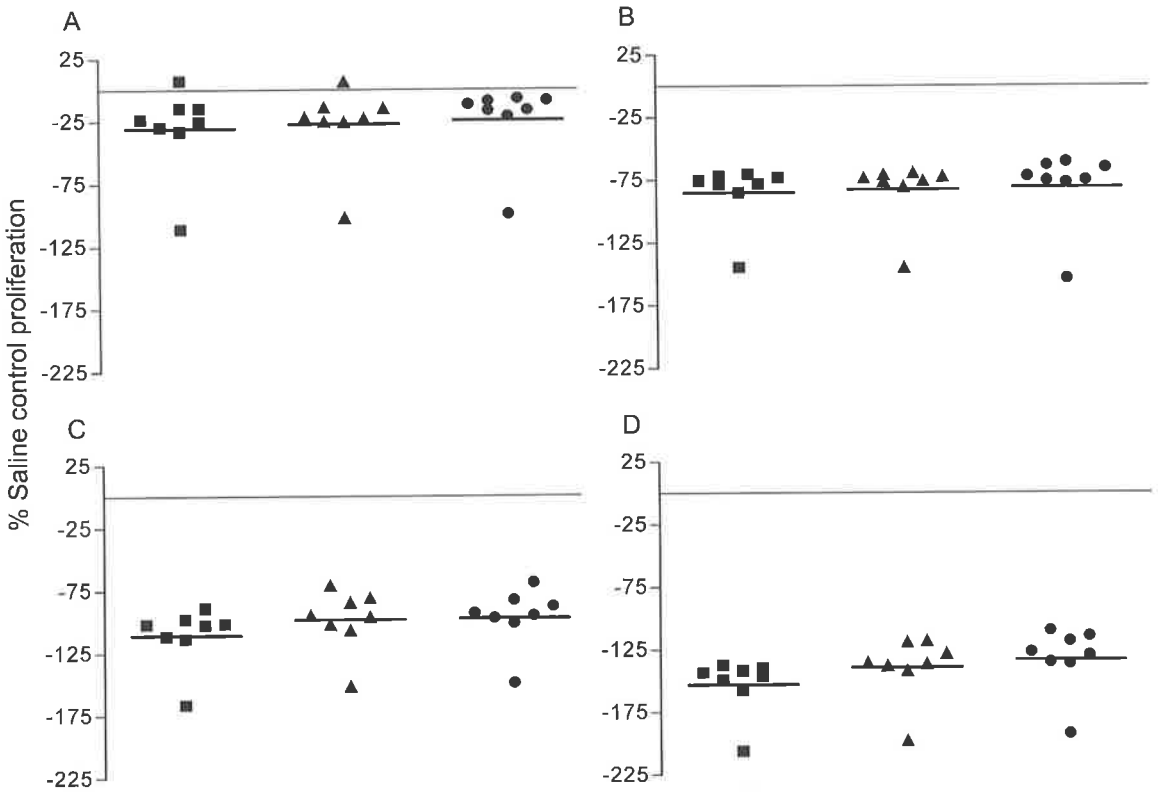


Figure 5.4-4 Comparison of the *ex vivo* proliferative response following *ex vivo* exposure to (R)-(-)-methadone (■), (S)-(+)-methadone (▲) and racemic methadone (●) in splenocytes from animals treated *in vivo* with saline (A), racemic methadone (B), (R)-(-)-methadone (C) and (S)-(+)-methadone (D).

5.5. Discussion

The chronic high dose regimen initially employed produced significant analgesia with partial tolerance developing on the fifth day to the analgesic effects of morphine and methadone. The proliferation of the splenocyte preparations following the chronic opioid treatment was substantially suppressed. However, the immune suppression was so great that *ex vivo* response to opioid exposure could not be assessed. Therefore, the high multi-dose regimen was supplemented with a single low dose regimen.

The acute exposure to racemic methadone and (R)-(-)-methadone caused significant increases in hot plate latencies and hence caused antinociceptive responses in animals 20 minutes after drug administration. In contrast, (S)-(+)-methadone was devoid of any such analgesic response as has been reported previously, due to its lack of μ opioid receptor affinity (Codd *et al.*, 1995; Kristensen *et al.*, 1995). This response of methadone was of a classical μ opioid receptor nature.

In stark contrast to the hot plate data, methadone *in vivo* and *ex vivo* acted non-stereoselectively to inhibit *ex vivo* concanavalin A-induced splenocyte proliferation. Moreover, *in vivo* (S)-(+)-methadone administration caused significantly greater inhibition than racemic or (R)-(-)-methadone. Therefore, despite antinociception being mediated via the neuronal classical μ opioid receptor, the immunomodulatory response appears not to be opioid related, since (S)-(+)-methadone had no antinociceptive effect, but caused substantial inhibition of splenocyte proliferation. This result was unexpected, since the magnitude of the response caused by the racemic dose (3 $mg.kg^{-1}$) was less than the sum of the responses of each enantiomer, indicating a negative "synergistic" interaction between the two enantiomers. Another possibility is that the *in vivo* dose response to methadone is not linear and instead is bell-shaped, such that increased doses of methadone result in less inhibition. Therefore, it appears that (R)-(-)-methadone dampens or eliminates the inhibitory potential of (S)-(+)-methadone.

These responses are likely to be via "indirect" mechanisms, such as activation of the HPA axis or autonomic nervous system, since the concentration of methadone achieved *in vivo* by the doses administered would be in the range where no *in vitro* immunomodulation occurred.

Therefore, this response was mediated via non-classical opioid receptors expressed at some higher centre, not on immune cells. Methadone is not only an opioid receptor agonist as it also possesses considerable antagonistic properties at NMDA receptors, equivalent to that of dextromethorphan (Ebert *et al.*, 1995). Therefore, (R)-(-)-methadone or racemic methadone administration would result in antagonism of NMDA and agonism of opioid receptors, whilst (S)-(+)-methadone administration alone would cause little or no opioid stimulation but with exclusive NMDA antagonism. Therefore, the mechanism for the difference in the responses between each enantiomer and racemic methadone could be explained by the stereoselective agonism and non-stereoselective antagonism of multiple independent receptors. Further research is required to elucidate the mechanisms that mediate this response as it has potential significant clinical relevance for the use of methadone in cancer patients and HIV positive or at risk injecting drug users.

Chapter 6. Immune system and pain control

6.1. Introduction

Pain serves an essential protective role by signalling the presence of tissue damage and often provoking a reflex reaction to prevent further damage, or motivating an individual to seek medical attention. Notwithstanding, pain can be a chronic, debilitating affliction associated with stress, anxiety and depression. The mechanisms involved in the perception of pain are complex and still not well understood. Electrical activity in small diameter primary afferent fibres of peripheral nerves is involved in the perception of noxious stimuli, or nociception. Afferent fibres are classified as A, B, or C-fibres with A-fibres further divided into subgroups α, β, δ and γ. Only the C- and Aδ-fibres carry nociceptive impulses (Markenson, 1996), they enter the spinal cord via the dorsal roots, and terminate in the superficial region of the dorsal horn, generally in laminae I and II. The cells of the substantia gelatinosa in the lamina II are predominantly short inhibitory interneurons, which project to lamina I and V, and regulate transmission between the primary afferent fibres and the spinothalamic tract transmission neurons.

Substantial interindividual variation in both clinical and experimental nociception have long been appreciated (Hardy *et al.*, 1967). Considerable attention has been paid to the impact of a wide range of variables that affect nociceptive response, including sex, age, ethnicity, cognitive factors and genotype (Elmer *et al.*, 1998). The potential importance of other biological factors in the variance observed in nociception (La Vincente *et al.*, 2003) is also gaining increased attention. The experience of pain is subject to modulation by chemical transmitters such as 5-HT, histamine and substance P in the nociceptive pathways. Modulation of nociception also occurs via the descending pathways (Fields & Basbaum, 1994), in which neurotransmitters and opioid peptides (endorphins, enkephalins, and

dynorphins) acting via opioid receptors on neuronal cells play an important role. Moreover, immune-derived signalling molecules including IL-1, IL-6 (Watkins *et al.*, 1995) and IL-8 (Boddeke, 2001) produced during infection, tissue or nerve damage have also been implicated in altered nociception (hyperalgesia) during these insults. Interestingly, exogenous opioids such as morphine, which are the most effective and frequently used class of analgesic drug for the treatment of moderate to severe acute pain (Yaksh, 1997) significantly alter immune function (Sacerdote *et al.*, 2000a).

The link between the immune system, central nervous system and opioids has been intensely researched since Wybran *et al.* (1979) demonstrated morphine could inhibit T cell function in a classical opioid fashion, that is, the response was reversed by naloxone, an opioid receptor antagonist. Opioid receptors are also expressed in the periphery and on some immune tissues (Pampusch *et al.*, 1998a; Pampusch *et al.*, 1998b). However, the action of opioids on these immune cells does not always follow classical opioid behaviour. For example, opioid agonists act non-stereoselectively (Pignatti *et al.*, 1998), display antagonist insensitivity (Roy *et al.*, 1997) and uncharacteristic rank order of effect (Iuvone *et al.*, 1995). Therefore, doubt has been cast over the participation of previously characterised classical opioid receptors, and elusive non-classical opioid receptors in these responses have been implicated. The physiological significance of these receptors is not understood. However, endogenous opioids potentially acting on these receptors, have been postulated to have a regulatory role in immune homeostasis and activation. Controversy also surrounds the relative importance of responses mediated via direct or indirect mechanisms. Specifically, opioids that act centrally at opioid receptors may cause alterations in endocrine pathways, such as the HPA-immune axis (Carr *et al.*, 1995), or the activation of neuron networks that innervate lymphoid tissue (Felten *et al.*, 1984), such as the sympathetic nervous system. Alternatively, opioids may act

directly on the cells of the immune system via receptors expressed on the cell membrane (Madden *et al.*, 2001).

