CNS Immune Signalling And Drug Addiction: The Role Of Interleukin-1 beta

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Doctor of Philosophy

by

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Abstract

Opioid and alcohol dependencies are significant public health problems worldwide. The causes of drug dependence are complex, comprising both genetic and environmental factors. Recent evidence from animal models has shown that opioid-/alcohol-induced proinflammation within the central nervous system (CNS) plays a contributing role in the development of dependence. Proinflammatory cytokines, such as interleukin-1 beta (IL-1β), may be involved in the development of opioid/alcohol dependence. Thus, genetic polymorphisms in the proinflammatory cytokine genes that alter their expression and/or function may alter the risk of dependence in humans. Previously, genetic polymorphisms in two genes, *IL-1B* and *IL-1RN*, which encode for IL-1β and IL-1 receptor antagonist (IL-1Ra), respectively, were shown to be associated with altered risk of alcohol dependence. However, this has yet to be examined in an opioid dependent population. Therefore, the first major aim of this thesis was to examine the possible association between genetic variability of *IL-1B* and *IL-1RN* in opioid dependent and healthy control populations. In order to confirm the previous study within the Australian context, this thesis also examined the variability of the *IL-1B* and *IL-1RN* genetic polymorphisms in an Australian alcohol dependent population. Genomic DNA from opioid dependent subjects (n = 60), alcohol dependent subjects (n = 99), and healthy non-dependent subjects (n = 60) were genotyped for the *IL-1B* -31T>C, -511C>T, and 3954C>T single nucleotide polymorphisms (SNPs) using PCR-RFLP assays, and *IL-1RN* VNTR polymorphism using a PCR assay. Significant linkage disequilibrium was observed between *IL-1B* -31 and -511 SNPs. *IL-1B* -511C and -31T alleles were more frequent in both

the opioid and alcohol dependent patients compared to the control group: odds ratio (OR, 95% confidence interval) = 1.91 (1.14 to 3.20), *P* = 0.014 and 1.89 (1.19 to 2.99), *P* = 0.007, respectively, for *IL-1B* -511C>T; and OR = 1.74 (1.02 to 2.97), *P* = 0.044 and 1.80 (1.13 to 2.87), *P* = 0.017, respectively, for *IL-1B* -31T>C. In contrast, no association was observed between: the *IL-1B* 3954C>T SNP and opioid dependence; and the *IL-1RN* VNTR polymorphism and opioid or alcohol dependence. This study has shown at least two susceptibility loci, -31T and -511C, in the *IL-1B* gene for opioid dependence, and have also confirmed their involvement in alcohol dependence.

With the findings of the significant association between *IL-1B* SNPs and opioid dependence, one important question raised is how drug-induced brain IL-1β expression affects the actions of these drugs, and thereby contributes to dependence. In an attempt to answer this question, I designed my second main study using different strains of mice. The primary aim of this study was to investigate whether the differences in opioid-induced brain IL-1β expression contribute to the differences in opioid tolerance and withdrawal behaviours. In addition, opioid-induced CNS glial activation is reported to occur via non-classical opioid mechanisms, engaging the toll-like receptor 4 (TLR4) and a series of downstream signalling pathways including MyD88-dependent pathways. However, the behavioural consequence of TLR4 and MyD88-dependent signalling pathways in opioid tolerance and withdrawal has yet to be examined. Therefore, this study also aimed to examine the role of TLR4 and MyD88-dependent pathways in opioid actions. Chronic morphine-induced tolerance and dependence were assessed in 3 inbred wild-type mouse strains (Balb/c, CBA, and C57BL/6) and 2 knockout strains (TLR4 and MyD88). Analysis of brain nuclei

(medial prefrontal cortex, cortex, brain stem, hippocampus, and midbrain and diencephalon regions combined) revealed that, of inbred wild-type mice, there were significant main effects of morphine treatment on IL-1β expression in the brain regions analysed ($P < 0.02$ for all regions analysed). A significant increase in hippocampal IL-1β expression was found in C57BL/6 mice after morphine treatment, whilst, a significant decrease was found in the medial prefrontal cortex region of wild-type Balb/c mice. Furthermore, the results of wild-type inbred strains demonstrated that the elevated hippocampal IL-1β expression was associated with withdrawal jumping behavior. Interestingly, knockout of TLR4, but not MyD88 protected against the development of analgesic tolerance. All these data support the involvement of opioid-induced brain IL-1β expression in dependence development.

Together, this thesis provides the first human evidence for the contribution of IL-1β release in opioid dependence, and animal data supporting the involvement of CNS immune signalling in the development of opioid dependence, and confirmed the *IL-1B* genetic findings in alcohol dependence.

Declaration

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Abbreviations

5-HT	Serotonin
AD_{50}	Half-maximal morphine analgesic dose
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ATP	Adenosine triphosphate
AV411	Ibudilast
bp	Base pair
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CPP	Conditioned place preference
CSF	Cerebrospinal fluid
CYPs	Cytochrome p450 family enzymes
DA	Dopamine
DβH	Dopamine β hydroxylase
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual of mental disorders, fourth edition
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase

GABA	γ-aminobutyric acid
$GABA_A$	GABA receptor type A
$GABA_B$	GABA receptor type A
GFAP	Glial fibrillary acidic protein
GWAS	Genome-wide association studies
IFN	Interferon
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-1B	Interleukin-1 beta gene
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IL-1RN	IL-1 receptor antagonist gene
iNOS	Inducible nitric-oxide synthase
IRF3	Interferon-regulatory factor
IRAK	IL-1R-associated kinase
LPS	Lipopolysaccharide
M-3-G	Morphine-3-glucuronide
M-6-G	Morphine-6-glucuronide
MAP	Mitogen-activated protein
MAPK	p38 mitogen-activated protein kinase
% MPE	Maximum possible analgesic effect
MyD88	Myeloid differentiation primary-response protein 88
NF-κB	Nuclear factor-κB

NMDA	N-methyl-D-aspartate glutamate receptor
OR	Odds ratio
mRNA	Messenger Ribonucleic acid
SEM	The standard error of the mean
siRNA	Small interfering RNA
SNPs	Single nucleotide polymorphisms
sTNFR	Soluble TNF receptor
TRIF	Toll/IL-1 receptor-domain-containing adapter-inducing interferon-β
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR4 KO	TLR4 knockout
TNF-α	Tumour necrosis factor alpha
UGTs	Uridine Diphosphate Glucuronosyltransferases
VNTRs	Variable number of tandem repeats

Chapter 1 Introduction

Drug dependence is a chronic, relapsing disorder that is characterised by compulsive drug craving, seeking, and drug consumption that persists even in the face of negative consequences. Drug dependence is associated with serious medical, legal, social, and psychiatric problems, and, as a result, it imposes large economic and social costs (UNDOC, 2010).

The causes of drug dependence are complex and include environmental and behavioural factors. However, not all individuals who experiment with a drug will become dependent on it, suggesting that genetic components also play a significant role. Unlike diseases that are caused by single gene mutations, vulnerability to drug dependence undoubtedly has a more complicated genetic basis. Previous genetic research based on current knowledge of drug pharmacology has provided positive information. However, other genetic traits responsible for drug dependence are still mostly undetermined. Identification of dependence-related genes will uncover information and mechanisms that may be exploited to improve and personalise the prevention and treatment of drug dependence (Khokhar et al., 2010).

Recent evidence has suggested that the immune-like-cells of the brain, glia, may modulate neuronal responses to drugs of dependence (Hutchinson et al., 2007). Drug-induced glial activation and release of proinflammatory cytokines, specifically, interleukin-1 (IL-1), have the potential to influence drug-induced reward, tolerance, and withdrawal behaviours (Hutchinson et al., 2009, Narita et al., 2006). As such, the

polymorphisms of *IL-1* genes, including *IL-1 beta* gene (*IL-1B*) and *IL-1 receptor antagonist* gene (*IL-1RN*), which encode for IL-1 beta (IL-1β) and IL-1 receptor antagonist (IL-1Ra), respectively, provide potential sources for the heritable risk of drug dependence (Saiz et al., 2009, Watkins et al., 2009).

As opioid and alcohol dependence are the focus of this thesis, this chapter commences with a general overview of opioid and alcohol dependence, and the classical/neuronal mechanisms.

1.1 Opioid and Alcohol Dependence

1.1.1 Opioid dependence

Opioid dependence is a significant public health problem. The United Nations estimated that approximately 16.5 million people abuse illicit opioids worldwide (UNDOC, 2010). In Australia, over 44 000 of the population above 14 years of age are estimated to be regular opioid users (AIHW, 2008).

1.1.1.1 Opioid pharmacology

Opiates are the drugs derived from opium and refer to all the naturally occurring and semisynthetic compounds, including morphine, codeine and their congeners (e.g. heroin). Opioids, on the other hand, refer to a more general class of drugs and consist of all natural and synthetic compounds with morphine-like activity, such as pethidine and methadone (Mattick et al., 2009, Somogyi et al., 2007).

The effects of opioids are mediated through the endogenous opioid system. There are three major classes of opioid receptors, mu (μ), delta (δ) and kappa (κ), located throughout the central nervous system (CNS), peripheral nerve terminals and many other organs (Dhawan et al., 1996, Waldhoer et al., 2004). Cloning has established that μ, δ and κ opioid receptors belong to the super family of seven transmembrane G-protein coupled receptors, which mediate the main pharmacological effects of opioids, as summarised in Table 1 (Narita et al., 2001, Raynor et al., 1994, Trescot et al., 2008). Opioids primarily interact with the μ opioid receptor, and therefore this is considered the most important receptor subtype for opioid dependence (Reisine, 1995).

Opioids produce analgesia by actions at several levels of the nervous system, in particular, presynaptic inhibition of neurotransmitter release from the primary afferent terminals in the spinal cord and postsynaptic activation of ascending and descending inhibitory controls. The rewarding effects of opioids are the most important factor for causing dependence, as it generally leads to repeated administration, resulting in neuronal adaptations and the development of tolerance and physical dependence. Reward is mainly mediated by μ opioid receptor signalling in the brain reward circuits, which inhibits γ-aminobutyric acid (GABA) signalling, resulting in disinhibition of the mesolimbic dopamine pathway and increased dopamine release in the nucleus accumbens, hippocampus, and prefrontal cortex, leading to sensations of reward and euphoria (Hyman et al., 2006, Johnson and North, 1992).

Table 1 Functional actions associated with the main types of opioid receptors (Summarised from Dhawan et al. 1996)

Receptors	Mu (μ)	Delta (δ)	Kappa (κ)
Pharmacological effects	• Analgesia • Respiratory depression • Pupil constriction • Sedation • Decreased gastrointestinal mobility • Euphoria • Physical dependence	• Spinal analgesia • Respiratory depression • Decreased gastrointestinal mobility	• Analgesia • Pupil constriction • Decreased gastrointestinal mobility • Sedation • Dysphoria • Diuresis • Physical dependence

1.1.1.2 Opioid tolerance and dependence

Repeated use of opioid drugs results in the adaptation of the cells and neuronal systems that are involved in opioid action. It is manifested by several core features, including tolerance, physical dependence (withdrawal), and psychological dependence (addiction).

Tolerance is defined as a loss of drug potency over time, which results in an increase of dose requirement to maintain the same magnitude of effect (Collett, 1998). Tolerance extends to most of the morphine pharmacological effects, including

analgesia, euphoria and respiratory depression, but does not develop to constipation or pupil constriction (Trescot et al., 2008). Tolerance is a general phenomenon of opioid receptor ligands, irrespective of which type of receptor they act on (Narita et al., 2001).

Physical dependence is manifested by a withdrawal syndrome that is typically observed after the cessation of drug administration or precipitation with an antagonist such as naloxone, and then followed by compulsive drug seeking and taking behaviour (psychological dependence) (van Ree et al., 1999). In laboratory animals, abrupt withdrawal of morphine after chronic administration for a few days caused increased irritability, loss of weight and a variety of abnormal behaviour patterns, such as body shakes, writhing, jumping and signs of aggression (El-Kadi and Sharif, 1994, Way et al., 1969). Humans often experience an abstinence syndrome when opioids are withdrawn after being repeatedly used, even for days or weeks, with symptoms of restlessness, runny nose, diarrhoea, shivering and piloerection; however, the intensity of the abstinence syndrome varies greatly between people (Johnson, 1997).

1.1.1.3 Opioid dependence pharmacotherapy

There are two general treatment paths for opioid dependence, opioid maintenance treatment and detoxification (Stotts et al., 2009). Most opioid dependent individuals engage in both during the course of therapy. Opioid detoxification aims to achieve a drug-free state within a relatively short time period to relieve acute withdrawal symptoms and increase abstinence. This treatment facilitates patients initiating maintenance treatment. α_2 adrenergic receptor agonist medications, such as clonidine, are commonly used for detoxification. A number of recent studies have provided

support for the role of detoxification in breaking the drug using cycle (Broers et al., 2000, Chutuape et al., 2001, Teesson et al., 2006b). For example, a US-based study found that 41% of those entering detoxification reported engaging in maintenance treatment post-detoxification and that 30% reported abstinence at 6-month follow up (Teesson et al., 2006a).

Maintenance treatment involves the substitution of a dangerous illicit opioid with controlled administration of a legally available, affordable opioid that does not require injection. The primary aim of maintenance treatment is to help patients to decrease their use of illicit opioids and stabilise the physiological and psychological state. The medications most commonly used include the opioid receptor agonists, methadone and buprenorphine (Mattick et al., 2009). Although the maintenance treatment is currently the most successful therapy for opioid dependent individuals, the yearly retention rates are limited to approximately 50-60% (Amato et al., 2005, Mattick et al., 2001). Therefore, further studies are needed to improve the effectiveness of the maintenance treatment for retention and sustained abstinence.

1.1.2 Alcohol dependence

Alcohol is one of the oldest known drugs in human history, with production dating back to as early as 8000 BC (Vallee, 1994). Alcohol abuse and dependence is a widespread disorder in the world, and the World Health Organization estimates that approximately 76.3 million people have alcohol use disorders (WHO, 2011). Alcohol is also the most widely used drug in the Australian community, as almost one in ten Australians over 14 years of age drank at levels considered ‗risky' or ‗high risk to health' in 2007 (AIHW, 2008).

1.1.2.1 Alcohol pharmacology

Alcohol acts as a general CNS depressant and produces a wide variety of behavioural and physiological effects. Alcohol can directly and indirectly affect most of the major neurotransmitter and neuromodulator systems. It has been shown that alcohol enhances activity of GABA receptor type A ($GABA_A$), neuronal α2β4 nicotinic acetylcholine, and glycine receptors, and inhibits N-methyl-D-aspartate glutamate receptor (NMDA) function (Tambour and Quertemont, 2007). Alcohol also induces presynaptic changes in neurotransmitter release, such as increased release of dopamine, serotonin and endogenous opioids (Vengeliene et al., 2008). These alterations in the brain are responsible for the major effects of alcohol, including sedation, euphoria, and motor in-coordination.

1.1.2.2 Alcohol tolerance and dependence

Chronic alcohol consumption affects the brain and alters its neurochemical properties, leading to alcohol dependence. As defined by the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV), alcohol dependence is characterised by three core criteria: alcohol tolerance, withdrawal, and compulsive alcohol consumption (American Psychiatric Association, 2000).

Tolerance to the effects of alcohol can occur over 1-3 weeks of continued alcohol administration, with a 2- to 3-fold reduction in potency (Tambour and Quertemont, 2007). The tolerance includes both enhanced elimination and tissue tolerance (Little, 1991), however the exact mechanisms are still not clear. Alcohol tolerance shows

cross-tolerance to many anaesthetic agents, and alcohol-dependent patients are often difficult to anaesthetise (Tambour and Quertemont, 2007).

Chronic alcohol administration produces various changes in the CNS neurons, which tend to oppose the acute effects of sedation, euphoria, and motor in-coordination, and contribute to the maintenance of alcohol dependence (Tambour and Quertemont, 2007). The neuronal changes include reduction in the density of $GABA_A$ receptors, and an increase of voltage-gated calcium channels and NMDA receptors (Charness et al., 1989).

Alcohol withdrawal is manifested by a well-defined physical abstinence syndrome. In humans, this syndrome can be defined by two stages (Charness et al., 1989): during the first stage, the main symptoms are tremor, nausea, sweating, fever, and sometimes hallucinations; whereas the second stage occurs over the following few days, with the patient becoming confused, agitated and often aggressive, and may suffer more severe hallucinations (Kosten and O'Connor, 2003, Romach and Sellers, 1991). A similar syndrome of central and autonomic hyperactivity can be produced in experimental animals during alcohol withdrawal (De Witte et al., 2003).

1.1.2.3 Alcohol dependence pharmacotherapy

Treatment of alcohol dependence generally commences with detoxification and acute withdrawal, followed by a rehabilitation program generally based on psychotherapy (Mann, 2004). Drugs for alcohol dependence have been developed from the basis of the biological mechanisms of dependence. Disulfiram, naltrexone and acamprosate are current treatments approved for the management of alcohol dependence (Heilig and

Egli, 2006, Kenna et al., 2004). Disulfiram, an irreversible inhibitor of aldehyde dehydrogenase (ALDH), blocks the breakdown of acetaldehyde, leading to an accumulation of acetaldehyde. This in turn causes a very unpleasant reaction, including flushing, shortness of breath, tachycardia, headache and nausea (Heilig and Egli, 2006, Mann, 2004). The objective of disulfiram treatment is thus to create an aversion to alcohol and help patients abstain from alcohol (Mann, 2004). Naltrexone is a μ opioid receptor antagonist that is thought to reduce the positive-reinforcing pleasurable effects of alcohol and to reduce craving (Mann, 2004). Acamprosate is a synthetic compound with a chemical structure similar to that of the endogenous amino-acid N-acetyl homotaurine. It has a number of effects on amino acid-mediated neurotransmission, however, the exact mechanism/s by which it affects drinking behaviour is not entirely clear (Soyka and Rösner, 2010). Although these treatments for alcohol dependence have been applied for decades, the relapse rate after long-term treatment is still high (> 50%) and may occur even after decades of abstinence, indicating a need for discovery of new treatments (Soyka and Rösner, 2010).

1.2 Pharmacogenetics of opioid and alcohol dependence

1.2.1 Genetic contribution to drug dependence

Drug dependence is a complex disorder of interacting genetic, physiological, environmental and socio-behavioural factors. A substantial genetic influence on drug dependence has been demonstrated by family, twin and adoption studies (Agrawal and Lynskey, 2008, Li and Burmeister, 2009). As opioids and alcohol are the main focuses of this thesis, here and in the following sections, the term drug dependence will refer to opioid and alcohol dependence only.

Alcohol dependence was the first addictive disorder to be shown genetically heritable (Oroszi and Goldman, 2004). Family studies have shown that a positive family history of alcohol dependence is a strong predictor of alcohol dependence within the offspring (Cloninger, 1987). Large-scale twin and adoption studies have estimated that the heritability of alcohol dependence ranges between 49 and 64% (Bohman et al., 1987, Heath, 1995, McGue, 1994). Opioid dependence has also been investigated in several large-scale twin studies. It has been estimated that the heritability of opioid dependence ranges between 50 and 60% (Kendler et al., 2000, Tsuang et al., 1998).

1.2.2 Pharmacogenetics and drug dependence

Pharmacogenetics is the science aimed at identifying genetic polymorphisms that influence the adverse reactions and physiologic responses to drug treatments (Roses, 2000). Genetic polymorphisms are differences in deoxyribonucleic acid (DNA) sequence between individuals, groups, or populations (http://genomics.energy.gov/). There are several types of genetic polymorphisms observed in humans, including single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), and microsatellites. SNPs are the most abundant genetic polymorphism, with over 10 million common SNPs in the human genome (http://www.genome.gov/11511175). Many polymorphisms have no effect on cell function, but others could predispose people to disease or influence their response to a drug (http://genomics.energy.gov/).

Unlike some diseases that are caused by single gene mutations, vulnerability to opioid or alcohol dependence undoubtedly has a more complex genetic basis. This is

complicated by the fact that addictive behaviours are both phenotypically and genetically heterogeneous, and also affected by environmental factors (Edwards, 2009, Saxon et al., 2005). Therefore, it is expected that there are multiple genes, with multiple polymorphisms in various combinations, which influence the manifestation of and variation in drug dependence.

There are several strategies which have been used to identify genetic variants involved in drug dependence. These mainly include genetic linkage studies, candidate gene association studies, and genome-wide association studies (GWAS). Linkage studies use families to provide evidence of how close a genetic marker is to an allele causing the phenotype under study (Kreek et al., 2005). Candidate gene association studies select genes that are likely to be involved in the physiological effect of the drug and/or have previously shown to be of interest. By comparing the allele/genotype frequencies between case and control groups, the possible genetic variants can be identified. GWAS is a relatively new approach, which can examine genetic variations across the whole genome and compare between case and control groups to identify gene candidates. Each of these approaches has advantages and limitations, which are summarised in Table 2 (Edwards, 2009).