The association between opioids, nociception and the immune system has become evident from studies demonstrating the expression and release of endogenous opioid agonist peptides by activated immune cells during inflammation (Cabot *et al.*, 1997a) and stress (Rittner *et al.*, 2001) and it is these products which are responsible for the analgesia experienced during these altered physiological states. Data have also been gathered that suggest some of the pharmacodynamic effects of opioids may be mediated via the immune system. For example, immune-derived cytokines are integral to the development of opioid tolerance (Raghavendra *et al.*, 2002), and immune status alters the analgesic efficacy of morphine (Kamei *et al.*, 1992) and the severity of opioid withdrawal (Dougherty *et al.*, 1990). These data suggest a substantially expanded role for the immune system than merely recognition of and attack upon external pathogens. It would therefore seem likely that if the immune system is involved in modulating sensitivity to noxious stimuli, that it may also be involved in the regulation of basal nociceptive thresholds. Therefore, we sought to identify whether a relationship exists between peripheral immune function and central pain processes. This relationship was investigated using an experimental pain induction technique, the cold pressor test, and an *ex vivo* immune function assay using isolated immunocompetent cells from healthy subjects. The cold pressor test is widely used in clinical investigations (Doverty *et al.*, 2001), and has been the subject of a recent normative study by our group (unpublished data). The cold pressor test is a tonic pain test, activating slow-conducting C-fibres, and is characterised by dull, aching pain. It is a sensitive test for opioids, including morphine (Jones *et al.*, 1988; Wolff *et al.*, 1966), dipipanone (Posner *et al.*, 1985), and codeine (De Jalon *et al.*, 1985), in which the response can be distinguished from both placebo (Jones *et al.*, 1988; Posner *et al.*, 1985) and other non-opioid analgesics (Jones *et al.*, 1988). However, the

response to this test is subjective (as are all pain responses) and to date there is no biochemical or objective measure of pain response. The immune marker investigated here involved quantification of the proliferation of isolated peripheral blood immune cells in the presence of a mitogen and various concentrations of opioids.

6.2. Aims

The aims of this study were to identify whether there is a relationship between the peripheral immune system and central pain processes and opioid systems in humans using the proliferation of peripheral blood mononuclear cells exposed *ex vivo* to opioids and *in vivo* cold pressor pain tolerance.

6.3. Material and Methods

Ethical approval to conduct these experiments was given by the Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia. Thirteen healthy Caucasian subjects (7 female, 6 male, mean age 26.6 ± 2.3 years) visited the testing centre on two separate occasions (screening session and testing day). Exclusion criteria included tobacco smoking, average alcohol consumption exceeding National Health and Medical Research Council guidelines, current or past history of chronic pain or substance abuse, a positive urine screen for illicit drugs, pregnancy and/or lactation, body mass index (BMI) outside the normal range (23 to 27 $kg.m^{-2}$), and the presence of any medical condition, including heart disease, hypertension, peptic ulcers, any other gastrointestinal disorder, psychiatric disorders, asthma, any other lung disease, Raynaud's Syndrome or any other condition associated with abnormal sensitivity to pain, any neurological disorder, abnormalities of the blood-forming organs, liver function and blood biochemistry abnormalities. Subjects were also excluded if regular use of

a medication or drug (excluding the contraceptive pill) which, for the purpose of this study, constituted more than once per week, and included both medical and recreational use of drugs. Subjects were instructed to refrain from taking any medication or drug (excluding the contraceptive pill) in the 24 hours prior to each testing session.

Using a cold pressor test previously described (Doverty *et al.*, 2001; La Vincente *et al.*, 2003), pain tolerance was assessed on the screening day, on the testing day following venepuncture and after a 30 minutes saline infusion. The saline infusion was part of a protocol for a different study that continued after this study's samples (baseline blood and cold pressor tolerance) were collected. Pain tolerance was quantified as the number of seconds the subject was able to keep their non-dominant forearm immersed in the cold-water bath (0.5-1.5 °C). Subjects were screened for tolerance and excluded from the study if outside the normal range (21 to 85 seconds), as determined by a yet to be published normative study. Subjects had 10 ml of venous blood drawn into EDTA tubes on the testing day prior to any pain stimuli. Peripheral blood mononuclear cells were isolated using Optiprep™ (Axis-Shield PoC AS, Oslo, Norway) as directed by the manufacturer, using the mixer flotation method. Isolated cells were diluted to 1×10^6 cells.ml^{-1} in enriched RPMI 1640 (10% foetal calf serum) and plated into 96 well plates (100 µl per well) followed by the addition of morphine (10^{-4} µM to 10^2 µM), (R)-(-)-, (S)-(+)- and racemic methadone (10^{-3} µM to 10^3 µM) and buprenorphine (10^{-4} µM to 10^2 µM) with a media control and concanavalin A 2.5 µg.ml^{-1} (final volume 200 µl, n = 12 for each concentration). Plates were incubated for 24 hours at 37 °C 5% CO_2 in a humidified environment, followed by the addition of 25 µl of a 1 in 5 AlamarBlue solution and a further 3.5 hours incubation. Media was then transferred (175 µl) to white 96 well plates and fluorescence quantified on a BMG Polarstar microplate (BMG Labtechnologies, Offenburg, Germany) reader (Excitation 545, Emission 590). Data were expressed as percent

of media control minus one hundred percent. A one-way ANOVA with Bonferroni post hoc test was used to determine changes in proliferation from baseline and a Pearson correlation was chosen to determine the relationship between pain tolerance and proliferative response.

6.4. Results

Pain tolerance from the three testing occasions (mean ± SD, range) was 47 ± 23 (22 to 83) seconds at screening, 39 ± 21 (19 to 84) seconds at baseline and 47 ± 28 (21 to 99) seconds post saline with no significant differences between nociceptive levels at each point ($P > 0.10$).

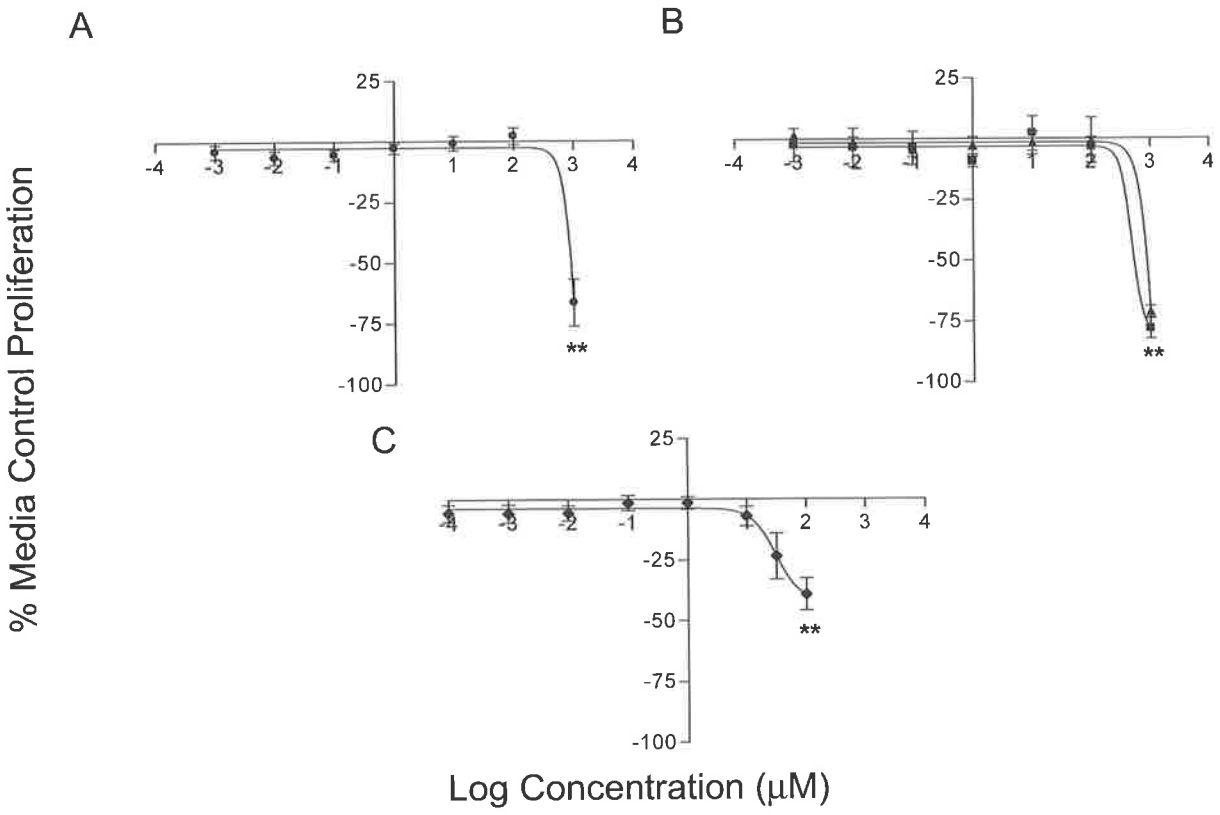


Figure 6.4-1 Ex vivo immunomodulation of human peripheral blood mononuclear cells.

Racemic methadone (A), (R)-(-)-methadone (■) and (S)-(+)-methadone (▲) (B) and buprenorphine (C) responses in 13 healthy subjects. Error bars are S.E.M. and statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Ex vivo exposure to buprenorphine, methadone and its' isomers caused significant inhibition at the highest concentration applied to the system (Figure 6.4-1). *Ex vivo* morphine exposure did not affect the peripheral blood mononuclear cell proliferation at any of the concentrations tested except for 100 μM, which caused a significant ($P < 0.001$) increase in proliferation compared to control (Figure 6.4-2).

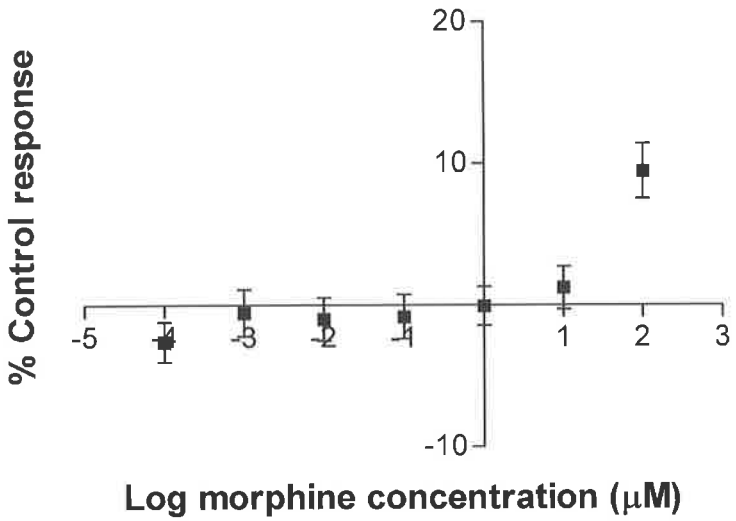


Figure 6.4-2 The mean proliferative response of 13 human peripheral blood mononuclear cells to 24 hours in vitro morphine exposure.

Error bars are S.E.M.

Significant positive correlations were found between the proliferative response to 100 μM morphine and the pain tolerance at each individual testing session (Pearson $r > 0.84$, $P < 0.0004$). In order to include variability from both the cold pressor test and the immunological assay, correlations were also conducted using grouped pain tolerance data, which identified a significant positive relationship (Pearson $r = 0.92$, $P < 0.0001$) (Figure 6.4-3). There was no correlation between pain tolerance and the basal proliferation of the cells or to any concentration of morphine or other opioid agonist applied to the system.

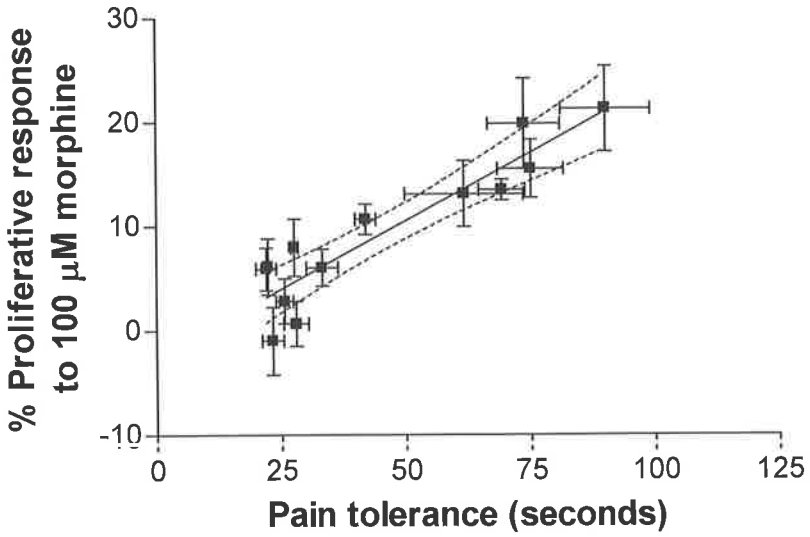


Figure 6.4-3 The correlation between proliferative response of peripheral blood mononuclear cells from 13 healthy subjects following exposure to 100 µM morphine and pain tolerances as assessed by a cold pressor test.

Error bars are S.E.M., Pearson r = 0.92, $P < 0.0001$. Dotted lines are the 95% confidence intervals of the linear regression.

6.5. Discussion

A potential link between the immune system and basal nociceptive thresholds in humans was demonstrated in the current study. The concept that the immune system is involved in controlling the basal nociceptive thresholds has recently been highlighted by Wolf *et al.* (2003) who demonstrated that IL-1 signalling influenced basal nociceptive thresholds in mice. However, our data suggests a mechanism that also incorporates opioids, which has not previously been reported.

The correlation between nociceptive threshold and *ex vivo* proliferative response to 100 µM morphine was very strong (Pearson r = 0.92, $P < 0.0001$, Figure 6.4-3). Given that the increase in proliferation of the isolated immune cells occurred at morphine concentrations that

are not generally observed clinically, it is unlikely that an increase in immune response would occur *in vivo*. However, the mechanism by which morphine induces proliferation appears to be associated with pain tolerance. The physiological, cellular and molecular reasons for these correlations at this point are unclear. Notwithstanding, this is the first objective marker that has been significantly correlated with pain perception. The difference in pain perception was not simply due to a more reactive immune system, as the basal proliferative data do not support this hypothesis. From the methadone and buprenorphine data generated using this model and from the array of opioid ligands applied to the animal model (Chapter 2 and Chapter 3) it is apparent that the induction of proliferation by 100 μM morphine is not mediated via a classical opioid receptor due to non-stereoselectivity, naloxone-insensitivity and uncharacteristic rank order of effect. Therefore, it is crucial that the receptor's identity and characteristics are elucidated and the downstream signalling pathways characterised, as this pathway appears to be fundamental to pain response. Interestingly, the human peripheral blood mononuclear cells required 10 fold higher concentrations of opioid agonist to achieve similar responses compared to those achieved in mouse splenocyte cultures (Chapter 2 and Chapter 3). This is not surprising due to the species and cell type differences, however this should be further investigated to elucidate the cause of this difference.

Variations in response to *in vitro* morphine exposure were also observed with a range of proliferative responses quantified (Figure 6.4-3 y axis). There are numerous physiological conditions which cause altered response to opioids, some of which involve the immune system. For example, in animal models of diabetes (Gul *et al.*, 2000) and immunologically deficient mice (Kimball & Raffa, 1989) morphine has been demonstrated to induce minimal analgesia. Moreover, opioid-dependent immunosuppressed animals display significantly reduced signs of withdrawal compared to control groups with normal immune function (Dougherty *et al.*, 1986a). Specifically, pro-inflammatory cytokines such as IL-1 appear to be

involved in increased opioid tolerance (Gul *et al.*, 2000) and lipopolysaccharide-induced hyperalgesia, whilst anti-inflammatory cytokines such as IL-10 inhibit such responses (Holguin *et al.*, 2003).

These data provide the first evidence of a biological marker for pain perception. Furthermore, the data support our hypothesis that while mechanisms involving the immune system contribute to pain sensitivity, they also appear to be involved in maintaining basal nociceptive thresholds. These mechanisms, which contribute to the central processes of nociception, were quantifiable following *ex vivo* exposure of peripheral blood immune cells to morphine, and variations in their function were measured. These findings warrant further investigation, as they illuminate a new area of pain research and provide new frontiers in the quest for superior pain management.

Chapter 7. Conclusion

Despite opioids being used for thousands of years, many aspects of their physiological effects remain a mystery, including a full understanding of their modulation of the immune system. The discoveries of opioid binding receptors on immune cells and the observation of changes in immune functions following *in vitro* and *in vivo* opioid exposure suggest substantial clinical significance and provide exciting opportunities for future research. Therefore, this research project was undertaken to further our understanding of the fundamentals of opioid immunomodulation in order for these findings to be used as a building block on which future research could be built.

Reports of both classical and non-classical opioid immunomodulation *in vitro* and *in vivo* can be found in the literature with increasing numbers of opioid ligands investigated. However, the number of opioids applied to each individual system is small. Therefore, I developed an *in vitro* mouse splenocyte mitogenesis assay in which the immunomodulation by a large number of opioids could be quantified. The assay is similar to those mitogenesis assays previously reported in the literature, however, there are a few significant and important differences, including the use of AlamarBlue to quantify proliferation and the use of the shortened incubation time. It can be assumed that the concentration of drug applied to the system causes the effect, since the *in vitro* availability of the drug was not affected by binding to plastic or serum. Furthermore, the stability of compounds applied to the system was accounted for, and in the case of diacetylmorphine, it was quantified. The stability of the compound of interest must be considered in future research as rapid degradation of the parent compound to both active and inactive molecules can occur, resulting in incorrect characterisation results since the quantified functional modulation may be due to the presence of an active degrative product.

Using the *in vitro* assay developed, the immunomodulatory capacity of forty-one 4,5-epoxymorphinans (n = 24) and non-4,5-epoxymorphinans (n = 17) was quantified. Several important characteristics were observed. Firstly, 4,5-epoxymorphinan immunomodulation could be divided into five major responses, whilst there were four non-4,5-epoxymorphinan immunomodulation responses: an inverted bell-shaped curve (e.g. oxycodone and fentanyl), a concentration-dependent inhibitory response curve (e.g. buprenorphine and methadone), an inverted bell-shaped curve with induction (e.g. morphine), a concentration-dependent induction response curve (e.g. oxymorphone and nor-binaltorphimine), an induction and inhibition response (e.g. tramadol), and absence of response (e.g. hydrocodone). The magnitude and efficacy of the immunomodulation by the opioids examined demonstrated non-classical opioid rank order of effect, implying mediation via different receptors than opioid receptors previously characterised on neuronal tissue. Low concentration oxycodone- and morphine-like inhibition of proliferation responses were opioid receptor antagonist sensitive. However, induction of proliferation at high morphine concentrations and the pronounced inhibition of proliferation at high concentrations of methadone and buprenorphine was opioid antagonist insensitive. Interestingly, morphine, codeine and methadone responses were non-stereoselective, suggesting non-classical opioid immunomodulation, which agrees with the opioid antagonist insensitivity results. However, the opioid receptor antagonist sensitivity observed for the low concentration inhibition responses does not agree with the obvious non-classical opioid rank order of effect. This emphasises the need to conduct multiple analyses using multiple opioid agonists and antagonists to determine the opioid nature of a response, especially when dealing with opioid modulation of these immune responses, as incorrect assignment of the responsible receptor may occur. Due to the large number of structurally similar 4,5-epoxymorphinans studied, a structure-effect relationship was developed. The analysis indicated that the R_2 functional group at position 6 of the 4,5-

epoxymorphinan structure determines inhibition of proliferation, whilst the R_1 at position 3 predicts induction of the proliferative response. This hypothesised structure-effect relationship is very different to that previously found for the classical μ opioid receptor, once again confirming the general conclusion of non-classical opioid immunomodulation. The differences between the opioid immunomodulation observed and the central opioid effects for the 4,5-epoxymorphinans may be found in the microenvironments in which immune cells operate. It is possible that the varied extracellular conditions that immune cells experience as they migrate through the body or as they take up residence in peripheral tissues, causes their opioid receptors to lose specificity due to altered tertiary structure or receptor co-localisation, resulting in the non-classical opioid responses. Alternatively, cytokines released by immune cells or other receptors expressed by immune cells may interact with opioid receptors and alter their affinity. The presence of splice variants of opioid receptors on immune cells may also explain the diversity of responses observed. Finally, a novel opioid binding receptor may be responsible for the changes in function. It is important that the characteristics of these receptors are further investigated, and the effect they have on opioid pharmacodynamics is elucidated, as the clinical relevance of these sites is likely to be significant.

In contrast to *in vitro* opioid immunomodulation where opioids act directly on the cells, *in vivo* opioid immunomodulation can occur directly via receptors on the immune cells and indirectly where opioids act centrally, triggering immunomodulation by endocrine or neuronal innervation pathways. Interestingly, some non-classical opioid responses *in vivo* have been reported. Therefore, in order to further characterise these *in vivo* responses, racemic methadone and its two enantiomers were administered *in vivo* to mice with a pharmacodynamic response (analgesia) and an immunopharmacodynamic response (splenocyte proliferation) quantified. Methadone was chosen for *in vivo* analysis since the opioid inactive isomer, (S)-(+)-methadone, was equally immunosuppressive *in vitro* and is

commonly administered clinically when methadone is prescribed in its racemate form (a 50/50 mix of the two enantiomers). *In vivo* a classic opioid analgesic response was observed with the inactive enantiomer, (S)-(+)-methadone, producing no analgesia. However, the immunopharmacodynamic effects did not reflect this, with (S)-(+)-methadone causing significant inhibition of immune function. Therefore, the *in vivo* immunopharmacodynamic did not mirror the pharmacodynamic response suggesting non-classical opioid immunomodulation. This immunomodulatory response is likely to be mediated via indirect mechanisms since the concentration of methadone achieved *in vivo* by the doses administered would be in the range where no *in vitro* immunomodulation occurred. Therefore, both *in vitro* and *in vivo* methadone immunomodulation appear to be mediated via non-classical opioid receptors. These sites are yet to be fully characterised and their physiological relevance is largely undefined, making them excellent targets for future research.