Table 2 Advantages and limitations of different gene identification methods (cited from Edwards et al. 2009)

Approach	Advantages	Limitations
Linkage Mapping	• Systematically scans the genome • Different markers can be linked in different families • Fewer markers are needed (compared to GWAS)	• Poor resolution—linkage peaks do not precisely localise genes • Not very powerful; need many family members
Candidate Gene Association	• Does not require large families with affected and unaffected members • Resolution is high • Relatively inexpensive	• Reliant on hypothesis • Assumes the same marker is associated across families
GWAS	• Systematic genome scan • Agnostic (not hypothesis-driven); can identify novel genes • Resolution is high	• More expensive; many markers needed • Multiple testing concerns; subject to false positives • Assumes the same marker is associated across families

1.2.3 Genes involved in drug dependence

By using the approaches listed above, significant progress has been made and several susceptibility genetic loci have been identified as associating with drug dependence.

Most of these genes are from the known mechanisms and pathways of drug action, including drug metabolism, transporters, receptors, and signalling pathways. As is well known, different drugs have distinct mechanisms of action and pharmacological effects; however, there are certain common effects and overlapping physiology and neurobiology activated by these drugs. Therefore, the genetic variations that influence the risk of dependence can be both non-specific (shared genetic traits) and specific for individual drugs.

1.2.3.1 Shared genetic factors for both alcohol and opioid dependence

There is considerable evidence, from animal and human models, that all drugs of dependence converge on common actions on the brain‘s reward circuits (Di Chiara et al., 2004, Nestler, 2005, Volkow et al., 2004). The monoaminergic neurotransmitter systems serve as the primary chemical messenger for the reward pathways in the human brain. Monoamines can be further divided into catecholamines (i.e., dopamine and noradrenaline) and serotonin. Dopamine is synthesised from the amino acid tyrosine by tyrosine hydroxylase (Molinoff and Axelrod, 1971). Dopamine can then be converted to noradrenaline by the dopamine β hydroxylase (DβH) enzyme (Kaufman and Friedman, 1965). Serotonin is biotransformed from tryptophan by tryptophan hydroxylase (Harper, 1964). Upon appropriate signalling, monoaminergic neurotransmitters are released by presynaptic neurons into the synapse where they bind to both pre- and post-synaptic receptors. The dopamine (DA), noradrenaline, and serotonin (5-HT) transporters then transport the unbound dopamine, noradrenaline, and serotonin, respectively, back into the presynaptic neuron for reutilisation or degradation. Interactions between these neurotransmitters and receptors affect drug reward and thereby influence the risk of drug dependence (Eisch and Mandyam, 2004,

Hyman et al., 2006, Nestler, 2005, Robbins et al., 2007). Therefore, genes involved in monoaminergic systems are believed to be associated with the vulnerability to drug dependence and common to multiple drug types. Examples of these include *DRD2*, *COMT*, *SLC6A4*, and will be discussed in greater detail below.

Dopaminergic system related genes

Dopamine D2 receptors are primarily expressed in the terminal regions of dopaminergic neurons (Di Chiara and Imperato, 1988). Dopamine D2 receptor protein is encoded by the *DRD2* gene, which is located at chromosome 11q22-23 (Grandy et al., 1989). The most well characterised polymorphism in the *DRD2* gene is the *TaqI* A, which is a C > T SNP located 10 kb downstream of the *DRD2* gene. This SNP has been associated with both opioid and alcohol dependence risk (Kohnke, 2008, Kreek et al., 2005). Blum et al. first reported an association between the *TaqI* A polymorphism and alcohol dependence, that the frequencies of A1 allele were significantly different between alcohol dependent (37%) and healthy controls (13%) (χ^2 = 9.75, P = 0.002) (Blum et al., 1990). This SNP has also been associated with opioid use, that the allele frequency of A1 was 19.0% in the opioid dependent patients compared with 4.6% in healthy controls (χ^2 = 6.79, P = 0.009) (Lawford et al., 2000). However, later replication studies have not always been consistent with these findings (Barratt et al., 2006, Doehring et al., 2009, Hallikainen et al., 2003, Noble, 1998), for example, in the Barratt et al. (2006) study, no significant differences in A1 allele frequency (%) were observed between: methadone (19.6%) and buprenorphine (18.0%) maintained opioid dependent, and healthy control (17.9%) groups (P > 0.7) (Barratt et al., 2006). Another frequently studied *DRD2* SNP is the *TaqI* B, which is located in the intron 2 of the gene. It was reported to be significantly associated with

multiple substance dependence in Caucasians ($\chi^2 = 5.28$, $P < 0.05$), but not in African Americans (O'Hara et al., 1993). In another study, the *TaqI* B polymorphism showed an association with opioid dependence in Chinese (odds ratio (OR) = 5.28, 95% confidence interval (95% CI) 1.29 to 3.82, $P < 0.0001$), but not in a German cohort ($P > 0.05$) (Xu et al., 2004). Therefore, a further study is required to investigate the ethnic influence of this SNP on the risk of drug dependence.

The dopamine transporter is another key regulator of dopaminergic neurotransmission (Giros et al., 1996). The *SLC6A3* gene, located on chromosome 5q15.3, encodes for the dopamine transporter. There is a 40 base pair (bp) VNTR polymorphism in the 3'-untranslated region of the *SLC6A3* gene. This VNTR polymorphism has been shown to affect dopamine transporter availability in the putamen (Heinz et al., 2000) and the A9 allele was associated with a higher risk of alcohol dependence in a German cohort (OR = 1.94, 95% CI 1.19 to 3.18, $P = 0.01$) (Kohnke et al., 2005). Taken together, these studies showed that genetic polymorphisms at multiple levels of the DA system could influence the heritability of drug dependence.

Catechol-O-methyl transferase gene

Catechol-O-methyl transferase (COMT) is an enzyme that metabolises catecholamines, terminating the action of these neurotransmitters in the synapse (Molinoff and Axelrod, 1971). A common polymorphism that exerts an effect on COMT function is *Val158Met* (1947 G>A SNP), which results in a valine to methionine mutation at position 158. The *Val158* allele is approximately three times more thermostable and has a 40% higher activity than *Met158* at normal body temperature (Lotta et al., 1995). This polymorphism has been associated with the risk

of both alcohol and opioid dependence. In a case-control study, Vandenbergh et al. showed that the *Val158* allele was more prevalent in poly-substance dependent (31%) than healthy controls (18%) among North American subjects (OR = 2.0, 95% CI 1.2 to 3.5; $P < 0.01$) (Vandenbergh et al., 1997). This was confirmed by a later study which showed that the frequency of the *Val158* allele was higher in opioid dependent than non-dependent (likelihood ratio (LR) = 4.48, $P = 0.03$) (Horowitz et al., 2000). In addition, it has been reported that the *Val158* allele frequency was significant higher in alcoholic patients than in healthy controls (OR = 2.51, 95% CI 1.22 to 5.19, $P = 0.006$) in a Finnish cohort (Tiihonen et al. 1999). However, several studies with negative results were later published. For example, no significant ($P > 0.10$) *COMT* allele frequency difference was found between alcohol dependent and healthy controls in a German population (Kohnke et al., 2003). A recent meta-analysis has failed to show an association between the *COMT Val158Met* and alcohol or opioid dependence (Tammimaki and Mannisto, 2010). Therefore, this *COMT* SNP may not affect or only play a minor role in the drug dependence.

Serotonin and serotonin transporter

The serotonin transporter mediates the reuptake of serotonin and is expressed on presynaptic terminals of serotonergic neurons. The serotonin transporter gene, *SLC6A4*, has a GC-rich 44 bp insertion (longer variant, *l* allele) or deletion (shorter variant, *s* allele) in the promoter region, which is referred as the 5-HT transporter gene–linked polymorphic region (5HTTLPR) (Collier et al., 1996, Lesch et al., 1996). The *s* allele has been shown to be associated with decreased transcriptional efficiency and lower gene expression compared to the *l* allele (Lesch et al., 1996). It has been reported that the *s/s* genotype is associated with higher risk of opioid dependence in

both Caucasian (OR = 1.45, 95% CI 1.03 to 2.04, $P = 0.025$) (Gerra et al., 2004), and Chinese populations (OR = 3.47, 95% CI 1.4 to 8.55, $P = 0.004$) (Tan et al., 1999). A recent meta-analysis study also revealed that the *s* allele contributes to the risk of alcohol dependence, especially in the subgroups of patients who have psychiatric co-morbidity or severe withdrawal symptoms (Feinn et al., 2005). However, in contrast to the above findings, the frequency of the *l* allele was shown to be higher in the alcohol dependent than in healthy controls in a Korea population ($\chi^2 = 19.1$, $P < 0.001$) (Kweon et al., 2005). Therefore, further study is needed to determine the exact role of this polymorphism in drug dependence.

1.2.3.2 Genetic variations specific for opioid dependence

Opioid receptors

As mentioned in the previous section, opioids produce effects through several types of opioid receptors, especially through the μ opioid receptor for the reward effect. Therefore, genes encoding for the opioid receptors have been treated as important genetic candidates for opioid dependence.

The μ opioid receptor protein is encoded by the *OPRM1* gene. The *OPRM1* 118A>G SNP is the most frequently studied polymorphism in association with opioid dependence. It is located in the coding region of exon 1 and causes an Asn40Asp substitution at a putative glycosylation site in the extracellular domain (Bond et al., 1998). The receptor encoded by the 118G allele did not show altered binding affinities for most opioid peptides and alkaloids, except for beta-endorphin, where the 118G allele was associated with approximately three times stronger binding than the 118A allele (Bond et al., 1998, Kreek and LaForge, 2007). This SNP occurs with a

frequency of between 10% and 40%, and varies between different ethnic groups (Gelernter et al., 1999, Kreek and LaForge, 2007). Numerous case-control association studies have attempted to identify underlying neurobiological correlates between the 118A>G variant and the risk of opioid dependence, but have so far provided contradictory results (Coller et al., 2009). Some studies have identified a significant positive correlation between the 118G allele and opioid dependence (Bart et al., 2004, Szeto et al., 2001); however, opposite and null findings have also been reported (Franke et al., 2001, Kapur et al., 2007). A recent meta-analysis, which included 5169 subjects (2324 of whom were opioid dependent), showed no evidence of a relationship (OR = 1.16, 95% CI 0.91 to 1.47, $P = 0.23$ for allele frequencies) between the *OPRM1* 118A>G SNP and opioid dependence (Coller et al., 2009).

The κ opioid receptor, encoded by the *OPRK1* gene, has also been implicated in response to opioids (Kreek et al., 2005). Preliminary evidence suggested that the 36G>T SNP may be associated with an increased risk for opioid dependence ($P = 0.016$) (Yuferov et al., 2004). The δ opioid receptor does not directly affect opioid dependence development, but it has been shown that it could modulate the interactions between the μ opioid receptor and opioids (Zhu et al., 1999). The 921T>C SNP of δ opioid receptor gene (*OPRD1*) has been reported to be associated with a greater risk for opioid dependence ($\chi^2 = 5.10$, $P = 0.03$) (Mayer et al., 1997), however, later studies were not able to replicate these results ($P > 0.7$) (Franke et al., 1999, Xu et al., 2002).

Interestingly, Levran et al. (2008) have examined multiple variants of all three opioid receptor subtypes. A combination of *OPRM1* (rs510769, a A>G SNP in the 5' intron

region) and *OPRD1* (rs2236861, a A/G SNP in the first intron) SNPs showed significant associations ($P = 0.0005$) with opioid dependence. Although none of these associations remained significant after adjustment for multiple testing, this study suggests the combination of several genes and variants of opioid receptors may reveal more information than individual SNPs.

Opioid metabolism enzymes

Uridine diphosphate glucuronosyltransferases (UGTs) glucuronidate morphine to the opioid inactive metabolite morphine-3-glucuronide (M-3-G) and the μ opioid receptor agonist morphine-6-glucuronide (M-6-G) (Trescot et al., 2008). A promoter region SNP, -161C>T, in the gene encoding UGT2B7 (*UGT2B7*) has been identified in individuals with altered rates of glucuronidation, and subjects with the *UGT2B7* -161C allele showed higher plasma morphine concentrations and reduced M-6-G/morphine ratios (Sawyer et al., 2003). Therefore, it was proposed that the *UGT2B7* -161T allele could be associated with higher risk of opioid dependence (Sawyer et al., 2003). However, there was no data supporting this hypothesis yet. There are other polymorphisms that have been identified in the *UGT2B7* gene, however, none significantly alter morphine clearance or M-6-G and M-3-G formation (Holthe et al., 2002), thereby not affecting risk of dependence.

Many opioid drugs, other than morphine and heroin, are generally metabolised by cytochrome P450 family enzymes (CYPs). Although CYP3A4 is involved in the metabolism of most of the opioids, CYP2D6 is of greater clinical interest, as CYP2D6 can convert the weaker opioids, such as codeine, oxycodone, hydrocodone, and tramadol, to their more potent hydroxyl metabolites, morphine, oxymorphone,

hydromorphone, and O-desmethyltramadol, which have much higher affinity (over 30-fold) for the μ opioid receptor (Somogyi et al., 2007, Trescot et al., 2008). The *CYP2D6* gene is highly polymorphic, with over 100 variants identified (Howard et al., 2002, Sim et al., 2011). The highly polymorphic nature manifests as varied levels of metabolism activities (Howard et al., 2002). It has been demonstrated that alleles with lower CYP2D6 activity (poor metaboliser phenotype) are associated with a lower risk for oral opiates dependence (OR > 7, $P < 0.05$) (Tyndale et al., 1997). However, no allele frequency difference was found between opioid dependent patients who are under methadone maintenance treatment and healthy controls in an Australian Caucasian cohort, which suggest no direct linkage between *CYP2D6* alleles and dependence risk (Coller et al., 2007). Therefore, further study is needed to verify the role of *CYP2D6* gene in the development of opioid dependence.

1.2.3.3 Genetic variations specific for alcohol dependence

Alcohol metabolism enzymes

Alcohol metabolism occurs predominantly in the liver through two steps: alcohol dehydrogenase (ADH) oxidizes alcohol to acetaldehyde, which is a toxic intermediate; and then, acetaldehyde is converted to acetate by ALDH (Agarwal, 2001). The accumulation of acetaldehyde during alcohol consumption, can cause a flushing response as well as headache, nausea, and palpitations (Edenberg, 2007). Therefore, it is hypothesised that people with genetic variants of *ADH* and *ALDH* leading to increased acetaldehyde levels would be less likely to have heavy alcohol consumption because of the discomfort of the acetaldehyde syndrome, and would be protected against the development of alcohol dependence (Agarwal, 2001).

Based on the structural and kinetic characteristics, the human ADH family has been grouped into five classes (Duester et al., 1999). Class I ADH has three separate gene loci with common allelic variants being found at *ADH1B* and *ADH1C*. *ADH1B* has three common alleles, **1*, **2* and **3*, whilst *ADH1C* has two, **1* and **2* (Agarwal, 2001). These genetic variants of *ADH1B* and *ADH1C* are associated with altered alcohol oxidation to acetaldehyde (Agarwal, 2001, Toth et al., 2010, Whitfield et al., 1998). The frequencies of these alleles vary greatly between ethnic groups; *ADH1B*1* allele is prevalent among Caucasians and American Indians (> 85%); *ADH1B*2* is predominant among East Asians, including the Han Chinese, Japanese and Koreans (> 60%); and *ADH1B*3* is prevalent in African populations (> 95%) (Goedde et al., 1992). *ADH1C*1* is predominant among Asians and African populations; whilst, *ADH1C*1* and *ADH1C*2* are equally distributed among Caucasians and American Indians (Agarwal, 2001). A study with a Han Chinese cohort showed that the frequencies of *ADH1B*2* and *ADH1C*1* alleles were significantly lower ($P < 0.001$) in alcohol dependent than in controls (Chen et al., 1999), which suggested these alleles protected against the development of alcohol dependence (Chen et al., 1999). Further haplotype analyses indicated that *ADH1C* alleles exert negligible effect on the risk of alcohol dependence due to linkage disequilibrium between the *ADH1C*1* and *ADH1B*2* (Chen et al., 1999). A meta-analysis of 15 studies in Asian populations further confirmed the protective role of *ADH1B*2*, that the *ADH1B*2* allele is associated with a 4-fold lower risk in the development of alcohol dependence (Luczak et al., 2006b).

Of ten gene families encoding for human ALDH, *ALDH2* plays the major role in the acetaldehyde oxidation (Sophos and Vasiliou, 2003). The *ALDH2*2* allele caused by a

G/A transition results in the substitution of glutamic acid by lysine at position 487, and occurs at a frequency of 16-24% in the Han Chinese, Japanese and Koreans but very rarely in Caucasians, Africans and American Indians (< 5%) (Sophos and Vasiliou, 2003). A study by Higuchi et al. surveyed 1300 Japanese alcohol dependent patients and found the frequency of *ALDH2*2* homozygote is zero, which suggested that homozygosity of *ALDH2*2* may protect against the development of alcohol dependence. In contrast, the frequencies of heterozygous *ALDH*1/*2* varied from 2.5 to 13% for the Japanese and from 10 to 18% for the Han Chinese alcohol dependent individuals, respectively (Chen et al., 1999, Higuchi et al., 1994). A meta-analysis of 15 studies indicated that possession of one *ALDH2*2* allele is associated with a 5-fold reduction in alcohol dependency ($P < 0.0001$), whereas homozygotes have a 9-fold reduction ($P < 0.0001$) (Luczak et al., 2006a). These findings suggest that the *ALDH*1/*2* heterozygosity only partially protects against the risk of alcohol dependence.

GABA receptors

Besides the dopaminergic system, GABA, as the major inhibitory neurotransmitter in the CNS, also has an influence on the behavioural effects of alcohol consumption and withdrawal symptoms (Buck, 1996). There are two types of GABA receptors, $GABA_A$ and $GABA_B$. $GABA_A$ is of particular interest because it is directly modulated by alcohol and is involved in the acute actions of alcohol, as well as in alcohol tolerance and dependence (Davies, 2003, Edenberg et al., 2004). The majority of $GABA_A$ receptor genes are found in clusters, including the subunit genes *GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1* on chromosome 4 (Kohnke, 2008). Edenberg et al. (2004) have evaluated the $GABA_A$ receptor gene cluster and found a strong association

between *GABRA2* encoding the α2 subunit of $GABA_A$, and alcohol dependence ($P < 0.05$). This association was also confirmed by later studies (Agrawal et al., 2006, Soyka et al., 2008). A linkage study also revealed a linkage between the *GABRB1* gene and alcohol dependence ($P < 0.001$) (Long et al., 1998), and this was confirmed by a later association study which showed a significant association between *GABRB1* and alcohol dependence ($P = 0.004$) (Parsian and Zhang, 1999).

Summary

Although great effort has been made, there is significant inconsistency in the literature with regard to the effect of a given gene on the risk of opioid and alcohol dependence. Moreover, the exact role of individual genes in these complex diseases is far from well characterised. The genes that have been targeted were mainly focused on neuronal and metabolism factors. Genes encoding immune system proteins have rarely been explored. There is growing evidence that the CNS immune competent cells - glia and the proinflammatory cytokines also modulate drug actions and contribute to the development of dependence; to be reviewed in the following section. Therefore, the genes encoding proteins of the immune signalling pathways could also be potential candidates for the heritability of opioid/alcohol dependence.

1.3 Glia, Proinflammatory Cytokines and Drug Dependence

1.3.1 Overview

Alterations in neuronal structure, biochemistry and function have been considered the basis for the initiation and maintenance of drug dependence (Hyman et al., 2006).

Therefore, most of the previous research on drug dependence has concentrated on the effects of drugs on neurons in the nervous system. Likewise, pharmacological therapies for drug dependence have been directed to neuronal mechanisms that are known to be affected by drug intake. Significant progress has been made in understanding the neuronal pathways and brain circuits that are altered in drug dependence and how these changes affect specific drug-induced behaviours. However, despite the central role of the neuronal system in the mechanisms of drug dependence, there are other components involved. Work during the past decade has challenged the classical views of neuronal-centred opioid and alcohol actions (Blanco and Guerri, 2007, Hutchinson et al., 2007). CNS immune-like cells, glia, are now recognised as another key player that contributes to drug dependence (Vijayaraghavan, 2009, Watkins et al., 2009). Opioid-induced glial activation and the ensuing proinflammatory response, particularly via the release of cytokines, counters the beneficial opioid actions and produces actions contributing to the development of tolerance and dependence. Recent findings have demonstrated that alcohol can also induce glial activation and the release of proinflammatory cytokines that cause brain damage (Alfonso-Loeches et al., 2010, Blanco and Guerri, 2007). The following sections will review the recent evidence for the involvement of glia and proinflammatory cytokines in both opioid and alcohol actions and the influence on dependence development.

1.3.2 Glia

Glial cells are non-neuronal immune-like cells in the central nervous system, which constitute approximately 70% of the total cell population in the brain and the spinal cord (DeLeo and Yezierski, 2001). Glial cells provide physical support and protection

for neurons. It has been recognised that glia are not only simply ―housekeepers" for neurons, but important neuromodulatory and neuroimmune elements in the CNS (Watkins and Maier, 2002). There are three types of glial cells in the CNS; microglia, astrocytes and oligodendrocytes (Figure 1) (Allen and Barres, 2009).