Although many mechanisms have been discovered that cause pain, a new molecular theory incorporating the immune system is gaining credence. Immunocompetent cells account for more than 85% of the cells in the brain. These cells are able to modulate pain sensations and decrease the efficiency of analgesic drugs, such as morphine. Therefore, if we could understand the activity status of these immune cells we might be able to predict pain tolerance. Hence, I sought to identify whether this relationship existed between the immune system and pain processes. Spinal and brain immune cells are difficult to access in humans, although the immune cells in the blood circulation are readily available by a simple venepuncture. Therefore, I isolated these immune cells from healthy volunteers and cultured them in the presence of morphine. Pain tolerance of the subjects was also quantified using the cold pressor test on three separate occasions: at screening, baseline and after a saline infusion. The proliferative response of these isolated immune cells to morphine was highly correlated with the pain tolerance of the subjects. Therefore, I have demonstrated, for the first time, an

objective pain tolerance test. The reason why this relationship exists is not clear, although the immune cells in the blood circulation may share characteristics with their spinal and brain counterparts, providing a window into the pain control centres. This discovery has potential to revolutionise pain treatment, as the immune system may become the target for future pain medications. These findings warrant further investigations into the link between the immune system and central nervous system and open the way for optimising the treatment of chronic pain.

Further investigation of opioid immunomodulation is required to fully realise the substantial physiological relevance this relationship may have. The potential clinical relevance of opioid immunomodulation is firstly evident since the populations of patients, such as people facing chronic pain, cancer, opioid dependence and surgery, who are commonly prescribed opioid pharmacotherapies are often immunocompromised or at risk of contracting infections. Therefore, any changes in immune function substantially impact on their clinical outcomes. While the effect opioids have on these infection and immunity aspects of immune function were not the direct focus of this research project, they are nonetheless vital to understand. The second area of clinical significance is in the developing area of neuroimmunopharmacology. This is where certain pharmacodynamic properties of opioids may be mediated or triggered, partially or fully, by the action of opioids on immunocompetent cells. The pharmacodynamic properties of opioids that may be influenced by the immune system include the development of tolerance, dependence and withdrawal. Obviously, a better understanding of these intricate communication pathways would further our comprehension and ability to treat pain and opioid dependence. Specifically, the manner in which opioid compounds change immune function needs to be further characterised both *in vitro* and *in vivo* with careful consideration taken to consider both classical and non-classical opioid action. Furthermore, the exciting possibility that the immune system may influence

the regulation of pain tolerance and the sensitivity and efficacy of opioid analgesic medications needs to be fully investigated. My research conducted using *in vitro* and *in vivo* animal models also needs to be replicated using human immune cells before its physiological significance can be fully realised and the results extrapolated to humans *in vivo*. Moreover, further characterisation of the specific cell types that responded in our mixed cell cultures is required.

Opioid neuroimmunopharmacology is a developing area of research with new discoveries being made using spinal and brain glial cultures. The potential for significant improvements in pain treatment resulting from research embracing these novel concepts, incorporating the immune system, are extremely exciting and could lead to a new era of pain research, pain treatment and analgesic pharmacology, which will be welcome news for pain suffers worldwide as this may lead to the better treatment of pain.

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↔ indicates no response, ↓ indicates inhibition and ↑ indicates an increase in the response was quantified. Responses were sensitive to antagonists if an antagonist is listed and were insensitive if an antagonist is listed and struck through.

8.1. *In vitro* splenocyte function modulated by opioids

Table 8.1-1 Summary of opioid effect on splenocyte calcium entry.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Concanavalin A induced calcium entry	Naltrindole ~~CTAP~~	Sharp *et al.* (1998)
DADLE	↑	Concanavalin A induced calcium entry		Shahabi *et al.* (1996) & Sharp *et al.* (1998)
DAMGO	↑	Unstimulated calcium entry		Molotkovskaya *et al.* (1999)
DAMGO	↓	Concanavalin A induced calcium entry	Naloxone	Molotkovskaya *et al.* (1999)
DAMGO	↑	T cell calcium entry	Naloxone	Radulescu *et al.* (1991)
DAMGO	↔	B cell calcium entry		Radulescu *et al.* (1991)
DAMGO	↔	Calcium entry		Sharp *et al.* (1998)
DSLET	↓	Concanavalin A induced calcium entry	Naloxone	Molotkovskaya *et al.* (1999)
Dynorphin A (1-13)	↑	Calcium entry	Naloxone	Molotkovskaya *et al.* (1999)
Leu-enkephalin	↑	Unstimulated calcium entry		Molotkovskaya *et al.* (1999)
Leu-enkephalin	↓	Concanavalin A induced calcium entry	Naloxone	Molotkovskaya *et al.* (1999)
Morphine	↔	Calcium entry		Sei *et al.* (1991b)
N-acetyl-β-endorphin	↔	Concanavalin A induced calcium entry		Sharp *et al.* (1998)

Table 8.1-2 Summary of the opioid effects on splenocyte cAMP production.

Compound	Effect	End point	Antagonist	Study
Morphine	↔	Splenocyte cAMP levels		Jessop *et al.* (1991a; 1991b)
Nociceptin	↔	Splenocyte cAMP levels		Pampusch *et al.* (2000)
β-endorphin	↓	Splenocyte cAMP levels		Carr *et al.* (1988b)
(-)-pentazocine haloperidol DTG (+)-PPP	↑	Stimulated splenocyte cAMP levels		Carr *et al.* (1992)
(+)-pentazocine	↔	Stimulated splenocyte cAMP levels		Carr *et al.* (1992)

Table 8.1-3 Summary of opioid effects on splenocyte immunoglobulin production.

Compound	Effect	End point	Antagonist	Study
(+)-pentazocine	↓	Lipopolysaccharide induced IgG and IgM		Carr *et al.* (1992)
(+)-pentazocine	↑	Pokeweed mitogen induced IgM production		Carr *et al.* (1992)
β-endorphin	↓	SRBC stimulated PFC response	Naloxone	Lin *et al.* (1994)
DAMGO	↓	Mishell-Dutton culture response	Naloxone Naltrexone	Taub *et al.* (1991)
DPDPE	↔	Splenocyte antibody response		Rogers *et al.* (1991)
Haloperidol	↓	Pokeweed mitogen stimulated IgG and IgM production		Carr *et al.* (1992)
Haloperidol	↑	Lipopolysaccharide stimulated IgM production		Carr *et al.* (1992)
Met-enkephalin	↓	IgM, IgG3 and IgG2a production	Naloxone (high concentrations only)	Das *et al.* (1997)
Met-enkephalin	↔	IgG1 production		Das *et al.* (1997)
Met-enkephalin	↓	High antigen dose induced suppression of PFC	~~Naloxone~~	Rowland *et al.* (1987)

Compound	Effect	End point	Antagonist	Study
Met-enkephalin	⇔	PFC in response to sub-optimal stimulation		Rowland *et al.* (1987)
Met-enkephalin	↓	Strong PFC response		Rowland *et al.* (1989)
Met-enkephalin	↑	PFC response with supra-antigen stimulation		Rowland *et al.* (1989)
Met-enkephalin	↓	SRBC stimulated PFC response		Yang *et al.* (1990)
Morphine	↓	Mishell-Dutton culture response	Naloxone	Pruett *et al.* (1992)
Morphine	↓	Mishell-Dutton culture response	Naloxone Mouse strain with no μ opioid receptors	Taub *et al.* (1991)
Morphine	↓	Splenocyte antibody response	Mouse strain dependent	Rogers *et al.* (1991)
MR 2034	↓	Splenocyte antibody response		Radulovic *et al.* (1995)
Naloxone	↓	Antibody response (IgM production)		Das *et al.* (1997)
Naltrindole	↓	Splenocyte antibody response		Carr *et al.* (1990)
Oxymorphindole	⇔	Splenocyte antibody response		Carr *et al.* (1990)
Pro-enkephalin	⇔	Production of IgM, IgG3		Das *et al.* (1997)
Pro-enkephalin	↑	IgG2a and IgG1 production	Naloxone (high concentrations only)	Das *et al.* (1997)
U50488	↓	Splenocyte antibody response	Naltrexone Naloxone Nor-BNI	Taub *et al.* (1991),
U50488	↓	Splenocyte antibody response		Guan *et al.* (1995)
U50488	↓	Splenocyte antibody response	Naloxone Nor-BNI	Guan *et al.* (1994)
U50488	↓	Splenocyte antibody response	Naloxone Nor-BNI	Rogers *et al.* (1991)
U50488	⇔	Splenocyte antibody response		Pruett *et al.* (1992)
U69593	↓	Splenocyte antibody response	Nor-BNI	Taub *et al.* (1991)
U69593	↓	Splenocyte antibody response		Rogers *et al.* (1991)

Table 8.1-4 Summary of the opioid effects on splenocyte mixed lymphocyte reaction.

Compound	Effect	End point	Antagonist	Study
Naltrindole	↓	Splenocyte MLR		Arakawa *et al.* (1993)
Naltrindole	↓	Splenocyte MLR	~~δ opioid receptor knockout mice~~	Gaveriaux-Ruff *et al.* (2001)
7-benzylidene-7-dehydronaltrexone	↓	Splenocyte MLR	~~δ opioid receptor knockout mice~~	Gaveriaux-Ruff *et al.* (2001)
Naltriben	↓	Splenocyte MLR	~~δ opioid receptor knockout mice~~	Gaveriaux-Ruff *et al.* (2001)

Table 8.1-5 Summary of opioid effects on splenocyte cytotoxic T lymphocyte production.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Generation of cytotoxic T lymphocytes	Naloxone	Carr *et al.* (1986)
Diacetylmorphine	↓	Generation of cytotoxic T lymphocytes		Thomas *et al.* (1995b)
Fentanyl	↓	Generation of cytotoxic T lymphocytes	~~Naltrexone~~	House *et al.* (1995)
Meperidine	↓	Generation of cytotoxic T lymphocytes	~~Naltrexone~~	House *et al.* (1995)
Met-enkephalin	↑	Generation of cytotoxic T lymphocytes	Naloxone	Carr *et al.* (1986)
Methadone	↓	Generation of cytotoxic T lymphocytes		Thomas *et al.* (1995b)

Table 8.1-6 Summary of the effect of opioids on splenocyte phagocytosis.

Compound	Effect	End point	Antagonist	Study
Met-enkephalin (high concentrations)	↓	Splenocyte phagocytosis	α-IFN-γ and α-IL-4 antibodies Naloxone (high concentrations only)	Singh *et al.* (1994)

Compound	Effect	End point	Antagonist	Study
Met-enkephalin (low concentrations)	↑	Splenocyte phagocytosis	α-IFN-γ and α-IL-4 antibodies Naloxone (high concentrations only)	Singh *et al.* (1994)

Table 8.1-7 Summary of opioid effects on splenocyte viability.