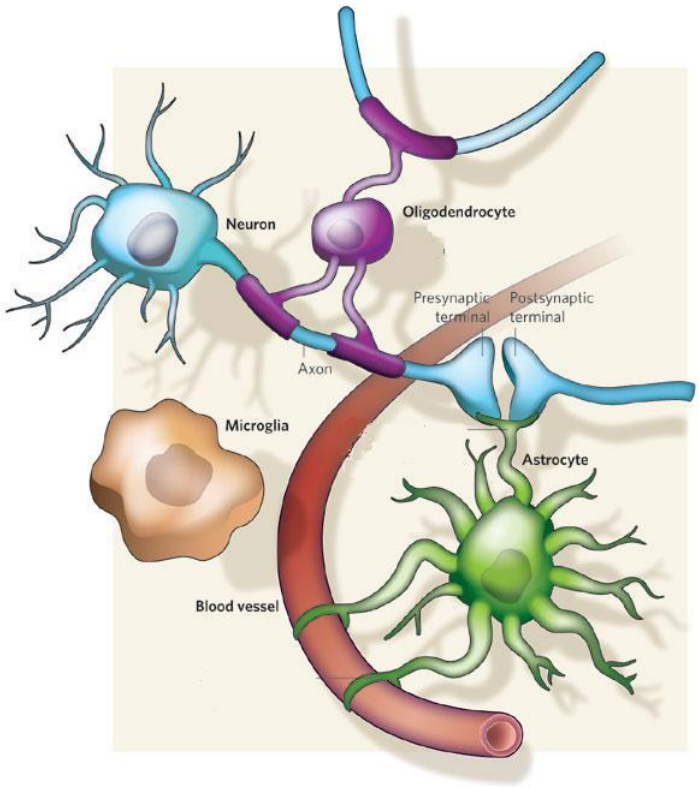


Figure 1 Three types of glial cells and their supportive roles for neurons (used with permission from Allen and Barres, 2009)

Thought of as the most reactive and mobile cells of the CNS, microglia (constituting 5 - 10% of all glia) are the specialised phagocytic representatives of the CNS, playing a similar role to that of peripheral macrophages (Graeber and Streit, 2010). Microglia perform various immune-related duties in the CNS. Under basal conditions, microglia form a network with a capacity for immune surveillance and control (Kreutzberg, 1996). A number of events, including injury, infection, ischaemia and neurodegeneration, can lead to the activation of microglia. Transformation from a

resting to an activated state occurs rapidly and results in changes in morphology, proliferation, receptor expression, and function (Watkins et al., 2007). The activated microglia can migrate to the injury sites, phagocytose debris, and release various substances, including proinflammatory cytokines, chemokines and nitric oxide.

Astrocytes (constituting 40 - 50% of all glial cells) encase most of the synapses in the CNS and modulate neuron-to-neuron synaptic communication (Watkins et al., 2007). Astrocytes express receptors for a wide variety of neurotransmitters and can release many neuroactive substances, as well as trophic factors (Barres, 2008). Under basal conditions, astrocytes provide neurons with nutritional support, regulate extracellular ions and neurotransmitters, and regulate neuronal survival, differentiation, and the formation of synapses (Perea and Araque, 2002). Activated astrocytes, which are triggered by injury and inflammation, upregulate the expression of glial fibrillary acidic protein (GFAP) and change in morphology and proliferation upon persistent activation (Aldskogius and Kozlova, 1998).

Oligodendrocytes provide the fatty myelin sheaths that insulate axons in the CNS (Baumann and Pham-Dinh, 2001). A single oligodendrocyte can extend its processes to 50 axons, wrapping approximately 1 mm of myelin sheath around each axon. Oligodendrocytes are closely related to nerve cells, and, like all other glial cells, provide a supporting role for neurons (Baumann and Pham-Dinh, 2001).

All three types of glial cells interact *in vivo*. They can activate each other via the release of proinflammatory cytokines, neurotransmitters and neuromodulators (Allen and Barres, 2009). Apart from neurotransmitters, such as glutamate, dopamine, and

substance P, glial cells can interact with neurons via immune signalling, such as cytokines, chemokines and complement components (Barres, 2008). For example, it has been shown that tumour necrosis factor alpha (TNF-α) plays a role in controlling normal function and plasticity of neural circuits *in vitro* and *in vivo* (Stellwagen and Malenka, 2006); complement component 1q was shown to be involved in the selective elimination of inappropriate synaptic connection (Barres, 2008). However, there is currently very limited understanding of the effects of immune signalling on neuronal activity, which requires further investigation.

Many studies to date suggest that activation of microglia and astrocytes contributes a significant portion to reduced opioid efficacy and the development of dependence and tolerance (Hutchinson et al., 2007, Narita et al., 2006, Watkins et al., 2007). Due to the lack of activation/expression marker(s), studying the involvement of oligodendrocytes has been hindered. As in most situations the relative roles of individual glial cell types are not clear, in this thesis, I will not specify the exact types, but use the term "glia" to refer to any type of glial cells.

1.3.3 Proinflammatory cytokines

Glial cells once activated by neurotransmitters, substances released by damaged, dying and dead neurons, immune signalling proteins, etc., release a number of proinflammatory products, one of the most important being cytokines. Cytokines are small non-structural proteins with molecular weights ranging from 8,000 to 40,000 Da (Dinarello, 2000). Cytokines are primarily involved in host responses to disease, infection, and alterations in homeostasis. Proinflammatory cytokines, including IL-1β, TNF-α, and interleukin-6 (IL-6), primarily function to induce inflammation.

It has long been appreciated that proinflammatory cytokines and their receptors exist in the CNS and are involved in the modulation of neuronal functions (Rothwell, 1991, Sawada et al., 1995, Stoll et al., 2000). However, the actions and the source of CNS proinflammatory cytokines have not been well understood. It was once believed that these cytokines were produced in the periphery by immune cells, which then enter the CNS (Blatteis, 1988). It is now understood that glia are a major source of the CNS proinflammatory cytokines (DeLeo and Yezierski, 2001, Watkins et al., 2003). In addition, neurons and blood-brain barrier endothelial cells are also possible sources of the CNS immune signalling molecules (Adler and Rogers, 2005, Simka, 2009), however, whether they release proinflammatory cytokines *in vivo* is still unknown.

In addition to proinflammatory cytokines, chemokines are also an important immune signalling protein involving in neuroinflammation (Watkins et al., 2007). Chemokines are a large family of small proteins that are involved in cellular migration and intercellular communication (Fernandez and Lolis, 2002). As proinflammatory cytokines are the main focus of this thesis, the detailed role of chemokines will not be discussed here.

1.3.4 Glia and proinflammatory cytokines regulate opioid actions

In the past decade, a series of discoveries have revealed a link between glia, proinflammatory cytokines and the pharmacological actions of opioids. In 2001, Song and Zhao first reported the involvement of glia in the development of opioid tolerance. After chronic systemic injection of morphine to rats, significant glial activation was observed in the spinal cord. Importantly, co-administration of fluorocitrate, a glial

metabolic inhibitor, with morphine significantly attenuated both glial activation and, surprisingly, morphine tolerance (Song and Zhao, 2001). Since then, work from several groups has strongly supported and extended these initial animal findings.

1.3.4.1 Opioids induce CNS glial activation and the release of proinflammatory cytokines

Chronic administration of morphine can activate CNS glia and upregulate proinflammatory cytokine expression. Raghavendra et al. (2002) investigated the impact of chronic morphine treatment on spinal glial activation and proinflammatory cytokine release in neuropathic and sham-operated rats. As evidenced by immunohistochemistry, spinal cord glia were activated in both neuropathic and sham-operated rats after chronic morphine administration. In the sham-operated rats, the messenger ribonucleic acid (mRNA) levels of IL-1β, TNF-α, and IL-6 in L5 lumbar spinal cord were significantly increased in the morphine-treated group compared to saline-treated animals ($P < 0.05$); whilst in the neuropathic rats, the chronic administration of morphine further enhanced the level of proinflammatory cytokines expression compared to sham-operated rats ($P < 0.05$) (Table 3). Furthermore, enhanced protein levels of IL-1β and TNF-α were found in all rats after chronic morphine treatment ($P < 0.05$) (Table 3). These observations have been confirmed by later studies using normal rats (surgery free), demonstrating that chronic morphine administration induced glial activation and enhanced proinflammatory cytokine levels in the spinal cord (Cui et al., 2006, Johnston et al., 2004, Raghavendra et al., 2004). In addition, glial activation and proinflammatory cytokine levels exhibited a temporal correlation with the expression of analgesic tolerance to morphine (Hutchinson et al., 2008a, Raghavendra et al., 2004).

Table 3 mRNA and protein levels of proinflammatory cytokines in L5 lumbar spinal cord after neuropathy, chronic morphine treatment, or a combination of both (Raghavendra et al., 2002)

mRNA levels	IL-1β	TNF-α	IL-6
Sham Morphine	1.6 ± 0.08*	1.73 ± 0.06*	1.4 ±0.11*
Neuropathic -Saline	2.6 ± 0.07*	3.0 ± 0.09*	2.25 ± 0.13*
Neuropathic -Morphine	3.1 ± 0.06*,**	3.09 ± 0.11*	3.06 ± 0.06*,**

Individual mRNA concentration in terms of fold activation was calculated as the ratio of their expression compared with sham saline-treated animals, in which normal values are weighed as 1. Values are mean ± standard error of the mean (SEM) (n = 4).

*P < 0.05 versus sham saline group; **P < 0.05 versus neuropathic saline-treated group.

Protein levels	IL-1β (pg/mg protein)	TNF-α (pg/mg protein)	IL-6 (pg/mg protein)
Sham Saline	43.5 ± 5.4	197.3 ± 17	25.8 ± 2.8
Sham Morphine	73.5 ± 9.2*	359 ± 62.4*	36.5 ± 5.6
Neuropathic -Saline	215 ± 20.7*	720.5 ± 56.5*	115.3 ± 8.2*
Neuropathic -Morphine	301.8 ± 16.2*,**	782.8 ± 28.7*	169 ± 10.9*,**

Values are mean ± SEM (n = 4).

*P < 0.05 versus sham saline group; **P < 0.05 versus neuropathic saline-treated group.

Hutchinson et al. (2009) have observed the elevation of both glial activation markers and proinflammatory cytokine protein levels in brain regions of rats with chronic morphine treatment. Glial activation markers GFAP (astrocyte) and CD11b (microglia) as assessed by densitometry were significantly elevated ($P < 0.05$) in several brain nuclei associated with opioid withdrawal, such as the ventral tegmental area (GFAP and CD11b: 2.5-fold and 3-fold increase, respectively), nucleus accumbens (GFAP: 3-fold increase), dentate gyrus (GFAP and CD11b: 1.5-fold and 2-fold increase, respectively) and general brain nuclei, including the medial prefrontal cortex (GFAP: 5-fold increase), ventral periaqueductal gray (GFAP and CD11b: 2-fold and 4-fold increase, respectively) and caudate putamen (GFAP and CD11b: 2-fold and 2.5-fold increase, respectively). Moreover, proinflammatory cytokine protein levels were significantly increased ($P < 0.05$) in several key brain nuclei, including the cornu ammonis of the hippocampus (2-fold increase for IL-1β) and the dorsal periaqueductal gray (1.5-fold increase for IL-1β).

1.3.4.2 Opioid induced glial activation alters opioid analgesia

Glial activation and the release of proinflammatory products have been shown to contribute to the development of opioid analgesic tolerance. This has mostly been demonstrated by findings that opioid tolerance was slowed or reversed by pharmacological inhibition or attenuation of glial activation. Table 4 at the end of this section summarises the findings from these studies.

In Song and Zhao‘s study (2001), co-administration of fluorocitrate, a specific and reversible glial metabolic inhibitor, significantly attenuated morphine analgesic tolerance development, with the maximum possible analgesic effect (% MPE) of

morphine alone versus morphine plus fluorocitrate of: 16.7 ± 13.7% versus 48.8 ± 3.6% MPE, $P < 0.05$. Chronic co-administration of propentofylline (1 μg), a glial modulator that depresses the activation of microglia and astrocytes, with morphine also significantly attenuated the development of tolerance in rats (2.5-fold increase in analgesic effect on day 6, $P < 0.001$) (Raghavendra et al., 2004). Similarly, administration of minocycline (20 μg/rat), a selective inhibitor of microglial activation, has been reported to attenuate morphine analgesic tolerance in rats (2-fold increase in analgesic effect on day 7) (Cui et al., 2008). Additionally, by testing several intrathecal doses of minocycline (20, 50, 100 μg/rat), Cui et al. (2008) demonstrated that minocycline attenuated tolerance in a dose-dependent manner, such that higher doses of minocycline were associated with lower tolerance development.

Inhibition of glial activation does not only reduce tolerance development, but also enhances acute opioid analgesia. Co-administration of minocycline with morphine, both intrathecally and systemically, caused a significant increase in acute analgesic potency (2 to 3 times longer, $P < 0.05$) (Hutchinson et al., 2007, Hutchinson et al., 2008b). Similarly, the acute analgesic effects of both morphine and oxycodone were significantly potentiated (significant leftward shifts in the dose response curve, $P < 0.001$) when co-administrated with a different glial attenuator, ibudilast (AV411) (Figure 2) (Hutchinson et al., 2009). Importantly, this observation suggested that the effects of glial inhibition were not restricted to simply morphine but rather extended to other opioids, such as oxycodone, as well.

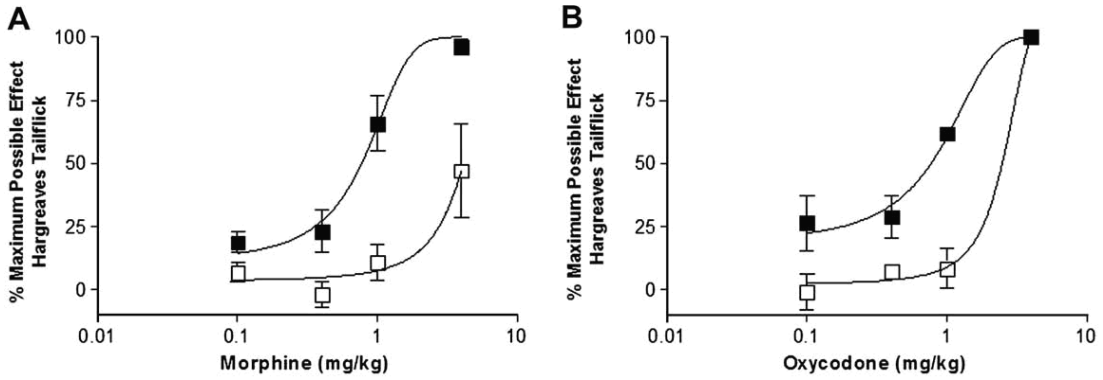


Figure 2 AV411 potentiates both morphine and oxycodone analgesia. (used with permission from Hutchinson et al., 2009).

AV411 (■, 7.5 mg/kg) significantly potentiated morphine (*P* < 0.0001) (A) and oxycodone (*P* < 0.001) (B) analgesia compared to vehicle treated rats (□). ()

1.3.4.3 Proinflammatory cytokines released from activated glia alter opioid analgesic effects

Activated glia communicate with neurons and other glial cells through the release of proinflammatory products, such as proinflammatory cytokines (IL-1β, IL-6, TNFα), anti-inflammatory cytokines, e.g. Interleukin-10 (IL-10), and chemokines, and modulation of neuropeptides, such as glutamate, adenosine triphosphate (ATP) and substance P (DeLeo and Yezierski, 2001, Watkins et al., 2007). In addition, recent evidence also suggests that there is also a need for a neuron to glia signal, as displayed by the finding that calcitonin gene-related peptide (CGRP) contributes to opioid tolerance by regulating several glial signalling cascades (Wang et al., 2010). Each of these components plays an important role in regulation of opioid actions. Since proinflammatory cytokines are the main focus of this thesis, the following sections will only summarise the evidence relating the involvement of proinflammatory cytokines in opioid actions.

Pharmacological and genetic blockade of proinflammatory cytokines has been shown to alter opioid pharmacological response. This was first observed following dynorphin exposure, which is an endogenous opioid peptide, that inhibition of proinflammatory cytokines in spinal cord reduced dynorphin-induced allodynia (a pain due to a stimulus which does not normally provoke pain) (Laughlin et al., 2000). Raghavendra et al. (2002) then showed that intrathecal administration of a cocktail consisting of IL-1Ra, soluble TNF receptor (sTNFR) and IL-6 neutralizing antibody restored the acute analgesic effect of morphine in morphine-tolerant rats ($P < 0.05$). This finding has been confirmed by several later studies. Johnston et al. (2004) demonstrated that blockade of IL-1β by IL-1Ra alone both potentiated the morphine acute analgesic effect and reversed established tolerance (1.5- and 2-fold increase in analgesic effect, respectively). Shavit et al. (2005) also reported that several IL-1β blockers (including IL-1Ra, alpha-melanocyte-stimulating hormone, and IL-1 tri-peptide antagonist) all successfully prolonged and potentiated opioid analgesia. In addition, genetic knockout of IL-1β signalling was also shown to unmask morphine analgesia (Shavit et al., 2005).

Hutchinson et al. (2008a) have further expanded the evidence that the actions of spinal proinflammatory cytokines oppose opioid analgesia. In their study, both soluble TNF-α receptor and IL-6 neutralizing antibody independently significantly unmasked morphine analgesia (1.5- to 2-fold increase in analgesic effect, $P < 0.05$). These data implicated the role of IL-6 and TNF-α, in addition to IL-1β, in opposing opioid analgesia. More recently, it has been shown that the expression of proinflammatory cytokine mRNA was significantly increased in the dorsal spinal cord of morphine-tolerant rats, such as TNF-α increased by 2.5-fold, IL-1β by 13-fold, and IL-6 by 113-

fold ($P < 0.05$) (Shen et al., 2011). Moreover, etanercept treatment, which is a TNF-α antagonist, preserved morphine analgesic effects and blocked the increase in TNF-α, IL-1β and IL-6 mRNA expression (Shen et al., 2011).

It has also been reported that anti-inflammatory cytokines enhance opioid analgesia and impede opioid-induced abnormal pain sensitivity. Intrathecal administration of IL-10 following dynorphin, prevented the dynorphin-induced allodynia in rats (Laughlin et al., 2000). Gene therapy to increase the anti-inflammatory cytokine IL-10 release in the spinal cord has been shown to significantly potentiate morphine analgesia (1.5-fold increase) and protect against the development of tolerance and withdrawal-induced pain enhancement (Johnston et al., 2004). Therefore, the balance between anti- and pro-inflammatory cytokines in the CNS may affect opioid analgesic effects, and further investigation is warranted.

1.3.4.4 Opioid-induced glial activation and proinflammatory cytokine release contributes to opioid dependence behaviours

The results from the animal models summarised above suggest that the glial activation and the ensuing proinflammatory signalling compromise the analgesic efficacy of opioids. Further evidence has shown that glia not only affect opioid analgesia but also contribute to opioid side effects.

Glial activation has been shown to be involved in the rewarding and reinforcing actions of opioids. Work by Narita et al. (2006) implicated glial activation in morphine reward through several behavioural tests in mice. In this study, the glial activation inhibitor, propentofylline, reduced (over 2-fold, $P < 0.01$) morphine-

induced conditioned place preference (CPP), an index of reward, in mice (Narita et al., 2008). This finding was confirmed by Hutchinson et al. (2008b) who showed that minocycline attenuated morphine-induced CPP in rats ($P < 0.05$). Glial modulation of opioid reward effects is partly via regulation of dopamine levels in the nucleus accumbens. It has been shown that morphine-induced dopamine level was significantly decreased (2-fold, $P < 0.05$) in AV411-treated than in saline-treated rats (Bland et al., 2009).

In addition to opioid reward, glial proinflammatory activation was also shown to have an impact on other opioid pharmacological effects. Hutchinson et al. (2008b) examined the effect of glial activation on other opioid-induced effects. They have explored whether inhibition of glial activation by minocycline could influence morphine-induced respiratory depression in rats. Systemic co-administration of minocycline (50 mg/kg) significantly attenuated morphine-induced respiratory depression ($P < 0.05$) in tidal volume (2-fold increase), minute volume (2-fold increase), inspiratory force (1.5-fold increase) and expiratory force (1.5-fold increase). In a later study, the same research group examined the effect of glial activation on opioid withdrawal in rats (Hutchinson et al., 2009). Both minocycline and AV411 have been shown to protect rats against naloxone-precipitated morphine withdrawal when co-administered during the development of morphine dependence. Moreover, they have also shown that AV411 protected rats against spontaneous withdrawal, as reflected by the suppression of withdrawal-induced spontaneous activity levels and weight loss. Importantly, these data, for the first time, implicated opioid-induced glial activation in the development of opioid dependence and the precipitation of withdrawal behaviours.

Table 4 Inhibition of glial activation or proinflammatory cytokines alter opioid actions in animal models (Modified from Watkins et al. 2009)

Model	Direction of effect	Intervention
Morphine-induced glial activation	suppressed	AV411(Hutchinson et al., 2009), (Mika et al., 2009), propentofylline (Romero-Sandoval et al., 2008), fluorocitrate (Song and Zhao, 2001)
Morphine-induced proinflammatory cytokines & chemokines	suppressed	Propentofylline (Romero-Sandoval et al., 2008), (+)-naloxone(Hutchinson et al., 2008c), AV411 (Hutchinson et al., 2009)
Opioid-induced acute analgesia	enhanced	minocycline (Hutchinson et al., 2008a) and(Hutchinson et al., 2008b), AV411 (Hutchinson et al., 2009), IL-10 (Johnston et al., 2004), IL-1Ra (Johnston et al., 2004) and (Shavit et al., 2005), IL-1 signaling KOs, IL-Ra over-expressing transgenics (Shavit et al., 2005), classic TLR4 antagonists (Hutchinson et al., 2010), (+)-naloxone (Hutchinson et al., 2010), (-)-naloxone (Hutchinson et al., 2010), sTNFR (Hutchinson et al., 2008a), anti-IL-6 (Hutchinson et al., 2008a)
Morphine analgesic tolerance	suppressed	IL-10 (Johnston et al., 2004), IL-1Ra (Johnston et al., 2004), fluorocitrate (Song

		and Zhao, 2001), minocycline(Cui et al., 2008), (+)-naloxone (Hutchinson et al., 2010), propentofylline (Raghavendra et al., 2004), IL-1 signaling KOs (Shavit et al., 2005), IL-Ra overexpressing transgenics (Shavit et al., 2005), IL-10 + IL-1Ra (Hutchinson et al., 2008a), IL-1 converting enzyme inhibitor + IL1Ra (Hutchinson et al., 2008a)
Morphine-induced pain enhancement	suppressed	IL-10 (Johnston et al., 2004), IL-1Ra (Johnston et al., 2004), propentofylline (Raghavendra et al., 2004), (+)-naloxone (Hutchinson et al., 2010), IL-10 + IL-1Ra (Hutchinson et al., 2008a), IL-1 converting enzyme inhibitor + IL1Ra (Hutchinson et al., 2008a)
Opioid withdrawal behaviours	suppressed	AV411 (Hutchinson et al., 2009), (+)-naloxone (Hutchinson et al., 2010), minocycline (Hutchinson et al., 2009)
Morphine-induced respiratory depression	suppressed	minocycline (Hutchinson et al., 2008a)
Morphine-induced dopamine release	suppressed	(+)-naloxone, AV411 (Bland et al., 2009)
Morphine-induced CPP	suppressed	minocycline (Hutchinson et al., 2008a), propentofylline (Narita et al., 2008)

1.3.4.5 IL-1β regulates opioid actions and consequences

Studies reviewed above demonstrated the involvement of glial-induced proinflammatory cytokines in the modulation of opioid actions. Among the three proinflammatory cytokines, the role of IL-1β has been emphasised. In this section, I will summarise the evidence for IL-1β in modulating opioid actions.

Proinflammatory cytokines, particularly IL-1β, contribute significantly to inflammatory pain and also recently was shown that involve in the generation and maintenance of pathological pain states, such as neuropathic pain (Marchand et al., 2005, Watkins et al., 2009). As the focus of this thesis is opioid dependence, the role of IL-1β in pain states will not be discussed in detail. However, the involvement of IL-1β in opioid action has firstly been shown in the development opioid-induced abnormal pain sensitivity in mice (Laughlin et al., 2000). In the Laughlin (2000) study, pretreatment of the mice intrathecally with IL-1Ra dose-dependently reduced the induction of dynorphin-induced allodynia, which indicated that IL-1 signalling could modulate opioid effects. Subsequent to this, Johnston et al. (2004) showed that chronic intrathecal morphine was associated with a rapid increase in IL-1β expression in the dorsal spinal cord (2.5-fold and 2-fold increase for IL-1β protein and mRNA level, respectively) and cerebrospinal fluid (CSF) in rats (3-fold increase for protein level). To determine whether IL-1β release modulated the effects of morphine, IL-1Ra was co-administered. This regimen significantly potentiated the morphine analgesic efficacy (1.5-fold increase, $P < 0.05$) and reduced the development of analgesic tolerance. Moreover, intrathecal injection of IL-1Ra to rats with established morphine tolerance reversed established tolerance and increased morphine analgesia.

A series of experiments performed by Shavit et al. (2005) have further demonstrated the role of IL-1β in countering opioid analgesia and enhancing tolerance development. Firstly, prior administration of a small dose of IL-1β (5 μg/kg, neither analgesic nor hyperalgesic) completely blocked the analgesic effect of morphine. Secondly, when the same morphine dose was applied to transgenic mice over-expressing IL-1Ra, the morphine analgesia was significantly increased (1.5-fold increase, $P < 0.05$) compared to their wild-type controls. Additionally, similar to the observation of Johnston et al. (2004), acute administration of IL-1Ra re-instated the opioid analgesia in tolerant mice.

Summary

In summary, as the evidence discussed above shows (summarised in Table 4), opioid-induced activation of glial cells and release of proinflammatory cytokines alter pharmacological actions of opioids. One of the key questions to ask is ~~h~~ow can glia be activated by opioids?". In the following section, I will discuss the current evidence showing the involvement of non-classical opioid receptors in activation of CNS glia.

1.3.5 Possible mechanisms of glial activation by opioids – through toll-like receptor 4

In the earlier years (before 2007), opioid binding to classical opioid receptors was assumed to be the initiating step of glial activation. However, following recent investigations, opioid-induced glial activation is recognised to occur via non-classical opioid mechanisms, engaging the innate immune receptors toll-like receptor 4 (TLR4)

and possibly toll-like receptor 2 (TLR2) (Watkins et al., 2009, Zhang et al., 2011). Most studies have been done on TLR4, and so this receptor is the focus of this thesis.

1.3.5.1 What is TLR4 and its downstream signalling?

TLR4 is an innate immune pattern recognition receptor well known for its sensitivity to bacterial lipopolysaccharide (LPS) (Medzhitov, 2001). LPS is a component of the bacterial cell wall, and is a potent activator of the innate immune response in humans. In addition to LPS, TLR4 is capable of recognising endogenous danger signals (Akira and Takeda, 2004). The activation of TLR4 induces a series of downstream signalling pathways and results in profound proinflammatory signalling (Figure 3) (O'Neill and Bowie, 2007). Stimulation of TLR4 facilitates the activation of two pathways: the myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathways (Akira and Takeda, 2004).

MyD88 and Toll/IL-1 receptor-domain-containing adapter-inducing interferon-β (TRIF) are critical in transducing MyD88-dependent and -independent signalling, respectively (Bell, 2003). The MyD88-dependent pathway involves the early phase of nuclear factor-κB (NF-κB) activation, which leads to the production of proinflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF3) and involves the late phase of NF-κB activation, both of which lead to the production of IFN and the expression of IFN-inducible genes (Akira and Takeda, 2004).

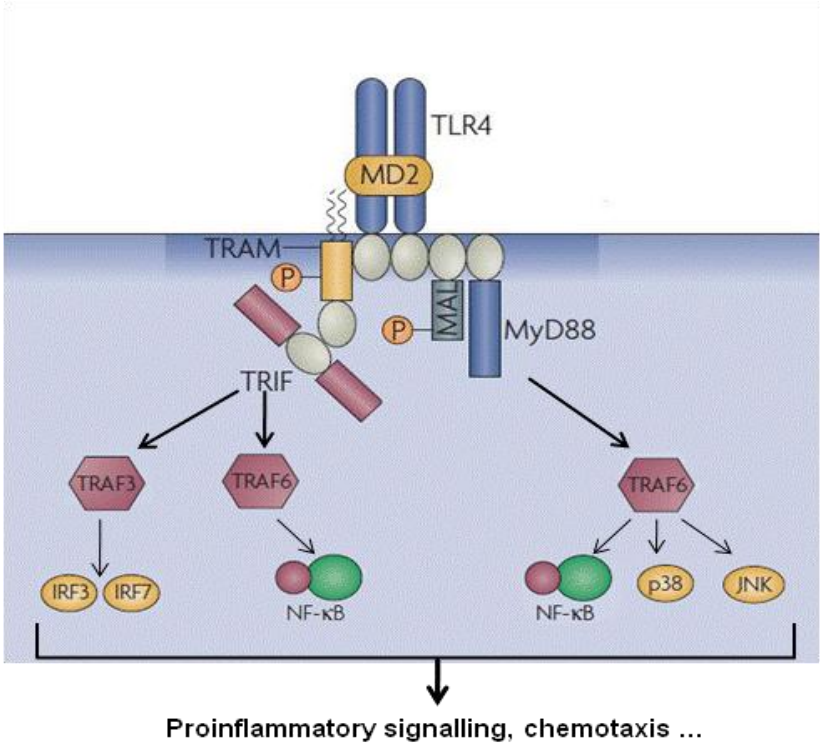


Figure 3 TLR4 signalling pathways: MyD88-dependent and -independent pathways (Adapted from O'Neill and Bowie, 2007).

1.3.5.2 Novel theory of TLR4 involvement in opioid-glial interactions

The theory that glial activation is via classical opioid receptors was questioned by the observed non-stereoselective effects of opioids. As it is well known, only (-)-opioid isomers bind to classical opioid receptors and activate the receptors (Raynor et al., 1994). However, several studies have reported that (+)-opioid isomers were also having effects *in vivo*. Takagi and colleagues (1960) first observed the induction of naïve tolerance/anti-analgesia by administration of (+)-morphine. Wu et al. (2007, 2005) have reported that the pre-treatment of (+)-morphine in the spinal cord reduced the (−)-morphine analgesic effect in mice. This research group also provided evidence to suggest that (+)-opioid isomers have glial effects and function independently of the μ opioid receptor, as: 1) administration of propentofylline blocked this effect (Wu et

al., 2005); and 2) this effect exists in μ opioid receptor knockout mice (Wu et al., 2006b). Furthermore, in μ, δ, and κ opioid receptor triple knockout mice, opioid-induced abnormal pain sensitivity, such as hyperalgesia (an increasing sensitivity to noxious stimuli), is still observed, which again suggests the existence of a non-classical opioid receptor (Juni et al., 2007, Waxman et al., 2009).

Hutchinson et al. (2007) first proposed the novel mechanism that opioids could activate glia through TLR4, after consolidating the evidence from various studies, including: 1) (+)-methadone, which possesses minimal classical opioid receptor activity, caused significant allodynia, hyperalgesia, and glial activation (Watkins et al., 2009); 2) co-administration of an (+)-opioid antagonist, such as (+)-naloxone, attenuated the reduction in opioid analgesia that occurred in response to glial activation by LPS (Wu et al., 2006a); and 3) early evidence that acute opioid exposure increased the expression of TLR4 both at the transcriptional and translational levels (Hutchinson et al., 2007). Then, through a series of *in vivo*, *in vitro*, and *in silico* studies, Hutchinson et al. (2010) have provided the evidence that opioid agonists, such as morphine, methadone, fentanyl, pethidine, and levorphanol, non-stereoselectively activate TLR4 and have behavioural consequences via TLR4 signalling. Moreover, they have shown that opioid antagonists such as naloxone and naltrexone non-stereoselectively block TLR4 signalling (Hutchinson et al., 2007, Hutchinson et al., 2010).

1.3.5.3 Role of TLR4 in opioid actions and possible mechanisms

The consequences of opioid-induced TLR4 activation are extensive and have been implicated in opposing the beneficial actions of opioids and contributing to opioid side

effects (Watkins et al., 2009). In a study by Hutchinson et al. (2010), blockade of TLR4 signalling, both pharmacological and genetic, significantly potentiated the magnitude and duration of opioid analgesia, and attenuated development of analgesic tolerance and opioid withdrawal behaviours.

The findings showing the detrimental effects of TLR4 activation in opioid actions suggest that the effects of opioids may actually partly result from the duality of opioid actions at TLR4 and other potential receptors, such as TLR2. Indeed this is the case and has been demonstrated by the different effects of two morphine metabolites, M-3-G and M-6-G (Hutchinson et al., 2010, Lewis et al., 2010). M-3-G is a morphine metabolite that is inactive at classical opioid receptors, and M-6-G is the opioid receptor active metabolite. An *in vitro* TLR4 agonist and antagonist assay using human embryonic kidney-293 cell line shows M-3-G has significant TLR4 activation, whilst M-6-G does not (Hutchinson et al., 2010). Importantly, intrathecal administration of M-3-G, but not M-6-G, to rats induced significant enhanced pain (Hutchinson et al., 2010, Lewis et al., 2010), suggesting a role for TLR4 in opposing opioid analgesic effect and contributing to opioid-induced pain enhancement.

With this novel understanding of TLR4 in opioid actions, the non-stereoselectivity of TLR4 provides a new approach to enhance the efficacy of opioids. For example, (+)-naloxone, as (+)-opioid agonist/antagonist isomers has no affinity for μ opioid receptors, would allow (-)-opioids to act neuronally on the mu receptor to suppress pain via their actions on classical opioid receptors, while preventing opioids from activating glial TLR4 (Figure 4) (Watkins et al., 2009). However, most of the studies to date have primarily focused on opioid-induced analgesic effects. The possible

involvement of TLR4 in opioid-induced side effects, such as reward and withdrawal, requires further investigation.

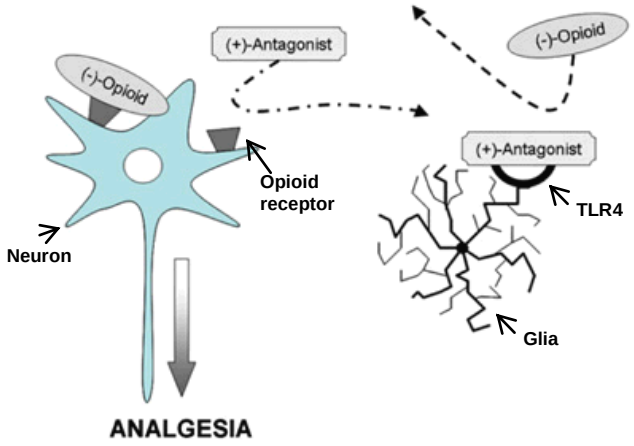


Figure 4 Possible approach to enhance opioid clinical efficacy by blocking TLR4. A combination of an opioid (+)-antagonist and an opioid (-)-agonist may enhance the opioid efficacy, as: 1) opioid (+)-antagonist block the nonstereoselective glial TLR4 preventing opioid-induced glial activation; 2) opioid (-)-agonist can act freely at the neuronal opioid receptor to produce opioid beneficial actions; and thereby increase the clinical efficacy (Adapted from Watkins et al., 2007).

As mentioned in the previous section, activation of TLR4 induces a series of downstream signalling, include MyD88-dependent and -independent signalling pathways. However, which downstream pathway is involved in the opioid-induced TLR4 signalling has yet to be studied. Therefore, further investigation is required.

1.3.6 Glia and proinflammatory cytokines involved in alcohol dependence

Alcohol is well known to affect the peripheral immune system. Chronic and even acute, moderate alcohol use can increase host susceptibility to infections caused by bacterial and viral pathogens (Szabo, 1999). Nevertheless, little is known about the potential action of alcohol on the CNS immune system. Recent studies have demonstrated that alcohol can induce CNS glial activation and proinflammatory responses contributing to alcohol dependence and brain damage.

1.3.6.1 Alcohol-induced glial proinflammatory response

In contrast to opioids, the involvement of glia and proinflammatory cytokines in alcohol actions is still largely unknown, and studies have only recently started to unmask this mystery. Several early studies have reported that chronic alcohol-induced glial abnormalities were observed in human and rat brains. Glial impairments in conjunction with astrocytic loss and death have been found in the prefrontal cortex and hippocampus regions of alcoholic patients‘ brains (Kril et al., 1997, Miguel-Hidalgo et al., 2002). Chronic alcohol administration was shown to increase the number of cerebellar glia before the appearance of brain atrophy in rats (Riikonen et al., 2002).

Recent studies have shown that similar neuroimmune changes were observed following both acute and chronic exposure to alcohol, which were time- and dose-dependent. Vallés et al. (2004) provided direct evidence showing that alcohol consumption increases glial release of proinflammatory cytokines and other inflammatory mediators in rat brain. They also showed that chronic alcohol treatment stimulated glial cell release of IL-1β *in vitro* (Vallés et al., 2004). Floreani et al. (2010) confirmed these findings by using primary human glial cells obtained from

second trimester human foetal brain tissues. Their results showed that alcohol exposure significantly enhanced the secretion of proinflammatory agents in these cells. In addition to the release of proinflammatory cytokines, alcohol exposure also induces multiple central immune signalling changes, including: increased expression of IL-1R1 mRNA; inhibition of C-X-C motif chemokine 10 production; increase in DNA binding of NF-κB; and increase in RhoE GTPase signalling (Blanco et al., 2005, Davis and Syapin, 2004a, Davis and Syapin, 2004b, Guasch et al., 2007). Although the functional consequences of these proinflammatory products to neuronal pathways are still unknown, it is believed that they initiate and facilitate the development of neuroinflammation and contribute the development of alcohol dependence (Alfonso-Loeches et al., 2010, Blanco and Guerri, 2007).

Finally, studies have also shown that alcohol could alter the central immunity to immune-challenges. For example, alcohol decreased the LPS-induced release of IL-1β and inducible nitric-oxide synthase (iNOS) expression in glial cell culture (Syapin et al., 2001); and pre-exposure of rat glial cells to low concentrations of alcohol, increased the glial sensitivity to TNF-α-induced toxicity (DeVito et al., 2000).

1.3.6.2 Involvement of TLR4 in alcohol-induced CNS neuroinflammation

Although the exact mechanisms of alcohol-induced glial activation in the CNS are presently unclear, recent data suggest that, like opioids, alcohol can activate TLR4 signalling, resulting in proinflammatory cytokine production. Blanco et al. (2005) have reported on the possible link between TLR4 and alcohol-induced glial activation. They have carried out experiments demonstrating firstly that acute alcohol treatment induces rapid activation of possible TLR4 signalling pathways in astrocytes, such as

phosphorylation of IL-1 receptor (IL-1R)-associated kinase (IRAK), extracellular signal-regulated kinase (ERK) 1/2, and p38 mitogen-activated protein kinase (MAPK), and secondly that blockade of the TLR4 binding site by using TLR4 antibody prevents the activation of these pathways (Blanco et al., 2005). These findings were extended to the other glial cell type, microglia, in a later study (Fernandez-Lizarbe et al., 2008). More recently, Alfonso-Loeches et al. (2010) provided further evidence supporting the critical role of TLR4 in alcohol-induced neuroinflammation. Their knockdown of TLR4 using small interfering RNA (siRNA) abolished the activation of mitogen-activated protein (MAP) kinase and NF-κB pathways and the production of proinflammatory cytokines. Moreover, glial cells from TLR4 knockout mice did not respond to alcohol treatment or produce proinflammatory mediators (Alfonso-Loeches et al., 2010).

The mechanism by which alcohol activates TLR4 signalling is thought to be via the modulation of lipid rafting in the cellular membranes of glial cells (Blanco and Guerri, 2007, Blanco et al., 2008). This appears to be dose-dependent, where lower concentrations of alcohol promote translocation and clustering of TLR4 into the lipid rafts and subsequently activation of the TLR4 signalling, whilst higher concentrations of alcohol perturb rafting to disrupt recruitment and signalling.

1.4 Genetic polymorphisms of the IL-1 gene family

Genetic variations that result in an altered structure or expression of a cytokine are likely to have pathological consequences (Tabor et al., 2002). Genetic polymorphisms of the *IL-1* gene family, especially the *IL-1B* gene that encodes for IL-1β, have been associated with a variety of diseases that have an inflammatory component, such as

rheumatoid arthritis (Harrison et al., 2008), inflammatory bowel disease (Vijgen et al., 2002), gastric cancer (El-Omar et al., 2000) and several neurological disorders including Alzheimer's disease (Di Bona et al., 2008) and Parkinson disease (Wahner et al., 2007). Based on animal studies, IL-1β is now believed to be an important mediator of opioid/alcohol dependence. Therefore, genetic variability in the *IL-1* gene family may alter the risk of opioid/alcohol dependence and could be among the genetic candidates that explain part of the heritability of drug dependence. To identify the possible candidate polymorphisms for investigation, the following section will summarise the common *IL-1B* and *IL-1RN* genetic polymorphisms, the functional impacts, and their association with drug dependence.

1.4.1 IL-1B gene

The human *IL-1* gene complex is located in a ~500 kb region of chromosome 2q.12 (Nicklin et al., 1994). The *IL-1* gene complex encodes for three proteins, IL-1α, IL-1β, and IL-1Ra. The gene encoding for IL-1β, *IL-1B*, is about 7.5 kb in length and has 7 exons. *IL-1B* is highly polymorphic, with more than 80 SNPs (http://www.genecards.org), three of the most common being -511C>T, -31T>C, and 3954C>T (Chen et al., 2006, Di Giovine et al., 1992, Pociot et al., 1992). Interestingly, significant linkage disequilibrium has been observed between the alleles of the -31 and -511 SNPs (*IL-1B* -31T linked with -511C, and *vice versa*), with this combination making up 99% of inferred haplotypes in Caucasians and Indians (Chakravorty et al., 2006, El-Omar et al., 2000, Hall et al., 2004). The allelic frequencies of these SNPs vary among different ethnic or geographic populations. For example, the frequencies of the *IL-1B* -511T and 3954C alleles are higher in Chinese than Caucasians (Trejaut et al., 2004), and the 3954C allele frequency is lower in

Caucasians than in African Americans (Zabaleta et al., 2008). Table 5 summarises the *IL-1B* SNP's allele frequencies of some ethnic and geographic populations.