Compound	Effect	End point	Antagonist	Study
Fentanyl	⇔	Cell viability		House *et al.* (1995)
Meperidine	⇔	Cell viability		House *et al.* (1995)
Morphine	⇔	Frequency of micronucleated cells		Sawant *et al.* (1995)
Morphine	↓	Cell viability		Luza (1992a)
Naloxone	↓	Cell viability		Jessop *et al.* (1991a)
Naltrexone	↓	Cell viability		Jessop *et al.* (1991a)
U50488	⇔	Cell viability		Taub *et al.* (1991)

Table 8.1-8 Opioid effect on splenocyte protein synthesis.

Compound	Effect	End point	Antagonist	Study
Morphine	⇔	Protein synthesis		Jessop *et al.* (1991a; 1991b)

Table 8.1-9 Opioid effect on splenocyte potassium conductancy.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↓	Potassium conductancy	Naloxone	Carr *et al.* (1988a)

8.2. *In vitro* peripheral blood mononuclear cell function modulated by opioids

Table 8.2-1 Summary of the effects of opioids on oxygen production.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↓	Oxygen and hydrogen peroxide generation	Naloxone	Peterson *et al.* (1987)
β-endorphin	↓	Neutrophil resting and respiratory burst oxygen use		Deitch *et al.* (1988)
Met-enkephalin	↑	Neutrophil respiratory bust oxygen consumption		Deitch *et al.* (1988)
Methadone	↓	Swine peripheral blood mononuclear cell oxygen production	Naloxone	Molitor *et al.* (1992b)
Morphine	↓	Oxygen and hydrogen peroxide generation	Naloxone	Peterson *et al.* (1987)
Morphine	↓	Oxygen production	~~Naloxone~~	Molitor *et al.* (1992a)
Morphine	⇔	Oxygen production		Ruud *et al.* (1988)
Morphine	↑	Neutrophil resting and respiratory burst oxygen consumption		Deitch *et al.* (1988)
Naloxone	↑	Neutrophil respiratory bust oxygen consumption		Deitch *et al.* (1988)
Naloxone	⇔	Swine peripheral blood mononuclear cell oxygen production		Molitor *et al.* (1992b)

Table 8.2-2 Summary of the effects of opioids on the MLR response.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	MLR response		Srinivasan *et al.* (1996)
Morphine-6-glucuronide	↓	MLR response		Srinivasan *et al.* (1996)
Codeine	↓	MLR response		Srinivasan *et al.* (1996)
Codeine-6-glucuronide	↓	MLR response		Srinivasan *et al.* (1996)

Table 8.2-3 Summary of the opioid effects on CTL response.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↓	CTL response		Prete *et al.* (1986)
Dynorphin	↓	CTL response		Prete *et al.* (1986)
Met-enkephalin	↓	CTL response		Prete *et al.* (1986)

Table 8.2-4 Summary of the opioid effects on rosette formation.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↓	Percent of active T rosettes	Naloxone	De Carolis *et al.* (1984)
Dextromoramide	↓	Percent of active T rosettes	Naloxone	Wybran *et al.* (1979)
Leu- & Met-enkephalin	↓	Percent of active T rosettes	Naloxone	De Carolis *et al.* (1984)
Levomoramide	⇔	Percent of active T rosettes		Wybran *et al.* (1979)
Met-enkephalin	↑	Percent of active T rosettes	Naloxone	Wybran *et al.* (1979)
Met-enkephalin	↑	Percent of active T rosettes		Bajpai *et al.* (1997)
Met-enkephalin ($ZnCl_2$ required)	↑	Percent of active T rosettes	~~Naloxone~~ Zinc chelator	Murgo *et al.* (1985)
Morphine	↓	Percent of active T rosettes	Naloxone	Wybran *et al.* (1979)
Morphine	↓	Percent of active T rosettes	Naloxone	De Carolis *et al.* (1984)
Naloxone	⇔	Percent of active T rosettes		De Carolis *et al.* (1984)

Table 8.2-5 Summary of the opioid effects on cell viability.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Cell viability		Luza (1992a)
Morphine	↑	Apoptosis		Nair *et al.* (1997)
Morphine	↑	T cell apoptosis	Naltrexone	Singhal *et al.* (1999)
Met-enkephalin	↑	Apoptosis		Sulowska *et al.* (2002)
β-endorphin	↑	Apoptosis		Ientile *et al.* (1997)
β-endorphin	↑	Apoptosis		Sulowska *et al.* (2002)
β-endorphin	↑	Apoptosis		Prete *et al.* (1986)

Table 8.2-6 Summary of opioid effects on NF-κB binding.

Compound	Effect	End point	Antagonist	Study
Morphine	⇔	Unstimulated NF-κB binding		Welters *et al.* (2000)
Morphine	↓	Lipopolysaccharide stimulated NF-κB binding	Naloxone L-NAME	Welters *et al.* (2000)

Table 8.2-7 Summary of opioid effects on nitric oxide production and cell rounding.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	Cell rounding	Naloxone CB1 antagonist LNAME	Stefano *et al.* (1998)
Morphine	↑	Nitric oxide release		Magazine *et al.* (1996)
Opioid peptides	⇔	Cell rounding		Magazine *et al.* (1996)
Morphine	↑	Membrane fluidity		Stoll-Keller *et al.* (1997)
Met-enkephalin	↑	Changes in neutrophil morphology		Sich *et al.* (1987)

Table 8.2-8 Summary of the opioid effect on iNOS expression.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	iNOS expression	Naloxone	Aymerich *et al.* (1998)

Table 8.2-9 Summary of the effects of opioids on amino peptidase activity.

Compound	Effect	End point	Antagonist	Study
DAMGO	⇔	Aminopeptidase activity		Balog *et al.* (1999)
DPDPE	⇔	Aminopeptidase activity		Balog *et al.* (1999)
Met-enkephalin	↓	Aminopeptidase activity		Balog *et al.* (1999)

Table 8.2-10 Summary of opioid effect on Substance P expression.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	Substance P expression	Naloxone	Li *et al.* (2000)
Morphine	↑	Substance P receptor (NK-1R) expression		Li *et al.* (2000)

Table 8.2-11 Summary of the opioid effects on HIV infection in peripheral blood mononuclear blood cells.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	HIV replication	Naltrexone	Li *et al.* (2002)
Methadone	↑	HIV infection		Li *et al.* (2002)

Table 8.2-12 Summary of opioid effects on phagocytosis.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Polymorphonuclear cell phagocytic activity		Luza (1992b)
Morphine	↑	Monocyte phagocytic activity		Luza (1992b)
Methadone	⇔	Antibody mediated phagocytosis		Molitor *et al.* (1992b)
Methadone	↓	Phagosomelysosome formation		Molitor *et al.* (1992b)
Naloxone (high concentrations)	↓	Antibody mediated phagocytosis		Molitor *et al.* (1992b)
Met-enkephlamide	↑	Release of β-glucuronidase and lysozyme		Sich *et al.* (1987)

Table 8.2-13 Summary of the effect of opioids on immunoglobulin production.

Compound	Effect	End point	Antagonist	Study
β-endorphin (10^{-11}M)	↑	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)
β-endorphin (above & below 10^{-11}M)	↓	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)
α-endorphin	↑	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)
α-endorphin	↓	Ovalbumin-specific IgM-PFC response		Heijnen *et al.* (1986)
ACTH 10^{-11} M	↑	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)
ACTH (10^{-13} M & 10^{-17} M)	↓	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)
DAMGO	↓	SAC-induced IgG-secreting cells	CTAP	Morgan (1996)
DPDPE	↓	SAC-induced IgG-secreting cells	ICI 174864	Morgan (1996)
Met-enkephalin (10^{-9} M)	↑	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)
Met-enkephalin (10^{-17} M & 10^{-7} M)	↓	Tetanus toxoid-stimulated antibody synthesis		Munn *et al.* (1989)

Morphine	⇔	Pokeweed mitogen stimulated IgG synthesis		Martinez *et al.* (1990)
U50488	↓	SAC-induced IgG-secreting cells	Nor-BNI	Morgan (1996)

8.3. *In vitro* influence of opioids on specialised immune cells

Table 8.3-1 Summary of the effects of opioids on specialised immune cells.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Keratinocyte migration	Naltrexone	Bigliardi *et al.* (2002)
β-endorphin	↑	Stimulated IL-1 β and IL-10 release by Langerhans cells		Hosoi *et al.* (1999)
DADLE	↑	Dendritic cell MLR		Makarenkova *et al.* (2001)
DADLE (10^{-7}-10^{-8} M)	↑	Dendritic cell MLR		Esche *et al.* (1999)
DADLE (high and low concentration)	↓	Dendritic cell MLR		Esche *et al.* (1999)
DAMGO	↓	Stimulated superoxide production by peritoneal neutrophils		Azuma *et al.* (2002)
DAMGO	↑	Dendritic cell MLR		Makarenkova *et al.* (2001)
Dynorphin	↑	Keratinocyte Migration		Bigliardi *et al.* (2002)
Dynorphin A	↓	Dendritic cell MLR	Nor-BNI	Kirst *et al.* (2002)
Dynorphin A	⇔	Antigen uptake and phenotypic maturation by Dendritic cells		Kirst *et al.* (2002)
Endomorphin 1	↓	Stimulated superoxide production by peritoneal neutrophils	β-funaltrexamine ~~Naltrindole~~	Azuma *et al.* (2002; 2000)
Endomorphin 1	↑	Un-stimulated superoxide production by peritoneal neutrophils		Azuma *et al.* (2000)
Endomorphin 1	↓	Constitutive peritoneal neutrophils apoptosis		Azuma *et al.* (2002)
Endomorphin 2	↓	Stimulated superoxide production by peritoneal neutrophils	β-funaltrexamine ~~Naltrindole~~	Azuma *et al.* (2002; 2000)
Endomorphin 2	↑	Un-stimulated superoxide production by peritoneal neutrophils		Azuma *et al.* (2000)
Endomorphin 2	↓	Constitutive peritoneal neutrophils apoptosis		Azuma *et al.* (2002)
Methadone	⇔	Alveolar macrophage TGF-β1 mRNA expression		Azuma *et al.* (2002)
Morphine	⇔	Alveolar macrophage TGF-β1 mRNA expression		Azuma *et al.* (2002)
U50488	↓	Dendritic cell MLR	Nor-BNI	Kirst *et al.* (2002)
U50488	⇔	Antigen uptake and phenotypic maturation of Dendritic cells		Kirst *et al.* (2002)

Table 8.3-2 Summary of the effects of opioids on peritoneal macrophage proliferation.