Table 5 Ethnic differences in *IL-1B* polymorphisms

Gene	Polymorphisms	Alleles/ genotypes	American Caucasian (n = 299)[a]	African American (n = 294)[a]	Indian (n = 130)[b]	Chinese (n = 145)[c]
IL-1B	-31	T	65.2%	41.0%		
		C	34.8%	59.0%		
	-511	C	65.4%	46.6%	37.3%	48.0%
		T	34.6%	53.4%	62.6%	52.0%
	3954	C	80.0%	87.4%	85.0%	99.0%
		T	20.0%	12.6%	15.0%	1.0%

[a] (Zabaleta et al., 2008); [b](Kaur et al., 2007); [c] (Trejaut et al., 2004)

IL-1B SNPs are believed to influence individual differences in IL-1β production but not protein structure. Most studies have focused on the effect of the *IL-1B* -31T>C and -511C>T SNPs on gene expression. This was firstly examined by El Omar et al. (2000) using electrophoretic mobility shift assay (EMSA) to compare the DNA-binding activity of the -31T and C alleles *in vitro*. The results showed that the synthetic oligonucleotides containing the -31T allele had a 5-fold preferential binding to the monocyte nuclear protein than the complex containing -31C allele. The authors proposed the mechanism behind this phenomenon was the creation by the *IL-1B* -31T allele of a TATA sequence in the promoter region, enhancing promoter activity, whilst the effect of the -511C>T SNP was simply due to its linkage disequilibrium with the

TATA box polymorphism (El-Omar et al., 2000). This finding has been confirmed by two later studies testing promoter activity and expression in different cell lines (Chakravorty et al., 2006, Lind et al., 2007). In both HeLa cells and the monocyte cell line U937, promoters with the -31T allele had an almost 10-fold increase in activity compared to those with the -31C allele. However, there are conflicting data generated from another study. Hall et al. (2004) reported that the *IL-1B* -31C and -511T alleles were associated with a 2- to 3-fold increase in IL-1β protein production as measured by an *ex vivo* blood stimulation. There is a major concern with this study that the IL-1β *ex vivo* stimulation assays were conducted with a fixed volume of blood, but without quantification of the cell numbers, which would likely lead to confounding results.

In addition to investigating individual SNPs, haplotype analysis investigating the combined effects of multiple SNPs, may reveal more information. A study by Chen et al. (2006) identified 4 *IL-1B* promoter SNPs that were active in luciferase reporter assays, including -31T>C and -511C>T, as well as two new SNPs, -1464G>C and -3737C>T (Chen et al., 2006). When analysed individually, the -1464G>C and -3737C>T SNPs did not show any effects on IL-1β expression, whilst -511T showed a small increase in transcriptional activity, and -31T demonstrated a significant increase in promoter activity ($P < 0.05$), which is in line with previous findings (El-Omar et al., 2000). Haplotype analysis of these four SNPs together revealed that the -3737C/-1464G/-511C/-31T combination resulted in an increase of IL-1β expression (~2-fold) than the -3737C/-1464G/-511C/-31C haplotype ($P < 0.05$), indicating that the -511 and -31 alleles may interact to influence gene expression. Interestingly, the occurrence

of the -511C/-31C haplotype is very rare (< 1%), but it reminds us that the uncommon haplotype may have significant clinical implications.

Few studies have investigated the functional impacts of the *IL-1B* 3954C>T SNP. An earlier study found that homozygousity for the *IL-1B* 3954T allele was associated with a 4-fold increase in the production of IL-1β when compared to the C allele homozygotes (Pociot et al., 1992). However, no significant association with *in vitro* IL-1β expression was found in a later study (Hall et al., 2004). Interestingly, the frequency of *IL-1B* 3954T allele is very low in Chinese population (< 5%); however, no study has found any significant functional consequences of this polymorphism in Chinese.

1.4.2 IL-1RN gene

IL-1Ra is an endogenous antagonist of IL-1. It binds to the same IL-1 receptor but does not activate the receptor and trigger signal transduction. The balance between IL-1 and IL-1Ra plays an important role in regulating the inflammatory process (McIntyre et al., 1991). The gene coding for IL-1Ra, *IL-1RN*, is located on chromosome 2 in close proximity to other *IL-1* genes (Steinkasserer et al., 1992).

The *IL-1RN* gene has a 86 bp VNTR in intron 2, consisting of at least 5 alleles (alleles *IL1RN *1*, *IL1RN *2*, *IL1RN *3*, *IL1RN *4*, and *IL1RN *5* representing four, two, five, three and six repeats, respectively) (Tarlow et al., 1993). The allelic frequencies of this polymorphism vary among different ethnic or geographical populations (summarised in Table 6). For example, the frequency of the **2* allele is significantly lower in Chinese, Africans and African Americans than in Caucasians (Trejaut et al.,

2004). However, in all populations, the **1* and **2* alleles are the most common, and the remaining alleles occur with a frequency of less than 5% in most populations. Interestingly, a sex difference in the *IL-1RN* VNTR polymorphism has been reported in a Jewish cohort, where the *IL-1RN*1* allele was more frequent in men (men vs. women: 69.9% vs. 62.5%, $P = 0.019$), and the *IL-1RN*2* allele was more prevalent in women (men vs. women: 22.7% vs. 33.3%, $P = 0.008$) (Bessler et al., 2007). However, no similar report was found in other populations.

Table 6 Ethnic differences in *IL-1RN* polymorphisms

Polymorphisms	Alleles	American Caucasian (n = 299)[a]	African American (n = 294)[a]	African (n = 46)[b]	Chinese (n = 145)[c]	Israeli Jewish (n = 638)
IL-RN VNTR	**1*	67.8%	87.6%	87%	93%	66.3%
	**2*	29.2%	9.2%	12%	6%	27.9%
	**3, *4 & *5*	3.0%	3.2%	1%	1%	5.8%

[a] (Zabaleta et al., 2008); [b] (Mwantembe et al., 2001); [c] (Trejaut et al., 2004)

Three potential protein-binding sites (an α-interferon silencer A, a β-interferon silencer B, and an acute phase response element) have been identified in the *IL-1RN* VNTR region; therefore, the variable copy number polymorphism appears to be of clinical significance (Tarlow et al., 1993). However, data regarding the functional effects of this polymorphism are currently controversial. Two studies have reported that the *IL-1RN*2* allele was associated with 2- to 4-fold enhanced IL-1Ra production *in vitro* after stimulation of mononuclear cells with granulocyte-macrophage colony-

stimulating factor (Danis et al., 1995) or phorbol dibutyrate (Santtila et al., 1998). It has been also shown that *IL1RN*2* allele carriers had a higher plasma IL-1Ra concentration than non-carriers among healthy volunteers (745 vs. 627 pg/ml, $P < 0.05$) (Hurme and Santtila, 1998). However, another study, which investigated mucosal inflammation, reported that *IL-1RN*2* was associated with significantly lower IL-1Ra expression in colonic mucosa (Andus et al., 1997). Another study has also found no correlation between *IL-1RN* genotypes and the circulating levels of IL-1Ra in plasma (Rider et al., 2000). Therefore, a further comprehensive study is needed to determine the function of the *IL-1RN* VNTR polymorphism.

1.4.3 Association with opioid and alcohol dependence

1.4.3.1 Association with alcohol dependence

Pastor et al. (2000) previously investigated the *IL-1* gene cluster polymorphisms and alcohol dependence in a Spanish cohort. Although their research was originally aimed at investigating genetic risks of alcohol-induced liver disease, they discovered several polymorphisms that were associated with susceptibility to alcohol dependence but not alcoholic liver disease (Pastor et al., 2000, Pastor et al., 2005). The studies showed that the frequencies of the *IL-1B* -511 C and *IL-1RN*1* alleles were significantly higher in alcoholic patients when compared to the control group (Tables 7 and 8), indicating that these two alleles could be risk factors for alcohol dependence.

Table 7 *IL-1B* -511 C>T allele frequencies comparison between alcohol dependent individuals and healthy controls in a Spanish population (Pastor et al., 2005)

		Healthy Control n (%)	Alcohol Dependent n (%)
IL-1B -511	C	92 (56.8)	200 (71.9)*
	T	70 (43.2)	78 (28.1)
	Total	80	180

*: OR = 2.08, 95% CI 1.17 to 3.69, $P < 0.02$;

Two later studies have confirmed the *IL-1RN* findings in both Spanish and Chinese populations, where the frequency of the *IL-1RN*1* allele was significantly higher in alcoholic patients than in controls (summarised in Table 8). However, the association between *IL-1B* -511C>T and alcohol dependence is yet to be replicated in other populations.

Table 8 *IL-1RN* VNTR allele frequencies comparison between alcohol dependent individuals and healthy controls of different ethnic populations (Chen et al., 2005, Pastor et al., 2005, Saiz et al., 2009)

		Healthy Control n (%)			Alcohol Dependent n (%)		
		Pastor et al. 2000	Saiz et al. 2009	Chen et al. 2005	Pastor et al. 2005	Saiz et al. 2009	Chen et al. 2005
		Spanish	Spanish	Chinese	Spanish	Spanish	Chinese
IL-1RN VNTR	**1*	61 (76.3)	595 (71.2)	100 (76.9)	171 (95.0)*	313 (78.6)#	286 (86.7)‡
	**2*	14 (17.5)	229 (27.4)	22 (16.9)	4 (2.2)	78 (19.6)	39 (11.8)
	**3, *4 & *5*	5 (6.3)	12 (1.4)	8 (6.2)	5 (2.8)	7 (1.8)	5 (1.5)
	Total	80	836	130	180	398	330

*: OR = 3.69, 95% CI 2.01 to 6.75, $P < 0.001$; #: OR = 2.32, 95% CI 1.45 to 3.84, $P < 0.01$; ‡: OR = 1.54, 95% CI 1.05 to 3.78, $P < 0.05$.

1.4.3.2 Does IL-1 genetics affect opioid clinical use?

To date, no immunological genetic variants, including those of the *IL-1* family, have been examined with respect to opioid dependence. However, Bessler et al. (2006) have examined the possible relationship between the *IL-1B* -511C>T and *IL-1RN* VNTR polymorphisms and opioid analgesic effect during the postoperative period (Bessler et al., 2006). They determined the genotypes of these polymorphisms in 76 women undergoing transabdominal hysterectomy. Based on their morphine consumption, patients were divided into three groups (low, medium, and high morphine consuming groups). No differences in *IL-1B* -511C>T genotype frequency were observed between the three groups, whilst *IL-1RN* VNTR genotype frequencies in the medium morphine consumer group were significantly different ($P < 0.03$) from the other two groups, where the incidence of *IL1RN*1/*2* heterozygotes was lower (medium versus low and high groups: 16% versus 42.3% and 46.2%, $P = 0.02$ and 0.01, respectively), and *IL1RN*2/*2* homozygotes significantly higher (24% versus 3.8% and 3.8%, $P = 0.01$ and 0.02, respectively), than in other groups. Although the subject numbers were small and the effect was only observed in the medium consumption group, these results revealed some information on the possible involvement of *IL-1* family genes in opioid action and raised the possibility of their influence on the risk of opioid dependence.

Summary

As IL-1β is now believed to be an important mediator in opioid/alcohol dependence development, genetic variability in the *IL-1* gene family that alters their expression may alter the risk of drug dependence and could be among the genetic candidates that

explain part of the heritability. Importantly, studies have shown that the *IL-1B* -511C and *IL-1RN*1* alleles are associated with the risk of alcohol dependence in Spanish and Chinese populations. Moreover, the *IL-1RN* genetic polymorphism has been related to variability in clinical morphine consumption. Taken together, this evidence raises the possibility of an association between *IL-1* gene cluster polymorphisms and opioid dependence.

1.5 Using multiple inbred mouse strains for opioid dependence research

Parallel to human research, animal models using mice or rats are valuable for drug dependence research (Shippenberg and Koob, 2002). Previous animal behavioural studies examining neuronal mechanisms of opioid action have demonstrated the heterogeneity of opioid-induced analgesic tolerance and dependence withdrawal severity among different inbred mouse strains (Kest et al., 2002a, Kest et al., 2002b). These strain-specific differences suggest that an animal model for the inheritance of opioid dependence could be utilised in the study of opioid action mechanisms.

1.5.1 Inbred mouse strains

Inbred mouse strains have been a valuable tool for many years in medical science research. Inbred mouse strain refers to a strain of mice that has been maintained by successive brother to sister mating over 20 generations, and can be traced back to a common ancestor (Beck et al., 2000). Within an inbred strain, same-sex animals are essentially monozygotic twins and have two identical copies of a single allele at each locus. Each inbred strain is isogenic (genetically identical), which makes it possible to

build up a genetic profile of the strain by genotyping an individual mouse (Beck et al., 2000).

Since the establishment of the first inbred strain, DBA, by Castle and Little from Harvard University in 1902, there are now several hundred inbred strains available to researchers. Large physiological and drug-response variability has been observed among different inbred strains, but is minimal within strains (Beck et al., 2000). Therefore, comparison between multiple strains of mice is a favourable model for studying the interaction between genetic background and behavioural responses, and also for mimicking human genetic variation among individuals (Beck et al., 2000, Crabbe et al., 1990).

1.5.2 Strain variations in opioid response

Many studies have employed comparisons of multiple strains of mice to study vulnerability to drugs of dependence. Examples of aspects of opioid actions that have been assessed between strains include: opioid analgesic effect, the development of tolerance, reward, physical dependence, and withdrawal. Using these methods, significant behavioural differences have been demonstrated between different strains.

1.5.2.1 Opioid analgesic sensitivity and tolerance development differences between strains

Oliverio & Castellano (1974) firstly compared opioid analgesic sensitivity and tolerance in three different strains of mice (C57BL/6J, Balb/c and DBA/2). They demonstrated that different strains experienced different morphine analgesic effects and developed different degrees of tolerance. Specifically, the analgesic effect was

less evident in C57BL/6 mice than in Balb/c and DBA/2 mice, whilst the degree of tolerance was higher in Balb/c than in C57BL/6 mice (Figure 5). Several later studies have reported similar observations when using other opioids such as heroin and ethylketazocine (Bergeson et al., 2001, Bryant et al., 2006, Gwynn and Domino, 1984, Ho et al., 1977).

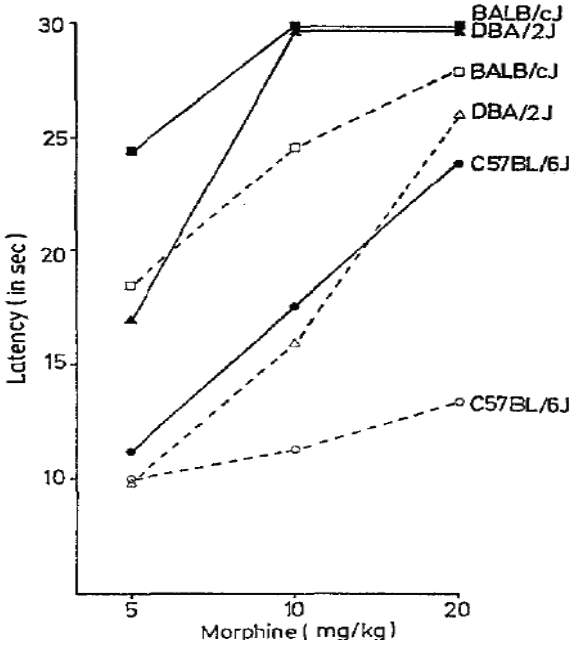


Figure 5 Morphine-induced analgesia (hotplate latency) in normal (solid line) and tolerant (broken lines) mice (C57BL/6J, Balb/c and DBA/2) on the 50 °C hotplate test (Oliverio and Castellano, 1974).

To more comprehensively study the genetic contribution to inter-strain differences, Kest et al. (2002a) compared morphine analgesic potency using the 49 °C tail-withdrawal test in 11 inbred mouse strains (129P3, A, AKR, Balb/c, C3H/He, C57BL/6, CBA, DBA/2, LP, SJL, and SWR) before and after chronic morphine treatment (using escalating dosage schedule 10 - 40 mg/kg for 3 days). The results showed a significant variation of morphine analgesia and tolerance between strains, that both the half-maximal morphine analgesic dose (AD_{50}) and the slopes of dose-

response curve were varied among strains. For acute morphine analgesia, at the lower dose (1 mg/kg), only Balb/c mice experienced morphine analgesic effect (~30% MPE); whilst, other strains did not obtain substantial analgesia (<10% MPE). However, at a higher morphine dose (21 mg/kg), most of the strains received maximal analgesic effect (> 90% MPE) but not C57BL/6 mice (< 80% MPE). Regarding the morphine analgesic tolerance, most of the strains developed significant tolerance, but not 129P3 and LP mice (Table 9). Finally, this study also demonstrated that there was no significant correlation between morphine analgesic sensitivity and the tolerance magnitude.

Table 9 Morphine analgesic potency before and after chronic morphine treatment in 11 inbred mouse strains (Kest et al., 2002a)

Strain	Half-maximal morphine analgesic dose (AD_{50}) Estimate: Morphine dose (mg/kg) and 95% CI		Potency shift [a]
	Day 1	Day 4	
129P3	13.7 (11.3 to 16.5)	11.4 (9.9 to 13.2)	0.8
A	4.4 (3.6 to 5.4)	8.4 (6.7 to 65.4)	1.9 *
AKR	13.0 (10.9 to 15.4)	42.3 (35.7 to 50.1)	3.3 *
Balb/c	2.8 (2.3 to 3.5)	12.5 (9.5 to 16.5)	4.5 *
C3H/He	5.4 (4.7 to 6.3)	42.8 (35.3 to 51.8)	7.9 *
C57BL/6	6.8 (5.5 to 8.6)	48.7 (39.3 to 60.4)	7.2 *
CBA	5.0 (4.4 to 5.8)	53.9 (43.1 to 67.4)	10.8 *
DBA2	13.8 (11.1 to 16.7)	39.6 (32.3 to 43.7)	2.9 *
LP	15.0 (11.9 to 18.8)	18.4 (14.4 to 23.5)	1.2

SJL	7.2 (5.6 to 9.3)	15.7 (12.5 to 19.7)	2.2 *
Swiss	18.7 (15.6 to 22.0)	93.8 (80.2 to 109.7)	5.0 *

[a]: Calculated as AD_{50} Day 4/AD_{50} Day 1 for that strain.

*: Significant reduction in analgesic potency compared to Day 1 ($P < 0.05$).

1.5.2.2 Opioid rewarding effect differences between strains

Genetic influences on the opioid reward effect have also been demonstrated using different mouse strains. Cunningham et al. (1992) compared morphine-induced CPP between C57BL/6 and DBA/2 strains. The results showed that morphine evoked a dose-dependent increase in activity during conditioning ($P < 0.05$), which was greater in C57BL/6 mice than in DBA/2 mice. Similar findings have been repeated by later studies, all of which displayed that C57BL/6 mice have the highest sensitivity to morphine‘s rewarding effects (Berrettini et al., 1994, Doyle et al., 2006, Orsini et al., 2005, Semenova et al., 1995). This finding has also been expanded to the rewarding effects of other opioids, such as heroin, where C57BL/6 mice have been shown to be more sensitive to heroin reward than other strains, such as Balb/c, CBA and DBA/2 (Bailey et al., 2010, Schlussman et al., 2008).

1.5.2.3 Opioid withdrawal intensity differences between strains

Finally, previous animal behavioural studies have also compared the chronic opioid-induced dependence withdrawal behaviours between different mouse strains. Physical dependence on opioids in mice can be inferred from multiple behavioural and physiological signs following precipitation of withdrawal with narcotic antagonists such as naloxone. The withdrawal symptoms of rodent animals include jumping, wet dog shaking, front paw tremors, changes in the thermoregulatory set point, autonomic

nervous system over-activity, increased gastrointestinal motility and stereotypy (El-Kadi and Sharif, 1994, Ritzmann, 1981). Of these, jumping is the most commonly tested and is widely considered the most sensitive and reliable index of withdrawal intensity in mice (El-Kadi and Sharif, 1994, Kest et al., 2002b, Metten et al., 2009, Ritzmann, 1981).

Kest et al. (2002b) studied both acute and chronic morphine-induced dependence in 11 inbred strains (129P3, A, AKR, Balb/c, C3H/He, C57BL/6, CBA, DBA/2, LP, SJL, and SWR) by comparing naloxone-precipitated withdrawal jumping responses. Three drug administration paradigms were employed in this study: acute (single injection), chronic injection (three daily morphine injections for 4 days), or chronic infusion (7 days via implanted osmotic minipumps). Most strains of mice displayed withdrawal jumping behaviour after naloxone injection (30 mg/kg). Although there were differences in the magnitude of withdrawal jumping between the three dosing paradigms, large and significant strain differences were also observed (range after acute, chronic injection and infusion: 0 to 70, 0 to 220, and 0 to 180 jumps, respectively). Figure 6 gives an example of chronic injection-induced withdrawal jumping. Importantly, the rank order of the jumping frequencies between strains was identical across all three administration methods.

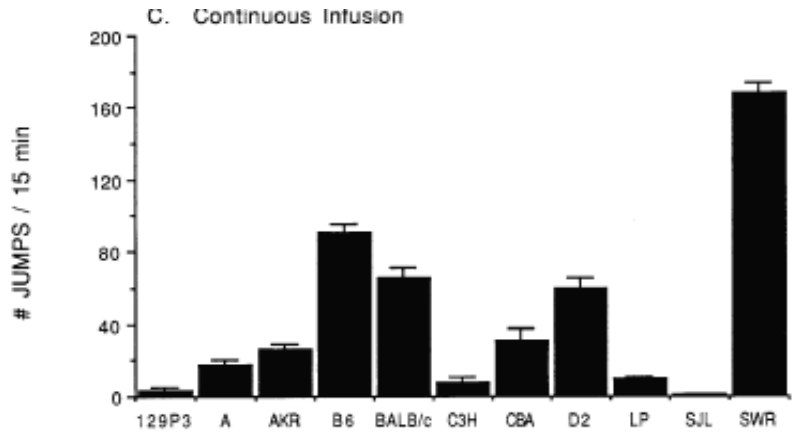


Figure 6 Mean naloxone-precipitated withdrawal jumping frequencies of 11 inbred strains of mice with chronic morphine injection (Kest et al., 2002b). Strain name abbreviated as: B6, C57BL/6; D2, DBA2. Error bars are SEM.

In addition to morphine treatment, heroin-induced dependence was also examined in 10 inbred mouse strains (129P3, A, AKR, Balb/c, C3H/He, C57BL/6, CBA, DBA/2, SJL, and SWR) (Klein et al., 2008). Naloxone-precipitated (50 mg/kg) withdrawal jumping was observed for most mouse strains (except for 129P3) after acute (50 mg/kg) or chronic heroin injection (escalating dose schedule, 5 to 20 mg/kg). There were significant jumping frequency differences between strains (range after acute and chronic heroin injection: 0 to 104 and 0 to 142 jumps, respectively; $P < 0.001$). Moreover, by comparing with morphine-induced dependence, the data showed that the rank orders of strain sensitivity for heroin and morphine were significantly correlated ($r = 0.75$ to 0.94, $P < 0.05$).

1.5.2.4 Implications of strain differences in opioid-induced behaviour for neuronal mechanism research

Several studies have used multi-strain models to investigate mechanisms that underlie opioid analgesia, tolerance, and dependence. Most studies to date have focused on neurobiological components, such as opioid receptors, dopamine systems, etc. Berrettini et al. (1994) have reported that strain differences (C57BL/6 and DBA/2) in μ opioid receptor density and regulation may be responsible for the distinct behavioural phenotypes in response to morphine. However, Doyle et al. (2006) failed to find any significant transcriptional regulation of the μ opioid receptor in these strains. A recent study has shown there are profound strain differences in μ opioid receptor G-protein activity. For example, a 2-fold higher μ opioid receptor stimulated [^{35}S]GTPγS binding was observed in the nucleus accumbens and caudate of saline-treated C57BL/6J mice when compared with DBA/2J, whilst heroin treatment decreased μ opioid receptor density in C57BL/6J mice but not DBA/2J mice (Bailey et al., 2010).

Strain differences in opioid-induced dopamine release in the striatum has also been examined. Early microdialysis studies did not find any evidence that strain differences in dopamine release could account for behavioural phenotypes (Fadda et al., 2005, Murphy et al., 2001). However, Bailey et al. (2010) have shown a higher density of dopamine transporters in the nucleus accumbens shell and caudate of heroin-treated DBA/2J mice when compared with C57BL/6J mice (Bailey et al., 2010). This suggests that, rather than dopamine itself, the dopamine transporter system may contribute to individual differences in opioid response.

1.5.3 Strain variations in immune profile

Parallel to the neurological heterogeneity between inbred mouse strains, individual strains display differing peripheral immune responses to infections (susceptibilities to disease onset and pathology), which suggests that the regulation of immune functions could also be strain-dependent. Several studies have examined the influence of the genetic background on the immune responses by comparing different mouse strains.

A research group from Russia has studied several peripheral immune parameters between different inbred mouse strains, including CBA, DBA/2, C57BL/6, and Balb/c strains (Gol'dberg et al., 2005, Masnaya et al., 2002). Their data showed that the immune functional activities, such as numbers of T cells in the bone marrow (DBA/2 versus C57BL/6 mean ± SEM: 54 ± 8 versus 45 ± 3, $P < 0.05$), and the levels of IL-2 and IL-10 production by splenic T cells (CBA versus C57BL/6 mean ± SEM: IL-2, 790.51 ± 3.31 versus 679.16 ± 30.77 pg/ml; IL-10, 273.57 ± 36.11 versus 141.60 ± 12.54 pg/ml, respectively), were lower in C57BL/6 mice than in CBA or DBA/2 mice. Although conflicting results have been observed in another study where C57BL/6 mice displayed 2- to 4-fold higher splenocyte proliferative activity than that of Balb/c mice when stimulated with ConA or LPS *in vitro* (Geiger et al., 2001), both of these studies demonstrated that the regulation of immune functions is different between mouse strains.

Wells et al. (2003) have assessed the transcriptional responses of macrophages to LPS stimulation in five inbred mouse strains, including BALB/c, C3H/ARC, C57BL/6 and DBA2. Macrophages from each strain responded to LPS in a strain-dependent manner, and the number, amplitude, and rate of induction of genes expressed varied greatly between strains. For example, upon LPS stimulation, 25% of genes were

expressed at the median level (normalised population median) in C57BL/6 mice, whilst only 12% and 15% of genes were expressed at the median level in DBA/2 and Balb/c mice, respectively. The responding time was shorter and the quantity of gene expression was higher in C57BL/6 mice than Balb/c mice, which consequently influenced the protein translation levels (Wells et al., 2003).

All the studies discussed above only assessed the peripheral immune factors' differences between different mouse strains. However, the CNS immune activity between different strains and whether there is any relationship between peripheral and CNS immune activity are still unknown. Therefore, further study is required.

Summary

In summary, the genetic heterogeneity between different inbred mouse strains leads to opioid-induced strain-dependent behavioural differences. The multiple mouse strains model has been valuable in examining the neuronal mechanisms of opioid action. Interestingly, a factor that has not been examined in these past opioid studies is the differing immune responses between different strains. With current evidence for the involvement of IL-1β in opioid actions, we could hypothesise that the differences of opioid-induced brain IL-1β expression could correlate with the opioid-induced behavioural differences between different mouse strains.

1.6 Summary, aims, and hypothesis

Genetic variations have been clearly shown to contribute to the risk of drug dependence. Pharmacogenetic studies have revealed some positive linkages between genetic variants and dependence, however, these findings only explain a small portion of the heritable risk, and more candidate genes need to be identified. Previous studies have primarily focused on the genes involved in the neuronal and metabolism pathways. One of the most important organs, the immune system, has rarely been investigated. Recent evidence has shown that opioid/alcohol-induced proinflammatory activation of immune signalling within the CNS plays a contributing role in opioid/alcohol reward, tolerance and dependence. Specifically, proinflammatory cytokines, the signalling messengers of the immune system, such as IL-1β, may be involved in the development of opioid/alcohol dependence. Therefore, the *IL-1* gene family could be a good candidate for drug dependence genetic research.

Parallel to human research, animal models using mice are valuable tools for drug dependence research. In particular, utilisation of multiple strains of mice could mimic the genetic variability between human individuals. This multi-strain model is a good tool for investigating the mechanisms behind the human genetic findings. Previous studies have demonstrated that different inbred mouse strains display different degrees of opioid-induced behaviours, such as analgesia, tolerance and withdrawal. Moreover, it has also been shown that the immune response to pathogenic factors varies significantly among different mouse strains. Therefore, the heterogeneity of the immune components between different strains, especially the opioid-induced glial-TLR4-IL-1β pathway, may contribute to the opioid-induced behavioural differences. This has not been examined.

Based on the existing literature, the major hypotheses to be tested in this thesis are;

Hypothesis 1: In humans, genetic polymorphisms associated with higher IL-1β or lower IL-1Ra expressions are associated with higher risk of opioid dependence;

Hypothesis 2: In humans, the genetic polymorphisms associated with higher IL-1β or lower IL-1Ra expressions are associated with higher risk of alcohol dependence;

Hypothesis 3: a mouse strain displaying more severe opioid tolerance or withdrawal behaviours will have higher opioid-induced brain IL-1β expression;

Hypothesis 4: genetic knockout of TLR4 or MyD88 will protect mice from developing opioid tolerance and dependence.

Therefore, the main aims of this PhD thesis are:

Aim 1: To investigate the association between genetic variability in *IL-1B* and *IL-1RN* and the risk of opioid dependence in Australian opioid dependent patients. The genetic polymorphisms examined include common *IL-B* SNPs (-31 T>C, -511 C>T, and +3954 C>T) and their haplotypes (Chapter 2), and the *IL-1RN* VNTR polymorphism (Chapter 3);

Aim 2: To investigate the association between *IL-1B* (Chapter 2) and *IL-1RN* (Chapter 3) genetic polymorphisms and alcohol dependence in Australian alcoholic patients.

The genetic polymorphisms examined including; *IL-B* SNPs (-31 T>C and -511 C>T) (Chapter 2), and the *IL-1RN* VNTR polymorphism (Chapter 3);

Aim 3: To investigate whether the heterogeneity in chronic opioid-induced brain IL-1β expression contributes to differences in opioid tolerance and withdrawal behaviours in three inbred mouse strains, Balb/c, CBA, and C57BL/6 (Chapter 4);

Aim 4: To investigate the role of opioid-activated TLR4 and its downstream signalling protein (MyD88) in opioid tolerance and withdrawal by using genetically modified mouse strains, TLR4 knockout (TLR4 KO) and MyD88 KO (Chapter 4).

Chapter 2 Association of IL-1B genetic polymorphisms with an increased risk of opioid and alcohol dependence

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The causes of drug dependence are complex, comprising both genetic and environmental factors and an interaction between both (Li and Burmeister, 2009). Recent evidence from animal models has shown that opioid- and alcohol-induced proinflammation within the CNS plays a contributing role in opioid and alcohol actions including dependence (Blanco and Guerri, 2007, Hutchinson et al., 2007). Specifically, opioids and alcohol proinflammatory activate glia, causing the release of proinflammatory cytokines, such as IL-1β, that involve in the development of dependence. Therefore, genetic polymorphisms that alter the expression and/or function of proinflammatory cytokine genes especially IL-1β may alter the risk of opioid or alcohol dependence in humans.

The gene encoding for IL-1β, *IL-1B*, is highly polymorphic (http://www.genecards.org). Several common SNPs have been identified, which may affect IL-1β expression, including *IL-1B* -511C>T (rs16944), *IL-1B* -31T>C (rs1143627), and *IL-1B* 3954C>T (rs1143634) (Smith and Humphries, 2009). In a previous study, *IL-1B* -511 C>T SNP was shown to be associated with the risk of alcohol dependence in a Spanish population in that the C allele frequency was significantly higher in alcoholic patients when compared with the control group (Pastor et al., 2005). However, this finding has yet to be repeated and expanded to other drugs of dependence. Therefore the aims of this study were to investigate: a) the association between genetic variability of the *IL-1B* and the risk of opioid dependence; b) the association between genetic variability of the *IL-1B* and the risk of alcohol dependence in Australian alcoholic patients in order to validate the previous Spanish findings.

We have found that the *IL-1B* -31T and -511C alleles were more frequent in the opioid dependent patients compared to the control group [OR, 95% CI = 1.91 (1.14–3.20), corrected *P* = 0.043, for *IL-1B* -511C>T; and OR, 95% CI = 1.74 (1.02–2.97), *P* = 0.066, for *IL-1B* -31T>C], which suggests an association between these SNPs and the risk of opioid dependence. This finding provides the first human evidence regarding the contribution of IL-1β to opioid dependence development. Similar to observations in opioid dependent patients, *IL-1B* -31T and -511C alleles were more frequent in the alcohol dependent patients compared to the control group, which indicates the *IL-1B* -31 and -511 SNPs are also risk traits for alcohol dependence. The latter result confirms the previous Spanish finding of Pastor et al. (2005) that also observed an association between the *IL-1B* -511C allele with the susceptibility to alcohol dependence.

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Chapter 3 Lack of association between IL-1RN VNTR polymorphism and risk of opioid dependence and alcohol dependence

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In the first study (Chapter 2), a genetic variability of *IL-1B* was discovered that may affect the risk of development of opioid dependence and confirmed its role in alcohol dependence in humans. I then continued to explore genetic candidates that could affect drug dependence risk. IL-1Ra, as the endogenous antagonist of IL-1 receptor, is also an important cytokine that modulates proinflammation. The *IL-1RN* gene has a VNTR polymorphism in intron 2, which was shown to affect both IL-1Ra and IL-1β production *in vitro* and **2* allele carriers may have a heightened and prolonged proinflammatory immune response (Hurme and Santtila, 1998, Santtila et al., 1998). Importantly, this polymorphism has also been associated with alcohol dependence in previous studies (Chen et al., 2005, Pastor et al., 2000, Pastor et al., 2005, Saiz et al., 2009). Moreover, a sex difference in *IL-1RN* VNTR polymorphism and the association between the *IL-1RN* genotype and alcohol dependence have been reported (Saiz et al., 2009). The aims of this study were to investigate: a) the association between genetic variability of the *IL-1RN* VNTR polymorphism and the risk of opioid dependence; b) the association between the *IL-1RN* VNTR polymorphism and the risk of alcohol dependence in Australian alcoholic patients in order to validate previous findings; c) sex differences in the *IL-1RN* VNTR polymorphism and its association with drug dependence; and d) the influence of image capture equipment to correctly classify the *IL-1RN* genotypes.

No association was found between the *IL-1RN* VNTR polymorphism and alcohol or opioid dependence. This study did not replicate previous findings (Chen et al., 2005, Pastor et al., 2000, Pastor et al., 2005, Saiz et al., 2009) that reported higher *IL-1RN*1/*1* genotype and **1* allele frequencies in alcohol dependent patients compared to healthy controls. No sex differences were observed in allele or genotype

frequencies within each group. Comparison of the results obtained from two image capture systems revealed that **1/*2* genotype was misclassified as **1/*1* genotype in images with low resolution (confirmed by sequencing analysis of random samples). This highlighted the importance of high resolution image acquisition when determining the presence of the *IL-1RN*2* VNTR allele and possibly other similar VNTR PCR assays. Therefore, until further investigations are made, past publications using potentially older imaging technology should be treated with caution.

Abstract

Aims: To investigate the association between the *IL-1RN* variable number of tandem repeats (VNTR) polymorphism and the risk of alcohol and opioid dependence, and the influence of image capture equipment on genotyping assignment.

Methods: 98 alcohol dependent, 58 opioid dependent, and 55 non-dependent healthy control subjects were genotyped for *IL-1RN* VNTR with polymerase chain reactions (PCR). Two image capture systems were used to document gel images.

Results: In low pixel PCR images, the *IL-1RN*1/*2* genotype was incorrectly classified. No difference was observed between allele or genotype frequencies in alcohol and opioid dependent groups compared to controls (allele: $\chi^2 = 5.86$, $P = 0.12$ and $\chi^2 = 3.36$, $P = 0.34$, respectively; genotype: $\chi^2 = 5.06$, $P = 0.28$, and $\chi^2 = 0.82$, $P = 0.93$, respectively).

Conclusions: In contrast to previous studies, no association was found between the *IL-1RN* VNTR polymorphism and alcohol or opioid dependence. The results highlight the importance of high resolution image acquisition when determining the presence of the *IL-1RN*2* VNTR allele and possibly other similar VNTR PCR assays.

Introduction

Interleukin-1 receptor antagonist (IL-1Ra) is an endogenous antagonist of the proinflammatory cytokine interleukin-1 (IL-1, IL-1α and IL-1β). It binds to the IL-1 cell surface receptor but does not activate the receptor or trigger signal transduction. Therefore as a competitive inhibitor, IL-1Ra plays an important role in regulating the inflammatory process (McIntyre et al., 1991).

The gene coding for IL-1Ra, *IL-1RN*, is located on chromosome 2 in close proximity to other *IL-1* genes (Steinkasserer et al., 1992). The *IL-1RN* gene has a variable number of 86 bp tandem repeats (VNTR) in intron 2, the number of which determines the allele nomenclature: alleles *IL-1RN*1*, *IL-1RN*2*, *IL-1RN*3*, *IL-1RN*4*, and *IL-1RN*5* consist of four, two, five, three and six repeats, respectively (Tarlow et al., 1993). The **1* allele is the most common (with frequency ~70% for Caucasians), followed by the **2* allele (with frequency ~20% for Caucasians) in all populations, with the remaining alleles occurring with a frequency of less than 10% in most populations (Hurme and Santtila, 1998; Tarlow et al., 1993). Evidence shows that the *IL-1RN*2* allele is associated with both enhanced IL-1Ra production and IL-1β expression *in vitro*, and **2* allele carriers may have a heightened and prolonged proinflammatory immune response (Hurme and Santtila, 1998; Santtila et al., 1998). As such, *IL-1RN* VNTR genotypes have been associated with several inflammatory conditions, such as ulcerative colitis, lupus erythematosus, and osteoporosis (Langdahl et al., 2000; Tjernstrom et al., 1999; Tountas et al., 1999).

Recent evidence has shown that drug-induced proinflammatory activation of immune signaling within the central nervous system (CNS) plays a contributing role in the development of drug dependence (Blanco and Guerri, 2007; Hutchinson et al., 2007). Specifically, animal data have shown that IL-1 signaling pathways are significantly

involved in opioid- and alcohol-induced behavioral response, for example: blockade of IL-1β signaling by administration of IL-1Ra altered the behavioral response to alcohol (Wu et al., 2011), and prolonged and potentiated opioid analgesia (Shavit et al., 2005); and chronic morphine-induced brain IL-1β expression were significantly associated with opioid withdrawal (Liu et al., 2011). Furthermore, LPS-induced activation of proinflammatory signaling promotes alcohol consumption in mice (Blednov et al., 2011). Moreover, genetic variations in proinflammatory cytokines have been associated with altered risk to drug dependence in humans (Pastor et al., 2005; Marcos et al., 2008; Liu et al., 2009; Saiz et al., 2009). A number of research groups have investigated the *IL-1* gene cluster polymorphisms and alcoholism in different ethnic populations and have identified that several genetic polymorphisms are associated with susceptibility to alcoholism (Chen et al., 2005; Pastor et al., 2000; Pastor et al., 2005; Saiz et al., 2009). The results showed that the frequencies of the *IL-1RN*1* and *IL-1B* (gene that encodes for IL-1β) *-511C* alleles were significantly higher in alcoholic patients when compared to controls (Chen et al., 2005; Pastor et al., 2000; Pastor et al., 2005; Saiz et al., 2009; Takamatsu et al., 1998). Recent work conducted by our group has confirmed the involvement of *IL-1B* polymorphisms (*IL-1B -511 C>T* and *-31 T>C*) in alcohol dependence (Liu et al., 2009). However, the association between *IL-1RN* VNTR and alcoholism is yet to be replicated in Australian populations.

In addition to the alcohol dependence associations, we have also observed an association between *IL-1B* polymorphisms and risk of developing opioid dependence (Liu et al., 2009), raising the possibility of a generalised association between opioid dependence with other *IL-1* gene cluster polymorphisms. Given that the *IL-1RN* genetic polymorphism has been related to the variation in post-operative morphine

consumption (Bessler et al., 2006), it is possible that the *IL-1RN* VNTR could also be a risk factor for development of opioid dependence. This association is yet to be investigated.

Finally, a sex difference in *IL-1RN* VNTR polymorphism and the association between the *IL-1RN* genotype and alcohol dependence has been reported; The *IL-1RN*1* allele was more frequent in men, and the *IL-1RN*2* allele was more prevalent in women (Bessler et al., 2007). While Saiz et al. reported a higher *IL-1RN*1/*1* genotype frequency being associated with alcohol dependence only in men (Saiz et al., 2009). These findings have not been replicated.

The aim of this study was to examine the association between genetic variability of *IL-1RN* VNTR polymorphism and the risk of opioid and alcohol dependence in an Australian Caucasian population. Furthermore, gender differences in association between the *IL-1RN* VNTR polymorphism and dependence was also examined. In addition, the influence of image capture equipment used during the standard polymerase chain reaction (PCR) amplification assay employed to correctly classify the *IL-1RN* genotypes was assessed during the course of the study.

Methods

Subjects

A retrospective case-control association study was designed to examine whether *IL-1RN* genetic variants contribute to an individual's susceptibility to opioid and alcohol dependence in an Australian population. All the subjects were from previous clinical studies conducted by the Discipline of Pharmacology at the University of Adelaide. Fifty-eight opioid dependent subjects who were receiving methadone maintenance therapy, 98 alcohol dependent subjects who were receiving naltrexone treatment and 55 non-alcohol/opioid dependent healthy controls were included (Table 1 shows demographic data). There was no comorbidity with alcohol dependence in the opioid dependent group and vice versa, and alcohol consumption in healthy controls was within the range stipulated in the Australian safe alcohol use guidelines (National Health and Medical Research Council of Australia). All studies were approved by the Royal Adelaide Hospital Research Ethics Committee and all subjects gave written informed consent.