Compound	Effect	End point	Antagonist	Study
U50488 (low concentrations)	↓	Stimulated proliferation	Naloxone	Guan *et al.* (1997)
U50488 (high concentrations only)	↓	Unstimulated proliferation		Guan *et al.* (1997)

Table 8.3-3 Summary of the effects of opioids on peritoneal macrophage cytokine production.

Compound	Effect	End point	Antagonist	Study
CGPM-9	↓	TNF-α production	CTOP	Hicks *et al.* (2001)
CTOP	⇔	TNF-α production		Hicks *et al.* (2001)
DAMGO	↑	Colony-stimulating factors production	Naloxone	Singh *et al.* (2000)
Deltorphin-1	↑	IL-6 production		House *et al.* (1996)

Compound	Effect	End point	Antagonist	Study
Diacetylmorphine	⇔	IL-6 production		Thomas *et al.* (1995b)
DPDPE	↓	Colony-stimulating factors production	Naloxone	Singh *et al.* (2000)
DPDPE	↑	IL-6 production		House *et al.* (1996)
DPDPE-trifluoroacetate	↑	IL-6 production		House *et al.* (1996)
D-TIPP	⇔	IL-6 production		House *et al.* (1997)
Endomorphin-1	↓	TNF-α, IL-10 and IL-12 production		Inui *et al.* (2002)
Endomorphin-1	↑	IL-1β production		Inui *et al.* (2002)
Endomorphin-2	↓	TNF-α, IL-10 and IL-12 production		Azuma *et al.* (2002)
Endomorphin-2	↑	IL-1β production		Azuma *et al.* (2002)
Fentanyl	↓	IL-6 production		House *et al.* (1995)
ICI 174864	↑ (Sporadic)	IL-6 production		House *et al.* (1997)
Leu-enkephalin	↑	Stimulated histamine release	Naloxone	Mediratta *et al.* (1988b)
Pethidine	↓	IL-6 production		House *et al.* (1995)
Met-enkephalin	↑	IL-6 production	~~Naloxone~~	Kowalski *et al.* (2000)
Met-enkephalin	↑	IL-1 production	~~Naloxone~~	Li (1998)
Met-enkephalin	↑	Stimulated IL-1 production		Marotti *et al.* (1994)
Met-enkephalin	↓	Stimulated TNF-α production		Marotti *et al.* (1994)
Met-enkephalin	↑	IL-1 production	~~Naloxone~~	Yang *et al.* (1989)
Met-enkephalin	↑	Stimulated histamine release	Naloxone	Mediratta *et al.* (1988b)
Methadone (Highest concentration)	↓	IL-6 production		Thomas *et al.* (1995b)
Morphine 10^{-4}-10^{-6} M	↓	Colony-stimulating factors production	Naloxone partially blocked	Singh *et al.* (2000)
Morphine 10^{-8}- 10^{-10} M	↑	Colony-stimulating factors production	Naloxone	Singh *et al.* (2000)
Morphine	↑	TGF-β mRNA and release		Singh *et al.* (1996)
Morphine	↑	IL-6 production		Thomas *et al.* (1995a)
Morphine	↑	Stimulated histamine release	Naloxone	Mediratta *et al.* (1988b)
Morphine (μM)	↓	IL-6 and TNF-α production	~~Naloxone~~	Roy *et al.* (1998)
Morphine (nM)	↑	IL-6 and TNF-α production	Naloxone	Roy *et al.* (1998)
Morphine-3-glucuronide	↑	IL-6 production		Thomas *et al.* (1995a)
Morphine-6-glucuronide	↓	IL-6 production		Thomas *et al.* (1995a)
Normorphine	↑ (Non-significant)	IL-6 production		Thomas *et al.* (1995a)
TIPP	⇔	IL-6 production		House *et al.* (1997)
U50488	↓	TNF-α & IL-1 production	Naloxone (partial)	Alicea *et al.* (1996)
U50488	↓	IL-6 production	Nor-BNI	Alicea *et al.* (1996)

Table 8.3-4 Summary of the effects of opioids on peritoneal macrophage phagocytosis and lysis.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	Phagocytosis	~~Naloxone~~	Ichinose *et al.* (1995a; 1995b)
β-endorphin	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
β-endorphin (1-27)	↑	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Dynorphin 1-13	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
Dynorphin A	↑	Phagocytosis	~~Naloxone~~	Ichinose *et al.* (1995a; 1995b)
Dynorphin A	↑	Stimulated phagocytosis	Naloxone	Foster *et al.* (1987)
Dynorphin A (1-13)	↑	Phagocytosis		Ichinose *et al.* (1995a; 1995b)

Compound	Effect	End point	Antagonist	Study
Dynorphin A (13-17)	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Dynorphin A (6-17)	↑	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Dynorphin A (9-17)	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Dynorphin B	↑	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Endomorphin-1	↔	Phagocytosis		Inui *et al.* (2002)
Endomorphin-2	↓	Phagocytosis of opsonized E. coli		Azuma *et al.* (2002)
Endomorphin-2	↔	Phagocytosis of non-opsonized E. coli		Azuma *et al.* (2002)
Leu-enkephalin	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Leu-enkephalin	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
Leu-enkephalinamide	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
Met-enkephalin	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
Met-enkephalin	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
Met-enkephalin	↓ (Slightly)	Phagocytosis		Marotti *et al.* (1994)
Met-enkephalin	↑	Stimulated phagocytosis		Marotti *et al.* (1994)
Met-enkephalinamide	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
Morphine	↓	Phagocytosis	Naltrexone	Rojavin *et al.* (1993)
Morphine	↓	Phagocytosis		Pagan *et al.* (2001)
Morphine	↓	Phagocytosis	Naloxone	Lazaro *et al.* (2000)
Morphine	↓	Phagocytosis		Tomei *et al.* (1997)
Morphine	↓	Phagocytosis	Naloxone	Casellas *et al.* (1991)
Morphine	↓	Phagocytosis	Naloxone	Renaud *et al.* (1996)
α-endorphin	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
α-endorphin (18-31)	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
β-endorphin (28-31)	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
β-endorphin (6-31)	↑	Phagocytosis		Ichinose *et al.* (1995a; 1995b)
γ-endorphin	↔	Phagocytosis		Ichinose *et al.* (1995a; 1995b)

Table 8.3-5 Summary of the effects of opioids on peritoneal macrophage adhesion.

Compound	Effect	End point	Antagonist	Study
Endomorphin-1	↑	Adhesion and expression of adhesion molecule MAC-1		Inui *et al.* (2002)
Endomorphin-2	↑	Adhesion and expression of adhesion molecule MAC-1		Azuma *et al.* (2002)

Table 8.3-6 Summary of the effects of opioids on peritoneal macrophage nitric oxide production.

Compound	Effect	End point	Antagonist	Study
CGPM-9	↓	Stimulated nitric oxide production	CTOP	Hicks *et al.* (2001)
DAMGO	↑	Stimulated nitric oxide production	Naloxone	Kowalski (1998)
DPDPE	↑	Stimulated nitric oxide production	Naloxone	Kowalski (1998)
Met-enkephalin	↑	Stimulated nitric oxide production	Naloxone	Kowalski (1998)
U50488	↑	Stimulated nitric oxide production	Naloxone	Kowalski (1998)
U50488 (high concentration)	↓	Stimulated nitric oxide production	~~Naloxone~~	Kowalski (1998)

Table 8.3-7 Summary of the effects of opioids on Bone marrow.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↓	Colony formation		Loh *et al.* (1993)
Bremazocine	↓	Chemotaxis		Kulkarni-Narla *et al.* (2001)
DAMGO	↓	Chemotaxis		Kulkarni-Narla *et al.* (2001)
DPDPE	↓	Chemotaxis		Kulkarni-Narla *et al.* (2001)
ICI 204488	↓	Chemotaxis		Kulkarni-Narla *et al.* (2001)
Morphine	↓	Killing of phagocytosed bacteria		Bhaskaran *et al.* (2001)
Morphine	↓	Colony formation	~~Naloxone~~	Loh *et al.* (1993)
U50488	↓	Chemotaxis	Naloxone	Kulkarni-Narla *et al.* (2001)
U50488	↓	β2-integrin and Mac-1 expression and adhesion to plastic	Naloxone	Kulkarni-Narla *et al.* (2001)

Table 8.3-8 Summary of the effects of opioids on lymph node lymphocytes.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↑	PGE inhibited proliferation	~~Naloxone~~	Hemmick *et al.* (1991)
β-endorphin	↑	Proliferation		Bidlack *et al.* (1990)
β-endorphin (1-27, human)	↑	Proliferation		Hemmick *et al.* (1990)
β-endorphin (1-31, rat)	↑	Proliferation		Hemmick *et al.* (1990)
HS 378	↓	Stimulated proliferation		Spetea *et al.* (2001)
Met-enkephalin	↑	PGE inhibited proliferation	~~Naloxone~~	Hemmick *et al.* (1991)
Met-enkephalin	↑↓ (Time dependent)	Proliferation	Naloxone	Dubinin *et al.* (1994)
Morphine	↑	Proliferation		Bidlack *et al.* (1990)
Morphine	↓	Proliferation	Naloxone	Wang *et al.* (2001)
Morphine	↓	IL-2 and IFN-γ production	Naloxone	Wang *et al.* (2001)
Morphine	↔	μ opioid receptor knockout lymphocyte proliferation		Wang *et al.* (2001)
Morphine	↔	μ opioid receptor knockout lymphocyte IL-2 and IFN-γ production		Wang *et al.* (2001)
Naloxone	↔	Proliferation		Bidlack *et al.* (1990)
Naltrindole	↓	Stimulated proliferation		Spetea *et al.* (2001)
Nor-BNI	↔	Proliferation		Spetea *et al.* (2001)
U50488	↔	Stimulated IL-2 production		Guan *et al.* (1997)

Table 8.3-9 Summary of the effect of opioids on Basophils and Mast cells.

Compound	Effect	End point	Antagonist	Study
Buprenorphine	↑	Histamine and tryptase release from lung mast cells		Marone *et al.* (1993)
Buprenorphine	↔	Histamine and tryptase release from skin mast cells		Marone *et al.* (1993)
Fentanyl	↔	Histamine and tryptase release		Marone *et al.* (1993)
Leu-enkephalin	↑	Histamine release by peritoneal mast cells		Mediratta *et al.* (1988a)
Morphine	↑	Histamine release by peritoneal mast cells		Mediratta *et al.* (1988a)
Morphine	↑	Tryptase release from skin mast cells		Marone *et al.* (1993)
Morphine	↔	Prostaglandin D2 release from skin mast cells		Marone *et al.* (1993)

8.4. *In vivo* effect of opioid administration on general immune function

Table 8.4-1 Summary of the effects of opioids on the *in vivo* antibody response.