Genotyping

Genomic DNA was isolated from whole blood using a QIAamp® DNA Mini kit (QIAGEN Pty Ltd, Doncaster, VIC, Australia). The VNTR of intron 2 of the *IL-1RN* gene were determined by PCR modified from that previously described (Tarlow et al., 1993) using the following primers: forward, 5'-CCC TCA GCA ACA CTC CTA TT-3'; reverse, 5'-CTG GTC TGC AGG TAA ATA GA-3'. PCR reactions were carried out in a 50 μl volume containing 100 ng genomic DNA, 0.4 μM of each primer, 0.1

mM of each dNTP, 1x ThermoPol buffer and 2.5 Units of Taq DNA Polymerase (New England Biolabs, Inc., distributed by Genesearch Pty Ltd, Arundel, QLD, Australia). The reactions were performed in a programmable thermal cycler (PTC-200TM MJ Research Inc., distributed by GeneWorks Pty Ltd, Thebarton, SA, Australia) under the cycling conditions of: denaturation at 95°C for 3 min; 36 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 35 sec, and extension at 72°C for 3 min; and a final extension step at 72°C for 5 min. The number of tandem repeats was determined by the size of PCR products (base pairs, bp) visualised on a 4 % 2:1 Omnigel-Sieve (Adelab Scientific Pty Ltd, Thebarton, SA, Australia) : Agarose I (AMERSCO, Inc., distributed by Adelab Scientific Pty Ltd) gel stained with ethidium bromide. A pUC19/HpaII DNA molecular weight marker (GeneWorks Pty Ltd) was used as reference. PCR products of the following lengths allowed allele assignment based on the number of tandem repeats: 239 bp - **2* (2 repeats), 325 bp - **4* (3 repeats), 411 bp - **1* (4 repeats), 500 bp - **3* (5 repeats) and 586 bp - **5* (6 repeats). Two image capture systems were used to document gel images, the Kodak Digital Science DC40 (0.38M Pixels, Kodak, Sydney, NSW, Australia) and the GE Healthcare ImageQuant 150 (10M Pixels, GE Healthcare, Adelaide, SA Australia). All assays were run with a negative (no DNA template) control to ensure that there was no contamination of assay reagents or non-specific amplification.

Ten randomly selected PCR products were sequenced to confirm specific amplification of the required portion of the *IL-1RN* gene and number of VNTRs for allele classification. Sequencing was performed using the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) together with the forward primer (0.27 μM) from the PCR assay, and analysed on the ABI Prism 3700 DNA Sequencer (Applied Biosystems).

Statistical analysis

Genotype distribution was examined for significant departure from Hardy-Weinberg equilibrium by χ^2 test. Allele and genotype frequencies between the groups and genders were compared using the χ^2 tests and the Fisher's exact test with Odds Ratios (OR) and 95% confidence intervals (95% CI) (GraphPad Prism 5.02, GraphPad Software Inc., San Diego, CA, USA). A *P*-value of ≤ 0.05 was considered to be statistically significant. The statistical power of the study was estimated by GraphPad StatMate 2.00 (GraphPad Software Inc., San Diego, CA, USA).

Results

The genotypes of opioid dependent and healthy control subjects were firstly determined by using Kodak Digital Science DC40 camera. Over the course of the study, the photographic equipment was upgraded and anomalies in the **1/*2* genotype were observed. As such, opioid dependent and healthy control samples were reanalysed using the new GE Healthcare ImageQuant 150. The genotypes of alcohol dependent subjects were only determined with the ImageQuant 150.

Comparison of results obtained with different image capture systems

Figure 1 shows the image of the same gel captured by the two cameras. *IL-1RN *1/*1*, **2/*2*, **1/*3*, and **1/*4* genotypes were readily detected on the images obtained from both of the cameras. However, the band of the **2* allele in **1/*2* heterozygotes was very faint. Hence, in the low pixel image (from DC40), the presence of the **2* allele was undetected resulting in incorrect classification of some individuals as a **1/*1* genotype. The allele and genotype frequencies based on image capture from both cameras are shown in Tables 2 and 3. The majority of the **1/*2* genotypes in opioid dependent and healthy control subjects were misclassified (confirmed by sequencing analysis) as these assays were assessed in the first instance when using DC40. All genotype assignment results were subsequently corrected using images captured by the ImageQuant 150.

Alleles and genotypes in opioid and alcohol dependent subjects, versus healthy controls

Only the results obtained by using the ImageQuant 150 were included for analysis. Four alleles (**1*, **2*, **3* and **4*) and seven genotypes (**1/*1*, **1/*2*, **2/*2*, **1/*3*. **3/*3*, **1/*4* and **4/*4*) were observed. Genotype frequencies were not significantly different from that expected under Hardy-Weinberg equilibrium ($P > 0.75$) in all three populations.

No significant differences in allele or genotype frequencies were observed between the opioid dependent and healthy control groups (allele: $\chi^2 = 3.36$, $P = 0.34$; genotype: $\chi^2 = 0.82$, $P = 0.93$). There was no difference in the **1*, **2* and **3* allele distributions between these two groups (OR (95% CI) = 1.04 (0.60 to 1.80), $P = 0.89$, 0.89 (0.50 to 1.60), $P = 0.77$, and 1.93 (0.56 to 0.59), $P = 0.38$ for the **1*, **2*, and **3* alleles respectively).

No significant differences in allele or genotype frequencies were observed between the alcohol dependent and healthy control groups (allele: $\chi^2 = 5.86$, $P = 0.12$; genotype: $\chi^2 = 5.06$, $P = 0.28$). There was no difference in the **1*, **2* and **3* allele distributions between these two groups (OR (95% CI) = 1.32 (0.80 to 2.18), $P = 0.30$, 0.93 (0.55 to 1.56), $P = 0.79$, and 0.13 (0.15 to 1.22), $P = 0.56$ for the **1*, **2* and **3* alleles respectively).

No significant sex differences were observed in allele or genotype frequencies within each group ($P > 0.5$), and with regard to the association between genotype and opioid and alcohol dependence for each sex ($P > 0.48$).

Based on the data from previous studies, the **1* allele differences between alcohol dependent and healthy control populations ranged from 8 to 19% (Pastor et al., 2000; Saiz et al., 2009). Therefore, based on a hypothesised 13% allele difference, statistical power of this study was more than 70% to detect an absolute 13% and less than 20% to detect an absolute 6% (observed in current study) **1* allele differences between the

alcohol dependent and non-dependent healthy control populations (using a two-tailed significance level of 0.05).

Discussion

This study examined the possible relationship between the *IL-1RN* VNTR polymorphism and the risk of alcohol and opioid dependence in an Australian population, and failed to detect an association.

With regard to alcohol dependence, this study did not replicate previous findings that reported higher *IL-1RN*1/*1* genotype and **1* allele frequencies in alcoholic patients compared to healthy controls in both Spanish Caucasian and Chinese populations (Chen et al., 2005; Pastor et al., 2000; Saiz et al., 2009). Comparing the data from Spanish studies with Caucasian populations, similar *IL-1RN*1* and **2* allele and **1* homozygote and heterozygote genotype frequencies were observed in our populations with Saiz et al. (2009) study (allele: **1* 71 - 79 versus 66 - 71, **2* 20 – 27 versus 28 - 29; genotype: **1/*1* 51 – 65 versus 42 – 50, **1/*2* 25 – 38 versus 40 - 41). Further, the allele and genotype frequencies from our work and Saiz et al. (2009) study are similar to previous reported in other Caucasian populations (**2* allele frequency 20 - 30%, **1/*2* genotype frequency 30 - 40%) (Hurme and Santtila, 1998; Tarlow et al., 1993; Zabaleta et al., 2008). However, a much higher frequency of **2* alleles and **1/*2* heterozygote genotypes was observed in the current study compared to Pastor et al. (2000) (allele: **2* 28 – 29 versus 2 – 17; genotype: **1/*2* 40 – 41 versus 0 – 17).

With regard to opioid dependence, this is the first study to investigate the *IL-1RN* VNTR polymorphism in this population. In our previous study, we reported a positive association between genetic variability of the *IL-1B* gene and the risk of developing

opioid dependence (Liu et al., 2009). However, the data from this study indicate that this association is not generalised to the *IL-1RN* VNTR polymorphisms.

This observation may appear to not to complement the animal findings demonstrating that IL-1Ra altered alcohol and opioid behavioral responses. However, it remains unknown if other as yet unidentified *IL-1RN* polymorphisms may have significant functional impact on IL-1Ra expression, and hence may be associated with drug dependence. In addition, genetic variation of other IL-1 pathway regulators, such as the IL-1 receptor and IL-1β converting enzyme (caspase-1) genes, has yet been examined. Therefore, further research is required to investigate possible associations between these candidate genes and drug dependence.

Sex differences in *IL-1RN* VNTR polymorphism allele and genotype frequencies have been reported in the Bessler et al. (2007) study. Moreover, Saiz et al. (2009) observed that *IL-1RN*1/*1* genotype was associated with alcohol dependence only in men. However, our study did not find any sex effect on *IL-1RN* polymorphism allele or genotype frequency distribution or on the association between the polymorphism and alcohol dependence in either sex.

One of the limitations of the current study however, is the sample size, with power analysis revealing that if there was a real association between the *IL-1RN* polymorphisms and opioid or alcohol dependence the current study would not have been adequately powered to detect this. However, due to no preexisting clinical opioid dependency population data, no *a priori* hypothesis could be formed, necessitating this study to assess any associations. Our data indicate that over 2000 subjects per group would be required to detect a doubling in allele frequency differences between the drug dependent and healthy control groups. Furthermore, due to the unbalanced number across the male: female ratio in healthy control and opioid dependent

populations, the sex effect could not be properly examined. Future study of patient cohort with balanced male: female ratio is required to assess the sex effect on *IL-1RN* VNTR polymorphism.

Due to a laboratory equipment upgrade, we used two image capture systems to document gel images. Genotyping of opioid dependent and healthy control subjects were conducted prior to this upgrade. When genotyping the alcohol dependent subjects using the new system, a much higher frequency of **1/*2* genotype and a low signal intensity for **2* PCR product was observed. Therefore, genotyping of the opioid dependent and healthy control subjects was repeated. The reanalysis revealed the misclassification of **1/*2* as **1/*1* genotype, and this was confirmed by sequencing analysis of random samples. For this *IL-1RN* VNTR PCR assay, because of lower signal intensity of the **2* PCR product and no clear intensity difference of **1* allele band between the homozygote and heterozygote, it is possible that the **2* allele in the **1/*2* heterozygote genotype could be missed in images with low resolution, and hence individuals misclassified as **1/*1* genotypes. For all other genotypes, such as **1/*3* and **1/*4* heterozygotes, no intensity variation was observed between the two bands. The reason for this phenomenon is not known and this problem may also exist for other PCR genotyping methods designed to detect VNTR polymorphisms.

This misclassification highlights the requirement of high resolution image capture equipment when genotype classification is based on the size of PCR products alone or in tandem with RFLP analysis dependent on the quality of image capture. Indeed, differences between our study and that of Pastor and colleagues, who observed similar allele and genotype frequencies to our study using the insufficiently powered DC40 camera, may be explained by misclassification in their study (Pastor et al., 2000). However, it was not reported what imaging equipment they utilised and this is a

common issue across previous literature. Therefore, until further investigations are made, past publications using potentially older imaging technology should be treated with caution and it would be advantageous that future research documents the resolution of image capture equipment.

In conclusion, no association was found between the *IL-1RN* VNTR polymorphism and opioid or alcohol dependence in an Australian population. The electrophoresis gel images from two capture systems highlight the importance of high resolution image documentation when classifying the **2* allele in the *IL-1RN* VNTR and possibly similar VNTRs.

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Tables

Table 1 Demographic Data

Subjects	Female: Male	Age (y) (Mean ± SD)	Daily Methadone/Alcohol use (Mean ± SD, Range)
Opioid Dependent	11 : 47	35.5 ± 5.6	122.1 ± 85.2 mg, 15 – 300 mg
Alcohol Dependent	42 : 56	42.6 ± 8.7	204 ± 131 g, 57 – 900 g
Healthy Control	19 : 36	34.7 ± 17.6	

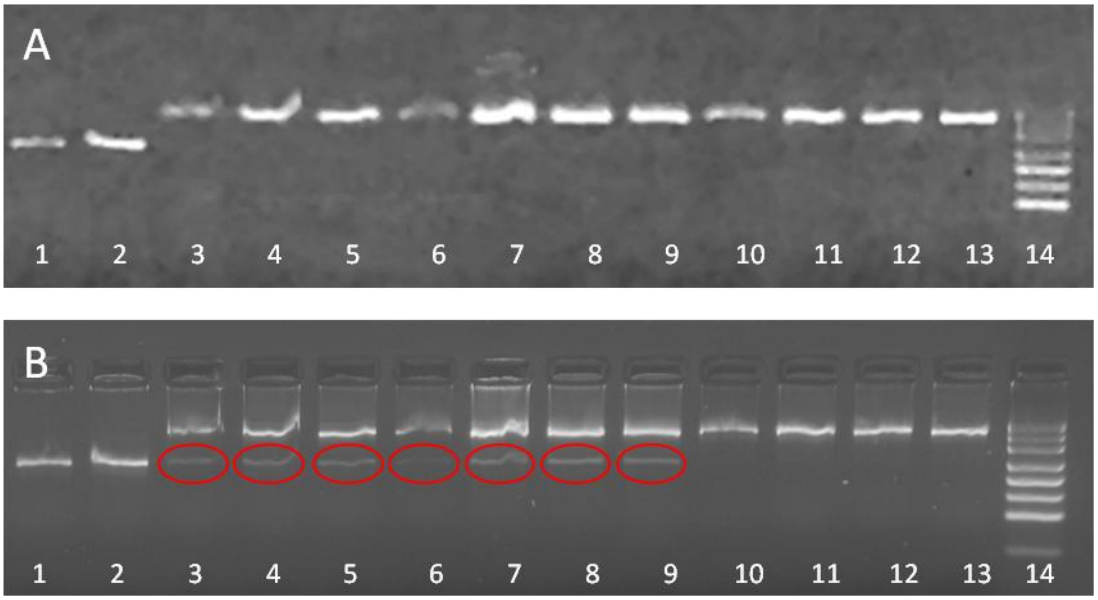
Table 2 *IL-1RN* allele frequencies (n (%)) in opioid (OD) and alcohol dependent (AD), and healthy control (HC) subjects determined with the Kodak Digital Science DC40 and the GE Healthcare ImageQuant 150 image capture systems. Odds ratios (95% CI) compare data from the GE Healthcare ImageQuant 150 system only.

	Kodak Digital Science DC40			GE Healthcare ImageQuant 150			Odds Ratio (95% CI), *P*-value	
Allele	Opioid Dependent	Alcohol Dependent	Healthy Control	Opioid Dependent	Alcohol Dependent	Healthy Control	OD vs HC	AD vs HC
**1*	96 (82.8)		89 (80.9)	77 (66.4)	140 (71.4)	72 (65.5)	1.04 (0.60 - 1.81), 0.89	1.32 (0.80 – 2.18), 0.30
**2*	12 (10.3)		15 (13.6)	31 (26.7)	54 (27.6)	32 (29.1)	0.89 (0.50 – 1.59), 0.77	0.93 (0.55 – 1.56), 0.79
**3*	8 (6.9)		4 (3.7)	8 (6.9)	1 (0.5)	4 (3.6)	1.93 (0.56 - 0.59), 0.38	0.13 (0.15 – 1.22), 0.56
**4*	0		2 (1.8)	0	1 (0.5)	2 (1.8)		

Table 3 *IL-1RN* genotype frequencies (n (%)) in opioid (OD) and alcohol dependent (AD), and healthy control (HC) subjects determined with the Kodak Digital Science DC40 and the GE Healthcare ImageQuant 150 image capture systems.

	Kodak Digital Science DC40			GE Healthcare ImageQuant 150		
Allele	Opioid Dependent	Alcohol Dependent	Healthy Control	Opioid Dependent	Alcohol Dependent	Healthy Control
**1/*1*	45 (77.6)		40 (72.7)	26 (44.8)	49 (50.0)	23 (41.8)
**1/*2*	0		5 (9.1)	19 (32.8)	40 (40.8)	22 (40.0)
**2/*2*	6 (10.3)		5 (9.1)	6 (10.3)	7 (7.2)	5 (9.1)
**1/*3*	6 (10.3)		4 (7.3)	6 (10.3)	1 (1.0)	4 (7.3)
others	1 (1.8)		1 (1.8)	1 (1.8) (**3/*3*)	1 (1.0) (**1/*4*)	1 (1.8) (**4/*4*)

Figure 1 Image comparison of the 4% 2:1 Omnigel Sieve: Agarose I gel with *IL-1RN* VNTR PCR products captured by the Kodak Digital Science DC40 (A) and the GE Healthcare ImageQuant 150 (B) systems.



In Fig 1A (genotypes in italicised font): Lanes 1 - 2: **2/*2*; Lanes 3 - 13: **1/*1*; Lane 14: pUC Marker;

In Fig 1B (genotypes in italicised font): Lanes 1 - 2: **2/*2*; Lanes 3 - 9: **1/**2; Lanes 10 - 13: **1/*1*; Lane 14: pUC Marker.

Circles indicates the presence of the **2* allele band of **1/*2* heterozygote genotype that were invisible in Fig 1A.

Chapter 4 Naloxone-precipitated morphine withdrawal jumping and brain IL-1β expression: comparison of different mouse strains

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The influences of genetic polymorphisms in the human *IL-1* gene family on the risk of drug dependence were assessed in the previous chapters. This chapter aimed to investigate the mechanisms behind these findings from the first two studies presented. Previous studies comparing rodent strains have demonstrated the important contribution of genetic background to individual differences in opioid tolerance and dependence. I designed an animal study using multiple mouse strains to further investigate the involvement of opioid-induced brain glia-TLR4-IL-1β pathway in opioid actions.

This study was based on previous animal studies examining neuronal mechanisms of opioid action, which have shown differences in opioid-induced analgesic tolerance and dependence withdrawal severity between different inbred mouse strains (Kest et al., 2002a, Kest et al., 2002b, Metten et al., 2009). A factor that has not been examined in these past studies is the differing proinflammatory responses of the different mouse strains, as it has also been shown that different mouse strains regulate immune function differently (Gol'dberg et al., 2005, Masnaya et al., 2002). Therefore, the model using multiple mouse strains can be a valuable tool for examining the role of opioid-induced brain IL-1β expression. Furthermore, it is well known that different brain regions contribute differently to opioid response and dependence (Kosten and George, 2002). However, opioid-induced IL-1β responses in different brain nuclei of different strains of mice, which have differing propensities to opioid dependence, are yet to be systematically investigated. In addition, the behavioural consequence of TLR4 and MyD88-dependent signalling pathways has yet to be examined in opioid tolerance and withdrawal. Therefore, the aims of this study were to investigate: a) whether the heterogeneity of chronic opioid-induced IL-1β expression contributes to

differences in dependence withdrawal behaviour; b) the role of the TLR4 signalling pathway and brain IL-1β expression in opioid-induced tolerance and withdrawal behaviours; and c) the genetic heterogeneity of IL-1β (*IL-1B*) and TLR4 (*TLR4*) genes for all mouse strains used by sequencing these genes.

The results demonstrated that there is main effect of morphine treatment on IL-1β expression in all brain nuclei analysed, including hippocampus, brain stem, midbrain and diencephalon regions, medial prefrontal cortex, and cortex. Comparison between wild-type strains of mice demonstrated that elevated hippocampal IL-1β levels contribute to withdrawal jumping behaviour. Genetic knockout of TLR4, but not MyD88 protected against the development of analgesic tolerance. Genetic differences of *IL-1B* and *TLR4* genes alone did not explain the heterogeneity of dependence behaviour between mouse strains. Together, these data further support the involvement of opioid-induced glia-TLR4-IL-1β signalling pathway in the development of opioid dependence and provide mechanistic evidence in support of my human genetic findings presented in chapter 2. Specifically, individuals with higher IL-1β expression alleles (i.e. *IL-1B* -31T/-511C) may have higher opioid-induced brain IL-1β expression, and thereby, may experience worse withdrawal, which could facilitate these individuals to administer opioids more frequently or for a longer time and finally lead to dependence.