Compound	Effect	End point	Antagonist	Study
(-)-Morphine	↓	Antibody production	~~Naltrexone methobromide~~	Lockwood *et al.* (1996)
(+)-Morphine	↓ (not significant)	Antibody production		Lockwood *et al.* (1996)
2-n-pentyloxy-2-phenyl-4-methyl-morpholine	↓	Antibody responses to SRBC, HGG-FITC and DEX-FITC, LPS antigens		Hadjipetrou-Kourounakis *et al.* (1989)
Buprenorphine	↓	Serum IgA concentrations		Van Loveren *et al.* (1994)
Buprenorphine	↑	Serum IgG concentrations		Van Loveren *et al.* (1994)
Buprenorphine	⇔	Serum IgM and IgE concentrations		Van Loveren *et al.* (1994)
Met-enkephalin	↑	Humoral and cellular immune responses		Jankovic *et al.* (1991)
Methadone	⇔	Antibody response (SRBC)		de Waal *et al.* (1998)
Methadone	↓	IgM antibody response		LeVier *et al.* (1995)
Methadone	↑	IgG concentration		LeVier *et al.* (1995)
Methadone	↑	IgG concentration		van der Laan *et al.* (1995)
Morphine	↓	Antibody response		Virsik *et al.* (1995)
Morphine	↓	Antibody response (SRBC)		de Waal *et al.* (1998)
Morphine	↓	IgM antibody response	Naloxone	Ishikawa *et al.* (1993)
Naloxone	↓	Serum IgG α-KLH antibody titers		Sacerdote *et al.* (2000b)
Naltrexone	↓	Antibody response		Virsik *et al.* (1995)

Table 8.4-2 Summary of the *in vivo* opioid effect on tumour growth.

Compound	Effect	End point	Antagonist	Study
Enkephalin	↑	Survival time following inoculation with tumour cells		Plotnikoff (1982)
Fentanyl	⇔	Survivability of the tumour ladended mouse model		Carr *et al.* (1996)
Methadone	↓	Growth of human lung cancer cells		Maneckjee *et al.* (1992)
Mirfentanil	⇔	Survivability of the tumour ladended mouse model		Carr *et al.* (1996)
Morphine	↑	Tumour growth	Naloxone	Ishikawa *et al.* (1993)
Morphine	↑	Survivability of the tumour ladended mouse model		Carr *et al.* (1996)
OHM3507	↓	Survivability of the tumour ladended mouse model		Carr *et al.* (1996)

Table 8.4-3 Summary of the *in vivo* opioid effects on viral mortality.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Viral induced mortality		Alonzo *et al.* (1999)
Morphine	↑	Replication of Salmonella	Naltrexone	Eisenstein *et al.* (2001)
Morphine	↑	Death rate		Eisenstein *et al.* (2001)
κ agonist	⇔	Death rate		Eisenstein *et al.* (2001)
δ agonist	⇔	Death rate		Eisenstein *et al.* (2001)

Table 8.4-4 Summary of the *in vivo* opioid effects on graft survival.

Compound	Effect	End point	Antagonist	Study
β-endorphin	↓	Graft rejection		Sacerdote *et al.* (1998; 2001)
Leu-enkephalin	↓	Graft rejection		Maric *et al.* (1987)
Met-enkephalin	↓	Graft rejection		Maric *et al.* (1987)
Morphine	↓	Graft rejection	Naloxone Adrenalectomy	Bryant *et al.* (1990)
Naloxone	↑	Graft rejection		Sacerdote *et al.* (2000a)
Naloxone	↑	Graft rejection		Sacerdote *et al.* (1998; 2001)
Naltrindole	↓	Graft rejection		Arakawa *et al.* (1992)

Table 8.4-5 Summary of the *in vivo* opioid effects on cytokine production.

Compound	Effect	End point	Antagonist	Study
2-n-pentyloxy-2-phenyl-4-methyl-morpholine	↓	IL-2 production	Naloxone	Hadjipetrou-Kourounakis *et al.* (1989)
2-n-pentyloxy-2-phenyl-4-methyl-morpholine	↔↓	IL-2 production	Naloxone	Hadjipetrou-Kourounakis *et al.* (1989)
Buprenorphine	↓	TNF-α production		Piersma *et al.* (1999)
Fentanyl	↓	IL-6 production		Shirouzu *et al.* (1998)
Fentanyl	↓	TNF-α production		Piersma *et al.* (1999)
Met-enkephalin	↑	IL-1 production		Yang *et al.* (1989)
Methadone	↓	IFN levels	~~Naloxone~~	Hung *et al.* (1973)
Methadone	↔	IFN levels		Lefkowitz *et al.* (1979)
Morphine	↑	Leukocyte endothelial adhesion		House *et al.* (2001)
Morphine	↓	TGF-β mRNA levels		Peng *et al.* (2001)
Morphine	↓	IFN levels		Geber *et al.* (1977)
Morphine	↓	IFN levels	~~Naloxone~~	Hung *et al.* (1973)
Morphine	↓	IFN levels	~~Nalorphine~~	Lefkowitz *et al.* (1979)
Naloxone	↓	IFN levels		Hung *et al.* (1973)

Table 8.4-6 Summary of the *in vivo* opioid effects on hypersensitivity.

Compound	Effect	End point	Antagonist	Study
Dihydroetorphine	↓	Delayed-type hypersensitivity		Wu *et al.* (1999)
Met-enkephalin (high dose)	↓	Skin hypersensitivity		Veljic *et al.* (1992)
Met-enkephalin (low dose)	↑	Skin hypersensitivity		Veljic *et al.* (1992)
Morphine	↓	Delayed-type hypersensitivity		Pellis *et al.* (1986)
Morphine	↓	Delayed-type hypersensitivity	Naloxone	Hendrickson *et al.* (1989)
Morphine	↓	Delayed-type hypersensitivity	Hypophysectomy	Kasahara *et al.* (1995)
Morphine	↓	Delayed-type hypersensitivity		Virsik *et al.* (1995)
Morphine	↑	Contact hypersensitivity		Nelson *et al.* (2001)
Morphine	↑	iNOS and IL-6 levels		Nelson *et al.* (2001)
Morphine	↔	IL-10 levels		Nelson *et al.* (2001)
Morphine	↓	Delayed-type hypersensitivity	Naltrexone	Bryant *et al.* (1990)
Morphine	↓	Delayed-type hypersensitivity		Schoolov *et al.* (1995)
Morphine	↓	Inflammation induced by 2,4-dintroflurobenozene		Molitor *et al.* (1992a)
Morphine (prior to challenge and sensitisation)	↔	Irritant contact sensitivity reaction		Nelson *et al.* (1999)
Morphine (prior to challenge)	↑	Irritant contact sensitivity reaction		Nelson *et al.* (1999)
MR 2034 (peripheral administration only)	↓	Adjuvant induced arthritis	(Strain dependent)	Antic *et al.* (1996)
Naloxone	↔	Delayed-type hypersensitivity		Hendrickson *et al.* (1989)
Naltrexone	↑	Delayed-type hypersensitivity		Virsik *et al.* (1995)
Tramadol	↓	Edema induced by yeast injection and carrageenin induced inflammation		Bianchi *et al.* (1999)

8.5. *In vivo* effect of opioid administration on splenocyte function

Table 8.5-1 Summary of the *in vivo* opioid effects on splenocyte receptor expression.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Mac-1 expression		Eisenstein *et al.* (1998)
Morphine	↓	MHC-II		Eisenstein *et al.* (1998)
Morphine	↔	Expression levels of surface Ig, CD3, CD4, and CD8		Portoles *et al.* (1995)

Table 8.5-2 Summary of the *in vivo* opioid effects on splenocyte cytotoxic T lymphocyte response.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	CTL activity	Naltrexone	Scott *et al.* (1996)
Morphine	↓	CTL activity	~~BNTX~~ ~~Naltrindole~~	Carr *et al.* (1995)
Morphine	↓	CTL activity	Naltrexone	Carpenter *et al.* (1995)
Morphine	↓	CTL activity	β-funaltrexamine	Carpenter *et al.* (1995)
Morphine	↓	CTL activity		Bhargava *et al.* (1995)

Table 8.5-3 Summary of the *in vivo* opioid effects on splenocyte rosette formation.

Compound	Effect	End point	Antagonist	Study
DSLET	↓	Rosette formation		Cheido *et al.* (1994)
ICI 174864	↑	Rosette formation	Haloperidol	Cheido *et al.* (1994)

Table 8.5-4 Summary of the *in vivo* opioid effects on splenocyte phagocytic activity.

Compound	Effect	End point	Antagonist	Study
Methadone	↓	Phagocytic function		LeVier *et al.* (1995)

Table 8.5-5 Summary of the *in vivo* opioid effects on splenocyte immunoglobulin responses.

Compound	Effect	End point	Antagonist	Study
(-)-Naloxone	↑	IgM production		Carr *et al.* (1991)
(+)-Naloxone	↔	IgM production		Carr *et al.* (1991)
Deltorphin-2	↓	PFC response	Naltriben	Rahim *et al.* (2001)
ICI 174864	↓	PFC response		Jankovic *et al.* (1988)
Leu-enkephalin	↓	PFC response		Jankovic *et al.* (1987)
Leu-enkephalin	↑	PFC response	~~ICI 174867~~ Naltrexone	Jankovic *et al.* (1988)
Leu-enkephalin (high)	↓	PFC response		Jankovic *et al.* (1991)
Leu-enkephalin (low)	↑	PFC response		Jankovic *et al.* (1991)
Met-enkephalin	↑	PFC response		Jankovic *et al.* (1991)
Met-enkephalin	↑	PFC response	~~Adrenalectomy~~	Marotti *et al.* (1992)
Met-enkephalin	↓	PFC response		Jankovic *et al.* (1987)
Met-enkephalin	↑	PFC response	ICI 174867 ~~Naltrexone~~	Jankovic *et al.* (1988)
Morphine	↓	PFC response	CTAP	Rahim *et al.* (2001)
Morphine	↓	Primary antibody response	IL-1 IL-6 IFN-γ ~~IL-2~~ ~~IL-4~~ ~~IL-5~~	Bussiere *et al.* (1992)

Compound	Effect	End point	Antagonist	Study
Morphine	↓	PFC response	μ opioid receptor knockout model	Eisenstein *et al.* (1993)
Morphine	↓	PFC response		Bussiere *et al.* (1993)
MR 2034	↓	PFC response	Nor-BNI Naloxone	Radulovic *et al.* (1995)
Naltrexone	↓	PFC response		Jankovic *et al.* (1988)
U50488	↓	PFC response	Nor-BNI	Rahim *et al.* (2001)

Table 8.5-6 Summary of the *in vivo* opioid effects on splenocyte calcium influx.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Stimulated calcium influx in $CD4^-$ $CD8^-$ cells	Naltrexone ~~Dexamethasone~~	Sei *et al.* (1991a)
Morphine	↓	Stimulated calcium influx in $CD4^+$	~~Naltrexone~~ Adrenalectomy Dexamethasone	Sei *et al.* (1991a; 1991b)
Morphine	↔	Stimulated calcium influx in $CD8^+$	~~Adrenalectomy~~ ~~Dexamethasone~~	Sei *et al.* (1991a; 1991b)
Morphine	↓	Stimulated calcium influx in $CD4^+CD8^+$ cells	~~Adrenalectomy~~	(Sei *et al.*, 1991a)
Morphine	↓	Stimulated calcium influx in B cells	~~Adrenalectomy~~ Naltrexone	(Sei *et al.*, 1991a)
Naltrexone	↔	Stimulated calcium influx		(Sei *et al.*, 1991a)

Table 8.5-7 Summary of the *in vivo* opioid effects on splenocyte nitric oxide production.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	Nitric oxide production		Fecho *et al.* (1994)
Morphine	↑	Nitric oxide production		Fecho *et al.* (1995a; 1995b)
Diacetylmorphine	↓	iNOS expression	Naltrexone	Lysle *et al.* (2000)
Diacetylmorphine	↓	Nitric oxide production	Naltrexone	Lysle *et al.* (2000)
Morphine-6-glucuronide	↓	iNOS expression	Naltrexone	Lysle *et al.* (2001)
Morphine-6-glucuronide	↓	Nitric oxide production	Naltrexone	Lysle *et al.* (2001)
Diacetylmorphine	↓	iNOS expression		Lanier *et al.* (2002)

8.6. *In vivo* effect of opioid administration on peripheral blood mononuclear cells function

Table 8.6-1 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell cytokine production.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	IL-6 levels	Naltrexone Adrenalectomy	Houghtling *et al.* (2000)
Morphine	↑	IL-2 production		Carr *et al.* (1993a)
Morphine	↑	IL-2 production		Palm *et al.* (1998)
Morphine	↑	IL-2 production		Carr *et al.* (1995)

Table 8.6-2 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell receptor expression.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	IL-2 receptor expression		Carr *et al.* (1995)
Morphine	↔	IL-4 and IL-7 receptor expression		Carr *et al.* (1995)
Morphine	↔	Fc receptor expression		Yeager *et al.* (1995)
Methadone	↓	Expression of CD11b/CD18 integrin receptors		Mazzone *et al.* (1994)
Diacetylmorphine	↓	Expression of CD11b/CD18 integrin receptors		Mazzone *et al.* (1994)

Table 8.6-3 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell rolling.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Rolling and sticking	LNAME	Ni *et al.* (2000)

Table 8.6-4 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell superoxide production.

Compound	Effect	End point	Antagonist	Study
Fentanyl	⇔	Superoxide production		Jacobs *et al.* (1999)
Morphine	↓	Superoxide production		Tubaro *et al.* (1985)

Table 8.6-5 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell phagocytosis and cytotoxicity.

Compound	Effect	End point	Antagonist	Study
Diacetylmorphine	↓	Antibody dependent cell cytotoxicity		Nair *et al.* (1986)
Fentanyl	↓	Antibody dependent cell cytotoxicity		Yeager *et al.* (2002)
Fentanyl	⇔	Phagocytic activity		Yeager *et al.* (2002)
Methadone	⇔	Phagocytic activity		Pacifici *et al.* (1994)
Morphine	↓	Antibody dependent cell cytotoxicity		Yeager *et al.* (1992)
Morphine	↓	Antibody dependent cell cytotoxicity		Yeager *et al.* (1995)
Morphine	↓	Phagocytic activity		Tubaro *et al.* (1985)
Morphine (high)	↑	Phagocytic activity		Pacifici *et al.* (1994)
Morphine (low 24 hours later)	↓	Phagocytic activity		Pacifici *et al.* (1994)

Table 8.6-6 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell HIV infectivity.

Compound	Effect	End point	Antagonist	Study
Morphine	⇔	HIV infection		Yeager *et al.* (1992)

Table 8.6-7 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell genetic damage.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	Frequency of micronucleated binuclear splenocytes	Adrenalectomy RU-486 Naloxone	Couch *et al.* (1995)
β-endorphin	↑	Frequency of micronucleated binuclear splenocytes		Couch *et al.* (1995)

Table 8.6-8 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell rosette formation.

Compound	Effect	End point	Antagonist	Study
Naloxone	↑	Opioid addict E-rosette formation		McDonough *et al.* (1980)
Methadone	↑(40% of patients)	E-rosette formation		Cushman *et al.* (1977)
Methadone	↓(27% of patients)	E-rosette formation		Cushman *et al.* (1977)

Table 8.6-9 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell chemotaxis.

Compound	Effect	End point	Antagonist	Study
Methadone	↓	Chemotaxis		Mazzone *et al.* (1994)
Diacetylmorphine	↓	Chemotaxis		Mazzone *et al.* (1994)

Table 8.6-10 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell immunoglobulin production.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	IgG and IgM production		Carr *et al.* (1993a)
Morphine	↑	IgG and IgM production		Carr *et al.* (1993b)
Morphine	↓	Immunoglobulin production		Palm *et al.* (1998)
Morphine	↓	Cholera toxin specific IgA and IgG	Naltrexone	Peng *et al.* (2001)

Table 8.6-11 Summary of the *in vivo* opioid effect on peripheral blood mononuclear cell cAMP production.

Compound	Effect	End point	Antagonist	Study
Morphine	↑	cAMP production		Carr *et al.* (1993a)

8.7. *In vivo* effect of opioid administration on various immune cell type functions

Table 8.7-1 Summary of the *in vivo* opioid effects on glial cell function.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Number of BrdU-labelled $GFAP^+$ cells	Naltrexone	Stiene-Martin *et al.* (2001)
Morphine	↔	Number of BrdU labelled $S100\beta^+$ cells	Naltrexone	Stiene-Martin *et al.* (2001)
Morphine	↑	GFAP immunostaining density in the spinal cord, posterior cingulate cortex and hippocampus		Song *et al.* (2001)
Morphine	↔	GFAP immunostaining density in thalamus		Song *et al.* (2001)
Naltrexone	↑	Number of BrdU cells		Stiene-Martin *et al.* (2001)

Table 8.7-2 Summary of the *in vivo* opioid effect on bone marrow function.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Colony formation	Naloxone	Loh *et al.* (1993)
Morphine	↓	Response to GM-CSF	Naloxone	Roy *et al.* (1991b)
Morphine	↓	Colony formation	Naloxone	Bhaskaran *et al.* (2001)
Morphine	↓	Migration		Patel *et al.* (2003)
Morphine	↑	Apoptosis		Patel *et al.* (2003)
		Stress induced reductions in colony formation	Naloxone Dexamethasone	Malacrida *et al.* (1997)
Morphine	↓	Proliferation	Dynorphin A-(1-13) Dynorphin A-(1-10) amide Naloxone	Roy *et al.* (1991a)
Methadone	↓	Proliferation	Dynorphin A-(1-13) Dynorphin A-(1-10) amide	Roy *et al.* (1991a)

Table 8.7-3 Summary of the *in vivo* opioid effects on peritoneal macrophage function.

Compound	Effect	End point	Antagonist	Study
Met-enkephalin	↑	Superoxide production	α-met-enkephalin antibodies Quaternary naltrexone	Vujic-Redzic *et al.* (2000)
Methadone	⇔	IL-1α and TNF-α levels		Pacifici *et al.* (1993)
Methadone	⇔	Phagocytosis and Killing		Tubaro *et al.* (1987)
Morphine	↑	Apoptosis	α-TGF-β antibodies	Singhal *et al.* (2000)
Morphine	↑	Apoptosis		Patel *et al.* (2003)
Morphine	↑	Bac and p53 expression		Singhal *et al.* (1998)
Morphine	↑	DNA fragmentation and apoptosis	L-NAME	Singhal *et al.* (1998)
Morphine	↑	Expression of IL-12 and TNF-α		Peng *et al.* (2000)
Morphine	↓	IL-1α and TNF-α levels		Pacifici *et al.* (1993)
Morphine	⇔	IL-10		Peng *et al.* (2000)
Morphine	↑	Injury of isolated cells and degradation of host defence	Naloxone	Bhaskaran *et al.* (2001)
Morphine	↓	Macrophage cytokine production (IL-10 and IL-12)	Naltrexone	Limiroli *et al.* (2002)
Morphine	↓	Macrophage migration		Patel *et al.* (2003)
Morphine	↑	Macrophage number in the peritoneal cavity	Naltrexone	Hilburger *et al.* (1997)
Morphine	↑	Macrophage number in the peritoneal cavity		Chang *et al.* (1998)
Morphine	↑	Nitric oxide production	Naloxone	Singhal *et al.* (1998)
Morphine	↓	Phagocytosis and Killing		Tubaro *et al.* (1987)
Morphine	↓	Proliferation	Macrophage depletion Indomethacin	Lu *et al.* (1996)
Morphine	↑	Tumour cell killing		Pacifici *et al.* (1995)
Morphine (24 hours after injection)	↓	Nitric oxide production	Naltrexone	Pacifici *et al.* (1995)
Morphine (immediately after injection)	↑	Nitric oxide production	Naltrexone Dexamethasone L-NAME	Pacifici *et al.* (1995)
SNC 80	↑	TNF-α and nitric oxide production		Gomez-Flores *et al.* (2001)

Table 8.7-4 Summary of the *in vivo* opioid effect on mast cells.

Compound	Effect	End point	Antagonist	Study
Enkephalins	↓	Mast cell degranulation		Maric *et al.* (1987)
Morphine	↓	Mast cell degranulation		Maric *et al.* (1987)

Table 8.7-5 Summary of the *in vivo* opioid effect on lymph node lymphocytes.

Compound	Effect	End point	Antagonist	Study
Buprenorphine	↑	Weight of popliteal and mesenteric lymph nodes		Van Loveren *et al.* (1994)
Methadone	↑	Weights of mesenteric lymph nodes		Van Loveren *et al.* (1995)
Morphine	↑	Weights of mesenteric lymph nodes		Van Loveren *et al.* (1995)
Morphine	⇔	Proliferation and cytokine production		Lysle *et al.* (1993a; 1993b)
Morphine	↓	Proliferation of axillary and inguinal lymph node cells	Naloxone	Ho *et al.* (1979)
Morphine	↓	Lymph node hyperplasia	Naltrexone	Maity *et al.* (1995)
Morphine	↓	Number of $CD4^+$ and Thy 1.2 lymphoid subsets	Naltrexone	Maity *et al.* (1995)

Table 8.7-6 Summary of the *in vivo* opioid effect on lung immune cells.

Compound	Effect	End point	Antagonist	Study
Morphine	↓	Proliferation and cytokine production		Coussons-Read *et al.* (2001)

8.8. Appendix 1 Additional References

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