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Chapter 5 Discussion

As shown by family, twin and adoption studies, genetic components contribute significantly to the risk of opioid and alcohol dependence (Agrawal and Lynskey, 2008). However, the identification of specific genetic traits has progressed slowly and limited by the incomplete knowledge of the drugs' mechanisms of dependencies. Recently, opioid- and alcohol-induced CNS immune signalling has been identified as contributing to the development of dependence, which is an important complement to the known neuronal mechanisms. Specifically, proinflammatory cytokines, such as IL-1β, and its associated signalling pathways may be involved in the development of opioid and/or alcohol dependence. Therefore, the *IL-1* gene family are potential candidates impacting on the heritable risk of drug dependence. Importantly, studies originally targeted to alcohol-induced liver disease have found significant evidence that genetic polymorphisms are associated with the risk of some inflammatory diseases are also associated with increased risk of alcohol dependence, especially for the *IL-1* gene family (Chen et al., 2006, Pastor et al., 2005, Saiz et al., 2009). This has yet to be studied in opioid dependence. As such, the major aims of this thesis were to investigate the association between *IL-1* genetic variability and the risk of opioid dependence, to validate previous *IL-1* genetic findings in alcohol dependence, and to investigate the mechanisms of how IL-1β and its associated signalling pathways contribute to opioid dependence behavioural consequences.

5.1 Human genetic study

5.1.1 Genetic association of IL-1B polymorphisms with risk of opioid dependence

The retrospective study presented in chapter 2 identified significant associations between *IL-1B* SNPs and the risk of opioid dependence. Higher frequencies of the *IL-1B* -31T and -511C alleles were found in opioid-dependent patients compared with healthy controls. However, these associations did not generalise to all *IL-1* gene family polymorphisms, as no allele or genotype frequency difference was observed between opioid-dependent and healthy control populations for *IL-1B* +3954 C>T SNP (chapter 2) and the *IL-1RN* VNTR polymorphism (chapter 3). These results provided the first human evidence regarding the contribution of IL-1β to opioid dependence development.

How does *IL-1* genetic variability influence opioid dependence? This is possibly caused by the individual differences in IL-1β production. Several common polymorphisms are shown contributing to individual differences in IL-1β expression (Bidwell et al., 1999, Hollegaard and Bidwell, 2006). *IL-1B* +3954 C>T, -511 C>T, and -31 T>C SNPs and *IL-1RN* VNTR polymorphisms are shown to be associated with a wide range of chronic inflammatory and autoimmune conditions; however, data regarding their functional effects is currently controversial. The data from this thesis are in agreement with the functional relevance of *IL-1B* -511 C>T, and -31 T>C SNPs, but not with the one of *IL-1B* +3954 or *IL-1RN* VNTR polymorphisms. A probable explanation for these observations is that the -31 position of the *IL-1B* gene occurs within the TATA sequence of the promoter region, an important functional sequence in the transcription initiation process, such that the T allele completes the TATA sequence and leads to higher *IL-1B* gene transcription and finally higher IL-1β

expression, whilst the C allele has an altered sequence and hence does not. As a result of the linkage disequilibrium, the functional impact of *IL-1B* -511C/T SNP is most likely to be linked to this TATA box polymorphism. With regard to the *IL-1B* +3954 and *IL-1RN* VNTR polymorphisms, we cannot simply exclude the functional consequences of these polymorphisms based on the data from this thesis, as they may be linked to other genetic variants or involved in some pathways of specific inflammatory conditions, therefore, further research is required to validate their functional implications.

In terms of the actual proinflammatory response to opioid stimulation, it is likely that individuals with *IL-1B* -31 T allele will have a higher CNS IL-1β expression compared with carriers of the C allele. Consequently, it can be hypothesised that opioid users who carry the -31 T allele will have higher expression of IL-1β and could experience more opioid reward, greater tolerance or worse withdrawal symptoms, and thereby, they may use more opioids and for a longer time, placing themselves at a higher risk of developing opioid dependence compared to people with lower IL-1β expression. Results from the study presented in chapter 4 partly supported this hypothesis, and will be discussed in a later section.

5.1.2 Genetic association of IL-1B polymorphisms with risk of alcohol dependence

The retrospective study presented in chapter 2 found significant *IL-1B* -31 and -511 allele frequency differences between alcohol-dependent and healthy control populations, with the -31T and -511C allele frequencies higher in the alcohol-

dependent group. However, the study presented in chapter 3 did not find any association between the *IL-1RN* VNTR polymorphism and alcohol dependence.

A number of research groups have investigated the *IL-1* gene cluster polymorphisms and alcoholism in different ethnic populations. Interestingly, most of these studies were originally aimed to examine the relationship between *IL-1* genetic polymorphisms and alcohol-induced liver disease rather than the alcohol dependence. They identified a positive association of *IL-1B* and *IL-1RN* gene variants with the susceptibility to alcoholism, but not with the risk of alcoholic liver disease (Chen et al., 2005, Pastor et al., 2000, Pastor et al., 2005, Saiz et al., 2009, Takamatsu et al., 1998). Results from these studies showed that the frequencies of the *IL-1B* -511C (Pastor et al., 2005) and *IL-1RN *1* (Chen et al., 2005, Pastor et al., 2000, Saiz et al., 2009) alleles were significantly higher in alcoholic patients when compared to controls. The finding of this thesis confirmed the genetic link between the *IL-1B* -511 SNP and alcohol dependence; however, in contrast to previous studies, we did not find any association between the *IL-1RN* VNTR polymorphism and alcohol dependence in our patient population.

Numerous *in vivo* animal studies and *in vitro* glial cell culture studies have revealed that alcohol can induce CNS glial activation and release proinflammatory cytokines. For example, Vallés et al. (2004) have provided direct evidence showing that chronic alcohol consumption increases glial release of proinflammatory cytokines and other inflammatory mediators in rat brain (Vallés et al., 2004). Floreani et al. (2010) have confirmed these findings by using human glial cells obtained from second trimester human foetal brain tissues, displaying that alcohol exposure elevated proinflammatory

cytokine release *in vitro*. In addition, mechanistic studies have shown that the IL-1R1/TLR4 signalling pathway plays a pivotal role in alcohol-induced CNS immune activation and neural proinflammation (Blanco and Guerri, 2007, Blanco et al., 2008). Adding to these data, the *IL-1B* genetic findings from this thesis and previous studies provide further human evidence supporting the role of neuroinflammation in alcohol dependence development.

5.1.3 IL-1B shared genetic traits for drug dependence?

Despite the disparate mechanisms of action and pharmacological effects, all drugs of dependence (e.g. opioids, alcohol, cocaine, nicotine, etc.) lead to certain common effects after drug exposure (Robbins et al., 2007). All drugs induce a reward effect, which promotes repeated drug intake and leads eventually to dependence. Furthermore, they also produce similar pathological features as a consequence of neuroadaption, such as tolerance and withdrawal symptoms. There is now considerable evidence, from both animal models of dependence and human studies, that all drugs of abuse converge on common pathways in the CNS, especially the brain's reward circuitries (Nestler, 2005). Genetic studies have also revealed that shared genetic factors affect all drugs of abuse, particularly genes involved in the brain reward pathways (Khokhar et al., 2010). As *IL-1B* genetic variants influence both opioid and alcohol dependence, I would speculate that *IL-1B* is a shared gene for drug dependence, at least for opioid and alcohol dependence. In addition, the genetic findings also indicate that the drug-induced proinflammatory cytokines could be involved in a shared pathway for drug dependence by affecting both drug reward and drug-induced withdrawal. Further study is required to validate this hypothesis.

5.2 Study of chronic morphine-induced brain IL-1β expression and the behavioural consequences using different strains of mice

As shown in the genetic association study (chapter 2), *IL-1B* genetic variants contribute to the risk of opioid dependence. To further investigate the mechanism behind the genetic findings, the study in chapter 4 examined the role of chronic morphine-induced brain IL-1β expression changes in withdrawal behaviours using different inbred and knockout mouse strains. The major findings of this study were: a) there are significant main effects of morphine treatment on IL-1β expression in the brain; b) the elevation of IL-1β expression in the hippocampus contributes to opioid withdrawal jumping in inbred wild-type mice; and c) TLR4 and non-MyD88 signalling pathways play an important role in opioid analgesia.

5.2.1 Chronic opioid-induced hippocampal IL-1β expression contributes to withdrawal jumping

5.2.1.1 Chronic opioid-induced IL-1β expression and withdrawal jumping

When comparing between inbred wild-type strains, significant main effects of morphine treatment on IL-1β expression were found for all five brain regions analysed, including hippocampus, brain stem, midbrain plus diencephalon, mPFC, and cortex. In addition, a significant association of the hippocampal IL-1β expression with withdrawal jumping severity was found. Specifically, mouse strains with higher hippocampal IL-1β expression experienced more severe withdrawal jumping behaviours. These data provide further evidence of opioid-induced proinflammatory

response in the CNS and demonstrate that IL-1β contributes to the development of opioid dependence.

In addition, this study also highlights the importance of the hippocampus in opioid dependence. Generally, the hippocampus plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. Recent evidence has shown that the hippocampus is involved in mediating both the acute reinforcing properties of opioids and the relapse to drug taking, and these behaviours are significantly associated with hippocampal neuroadaption after drug exposure (Eisch, 2002, Eisch and Mandyam, 2004, Everitt et al., 2001). Although the mechanism behind the relationship of hippocampal IL-1β and dependence withdrawal behaviours is unclear, other lines of evidence have shown that glial-induced IL-1β within the brain modulate hippocampal neurogenesis (Goshen and Yirmiya, 2009). Taken together, it can be speculated that chronic opioid-induced elevation of hippocampal IL-1β may facilitate hippocampal neuroadaption and thereby contribute to the development of opioid dependence.

5.2.2 Role of TLR4 and MyD88 pathway in opioid tolerance

In comparison with the wild-type Balb/c mice, TLR4 KO mice did not develop significant analgesic tolerance after chronic morphine treatment; however, MyD88 KO mice developed a similar degree of tolerance to wild-type Balb/c mice. The TLR4 KO mice result is consistent with findings from the Hutchinson et al. (2009) study, that knockout of TLR4 potentiates morphine analgesia in mice. TLR4 has two signalling pathways, MyD88-dependent and TRIF-dependent pathways (Bell, 2003). Recent studies have implicated a cross-talk mechanism between the two pathways

(Selvarajoo, 2006, Selvarajoo, 2011). As MyD88 KO mice still developed opioid tolerance, this indicates that the MyD88-independent pathway, TRIF-dependent pathway or the cross-talk mechanism between the two pathways, maybe critical for the development of opioid tolerance. Further studies are required to examine the role of the TLR4-TRIF pathway in the development of tolerance.

5.3 Messages from the two studies

Adding to the growing knowledge of opioid-induced proinflammatory responses in the CNS, the findings in this thesis, through both human and animal experiments, provided further evidence regarding the contribution of IL-1β to opioid dependence development. Furthermore, the data from the animal study (Chapter 4) offered mechanistic support for the human genetic findings.

In the human study, people with *IL-1B* -31T/-511C alleles are under higher risk to develop opioid dependence than others. As hypothesised in the previous section, this phenomenon could be explained by the higher opioid-induced IL-1β expression which leads to more opioid reward or worse withdrawal, and consequently, placing the subjects at a higher dependence risk. The mice study carried out in chapter 4 has partly verified this hypothesis. Firstly, chronic morphine treatment has a significant main effect on brain IL-1β expression in several brain regions of different mouse strains, especially in the hippocampus. This may be the same case in humans that chronic opioid intake induces IL-1β in the brain. Secondly, mouse strains that have greater LPS-induced IL-1β expression also respond substantially to morphine and with higher IL-1β expression in the hippocampus after chronic morphine treatments. Multiple strains of mice could mimic the genetic variability between human

individuals; therefore, humans with the higher IL-1β expression alleles (-31T/-511C) would also have higher drug-induced IL-1β expression in the CNS. Thirdly, the amount of hippocampal IL-1β expression contributes to withdrawal severity in mice. This suggests that humans with *IL-1B* -31T/-511C alleles would experience worse withdrawal than people with other alleles. However, one major question, raised from the human genetic studies that still cannot be answered by the current thesis, is how IL-1β signalling alters the risk of dependence. Is it because of the greater drug reward that attracts people to use more opioids, or because of the worse withdrawal that facilitates individuals to administer opioids more frequently or for a longer time, or both? Further opioid reward-related behavioural studies, such as mouse conditioned place preference models, are required to find the answer.

5.4 How do drug-induced IL-1β changes impact on neuronal functions that contribute to drug dependence?

The work in this thesis has provided further evidence of the central immune signalling pathways in modulation of drug dependence. The theory of drug-induced neuroinflammation is an important complement to the established neuronal mechanisms. Importantly, the actions of the IL-1β/TLR4 pathways studied in this thesis do not act alone to produce dependent behaviours, but contribute to the dysregulation of neuronal functioning.

The neuronal consequences of glial activation and proinflammatory cytokine release are complex and still largely unknown, however, opioid-/alcohol-induced proinflammatory immune signalling, especially IL-1β, appears to impact on

neuroexcitability via regulation of important neuronal mechanisms, such as glutamate homeostasis, NMDA and dopamine systems. IL-1β alone was shown to induce a dose-dependent suppression of astrocyte glutamate uptake activity in human foetal astrocyte culture (Hu et al., 2000), whilst blockade of IL-1β release prevented the loss of glutamate transporter expression (Prow and Irani, 2008). The dysregulation of glial glutamate transporter activity leads to an increase of synaptic glutamate concentration and results in enhanced neuroexcitability. Furthermore, IL-1β is implicated in NMDA receptor functions. Using primary cultures of rat hippocampal neurons, Viviani et al. (2003) showed that IL-1β (0.01 – 0.1 ng/ml) dose-dependently enhanced NMDA-induced Ca^{+2} influx, and this is partly through increased phosphorylation of NMDA receptor subunits 2A and 2B. This IL-1β-related effect contributes to the hyper-activity of neuronal NMDA systems in drug abuse models. In addition, it has also been found that IL-1β could impact on dopamine release by involvement in the differentiation of dopaminergic neurons (MohanKumar et al., 1998). However, this was only studied in the paraventricular nucleus region. Whether glial-induced IL-1β affects dopamine release in other drug-related brain nuclei requires further investigations. In addition to IL-1β, other opioid-/alcohol-induced glial proinflammatory products also have significant neuronal impacts via modulation of neurotransmissions. For example, TNF-α increases the conductivity of glutaminergic AMPA receptors (De et al., 2003) and potentiates inward currents in neuronal tetrodotoxin-resistant sodium channels (Jin and Gereau, 2006). TNF-α and IL-1β upregulate the neuronal cell surface expression of both AMPA and NMDA receptors while downregulating cell surface expression of GABA (De et al., 2003). Taken together, proinflammatory products exert multiple effects with the end result being neuroexcitation.

Parallel to the influence on neuroexcitation, glial activation and the release of the proinflammatory products may impact on neuronal functions via regulation of neurogenesis, proliferation and neuron survival. Brain IL-1β was shown to reduce hippocampal neuronal neurogenesis (Goshen and Yirmiya, 2009) either resulting from microglial activation (Kempermann and Neumann, 2003) or direct TLR4 activation (Ekdahl et al., 2003, Monje et al., 2003). Furthermore, drug-induced glial activation could decrease the expression of brain neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF) (Carnicella and Ron, 2009). Since GDNF plays an important role in the development of the nervous system and a unique role in negatively regulating the actions of drug dependence (Carnicella and Ron, 2009), this glial regulation of the neurotrophic factors could also contribute to the dependence development. However, future research is warranted.

5.5 Study limitations and future directions

5.5.1 The human genetic study

As with many studies, there are limitations to the work that has been conducted in this thesis which should be addressed in the future. For the human genetic study, the retrospective nature of the study warrants that further prospective investigations should be carried out on larger populations. Furthermore, there are several other components that could influence the expression of IL-1β, such as IL-1β converting enzyme (caspase-1), IL-1 receptors, etc. Therefore, genes encoding for those proteins could also be future investigation targets. In addition, other proinflammatory cytokines, such as TNF-α and IL-6, could also be involved in the opioid-induced CNS

proinflammatory response. Moreover, interaction between proinflammatory cytokines can also modulate the expression of IL-1β, e.g. the inhibition of TNF-α by etanercept down-regulates morphine-induced IL-1β expression (Shen et al. 2011). Thereby, the polymorphisms which alter the expression of these cytokines should also be considered as genetic risk factors for drug dependence.

5.5.2 The animal study

Opioid-induced reward effect and the relationship with IL-1β expression was not studied in this thesis. To further elucidate the mechanism behind the human *IL-1B* genetic findings, future studies of the involvement of brain IL-1β expression and reward-related behaviour is warranted. Furthermore, there are limitations in the animal study presented in this thesis. Firstly, only withdrawal jumping behaviour was quantitatively recorded in the experiment. Opioid-induced neuroinflammation may also affect other withdrawal signs (such as wet dog shaking or diarrhoea). However, this study was not designed to record other behaviours initially, therefore, further investigation will be required. In addition, chronic opioid tolerance behaviour was measured in the mice study. Since expression of IL-1β in spinal cord is involved in tolerance development (Shavit et al., 2005), future examination of spinal tissue IL-1β expression is necessary and may reveal whether IL-1β expression differences between strains contribute to different magnitudes of tolerance.

Chapter 6 Conclusion

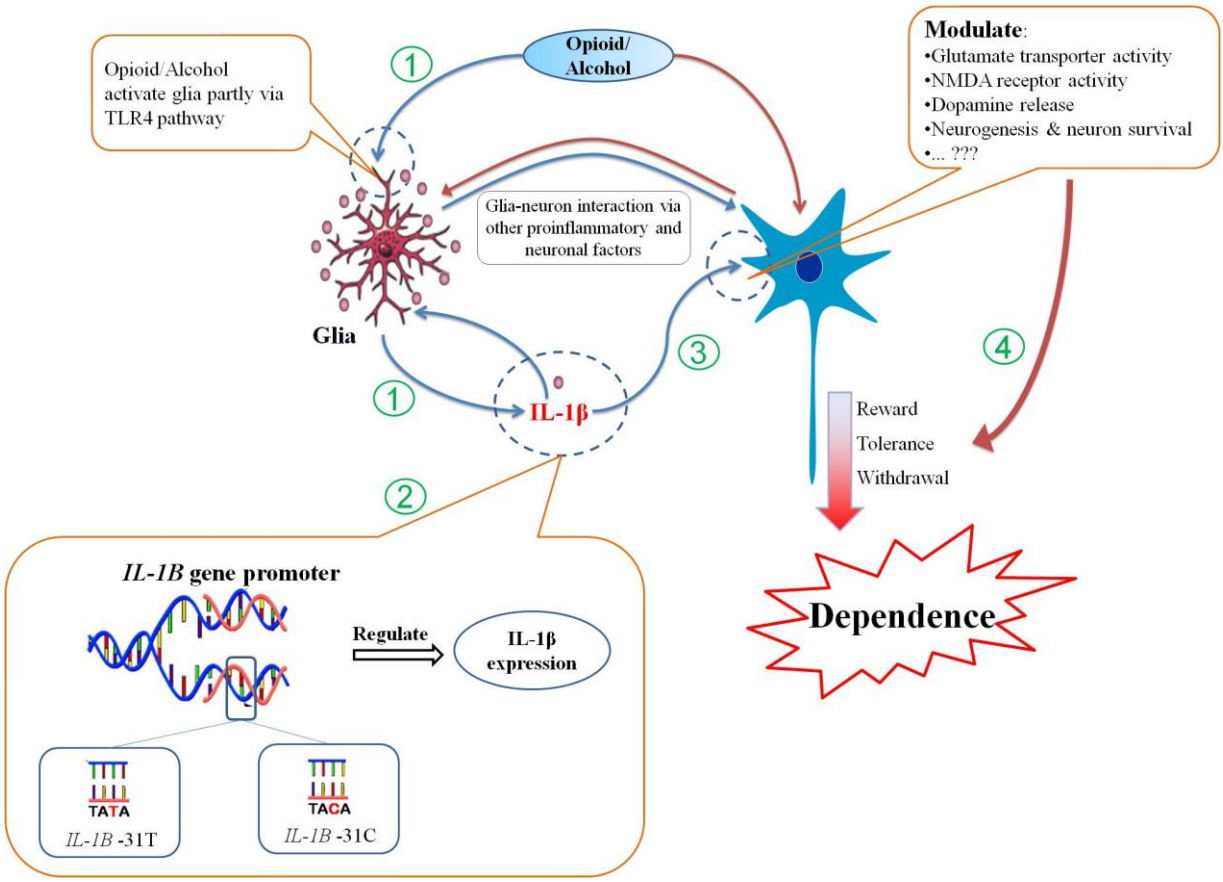


Figure 7 Summary of the possible mechanisms behind the impact of *IL-1B* genetic polymorphisms on opioid/alcohol dependence based the findings of this thesis and previous studies.

In conclusion, the studies presented in this thesis have identified that two *IL-1B* SNPs, -31 T>C and -511 C>T, are associated with altered risk of opioid dependence, and have confirmed their involvement in alcohol dependence. The animal study using different inbred mouse strains showed that chronic opioid-induced brain IL-1β

expression contributes to opioid withdrawal severity, which provides mechanistic evidence supporting the *IL-1B* human genetic findings. Figure 5-1 summarises the possible mechanisms of how IL-1β and its genetic polymorphisms impact on the development of drug dependence:

1. opioid/alcohol activate glial cell leads to release of IL-1β;

2. the level of IL-1β expression is regulated by the *IL-1B* genotypes;

3. IL-1β acts on the neuronal system and induces a series of neuronal events;

4. these events result in increased withdrawal severity and possibly increased drug reward, and finally they contribute to the development of dependence.

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