THE NEUROIMMUNOPHARMACOLOGY
OF ALCOHOL

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Discipline of Pharmacology

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A thesis submitted for the degree of Doctor of Philosophy
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Abstract

Background and purpose

Alcohol exposure induces glial toll-like receptor 4 (TLR4) signalling, while morphine administration leads to both TLR2 and TLR4 signalling in the central nervous system. However, the acute behavioural consequences of such immune activation remain unknown. This thesis aimed to examine: (a) the role of microglia, TLR2, TLR4, MyD88, and IL-1 receptor signalling in sedation and motor impairment following acute alcohol administration in mice; (b) the relationship between these observed behavioural effects and the changes in central and peripheral alcohol pharmacokinetic profiles; (c) the effect of alcohol on MAPK (ERK, JNK, and p38) and NFκB (IκBα) pathways and the alteration of such effects by attenuating microglial, TLR4, MyD88, and IL-1 receptor signalling \textit{ex vivo} and \textit{in vitro}; (d) the role of TLR2, TLR4, MyD88, IL-1 receptor, and µ opioid receptor (MOR) in the interaction between alcohol and morphine as assessed by sedation in mice; and (e) the association between the \textit{TLR4} Asp299Gly SNP and opioid or alcohol dependence in humans.

Experimental approach

In mouse studies and mouse cellular studies, pharmacological blockade of microglial signalling, TLR4, IL-1 receptor, or both MOR and TLR4 by minocycline, (+)-naloxone (the MOR-inactive isomer), IL-1 receptor antagonist, or (-)-naloxone (the MOR-active isomer), respectively, was utilised. Mice deficient in TLR2, TLR4, both TLR2 and TLR4, or MyD88 were used. The sedative effect of alcohol and the interaction between alcohol and morphine were assessed by the sleep time (loss of righting reflex) test, and alcohol dose-induced motor impairment was
determined by the rotarod test. The activation of MAPK cascade was determined by ERK, JNK, and p38 phosphorylation using a cytometric bead array assay, and the alteration in NFκB cascades was characterised via cellular IκBα protein levels utilising western blotting experiments.

In the human pharmacogenetic study, TLR4 Asp299Gly SNP genotypes were determined by a polymerase chain reaction (PCR)-restriction fragment length polymerase (RFLP) assay in 99 opioid dependent subjects, 100 alcohol dependent subjects, and 56 non-dependent healthy controls.

Key results

Pharmacological or genetic inhibition of microglial activation, TLR2, TLR4, both TLR2 and TLR4, MyD88, or IL-1 receptor signalling attenuated alcohol dose-induced sedation and motor impairment in mice. The modification of IκBα protein levels by alcohol exposure in vitro was time-dependent, and the increase in such protein levels was attenuated by inhibiting proinflammatory microglial activation, TLR4, MyD88, or IL-1 receptor signalling. In contrast, blocking the activities of TLR2, both TLR2 and TLR4, and MyD88, but not TLR4 or IL-1 receptor, inhibited the enhancement of alcohol’s sedative effect by morphine. The human genetic data showed a lack of association between alcohol or opioid dependence and TLR4 Asp299Gly polymorphisms.

Conclusions and implications

Collectively, these data suggest that in mice, alcohol activates microglial and TLR2- and TLR4-MyD88-NFκB-IL-1 receptor signalling rapidly, and this activation subsequently contributes to sedation and motor impairment induced by alcohol administration. However, TLR2-MyD88,
not TLR4 and IL-1 receptor, cascade is involved in the interaction between alcohol and morphine. Such behavioural preclinical data provide novel insights into the immune mechanisms of the effects of alcohol and opioids.
Declaration

I, Yue Wu certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Yue Wu

Date
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Statement of Authorship

Attenuation of microglial and IL-1 signaling protects mice from acute alcohol-induced sedation and/or motor impairment


Impact Factor: 5.061

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Signed  Date  18/07/2011

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Signed                      Date       20/07/2011

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Contributed to experimental design and manuscript evaluation

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Signed                      Date       29/07/2011

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Provided critical evaluation of article

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Inhibiting the TLR4-MyD88 signalling cascade by genetic or pharmacologic strategies reduces acute alcohol dose-induced sedation and motor impairment in mice

Br J Pharmacol (2011): accepted paper

Impact Factor: 4.925

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Watkins LR

Provided critical evaluation of article

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TLR2 and MyD88 mediate the sedative effect of alcohol and interaction between alcohol and morphine in mice


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Signed  Date  18/07/2011

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Signed                      Date          29/07/2011
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B2M</td>
<td>β-2-microglobulin</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine (C-C) motif ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>chemokine (C-C) motif ligand receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CTSF</td>
<td>cathepsin F</td>
</tr>
<tr>
<td>CTSS</td>
<td>cathepsin S</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine (C-X-C) motif ligand</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1RI</td>
<td>interleukin-1β receptor type I</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>IRAK4</td>
<td>interleukin-1 receptor-associated kinase 4</td>
</tr>
<tr>
<td>IRF3</td>
<td>IFN regulatory factor 3</td>
</tr>
<tr>
<td>IκBα</td>
<td>NFκB inhibitor α</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>LORR</td>
<td>loss of righting reflex</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAL</td>
<td>myelin and lymphocyte protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>myeloid differentiation factor 2</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOR</td>
<td>µ opioid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TIR</td>
<td>toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>toll/IL-1R domain containing adaptor inducing interferon-β</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
</tbody>
</table>
Human gene symbols

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ABCB1</em></td>
<td>ATP-binding cassette, sub-family B, member 1</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td><em>ADH1B</em></td>
<td>alcohol dehydrogenase 1B (class I), beta polypeptide</td>
<td>ADH1B</td>
</tr>
<tr>
<td><em>ADH4</em></td>
<td>alcohol dehydrogenase 4 (class II), pi polypeptide</td>
<td>ADH4</td>
</tr>
<tr>
<td><em>ALDH1A1</em></td>
<td>aldehyde dehydrogenase 1 family, member A1</td>
<td>ALDH1A1</td>
</tr>
<tr>
<td><em>ALDH2</em></td>
<td>aldehyde dehydrogenase 2 family (mitochondrial)</td>
<td>ALDH2</td>
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<tr>
<td><em>CYP2B6</em></td>
<td>cytochrome P450, family 2, subfamily B, polypeptide 6</td>
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<td>cytochrome P450, family 2, subfamily E, polypeptide 1</td>
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<tr>
<td><em>CYP3A4</em></td>
<td>cytochrome P450, family 3, subfamily A, polypeptide 4</td>
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<tr>
<td><em>GABRA6</em></td>
<td>gamma-aminobutyric acid (GABA) A receptor, alpha 6</td>
<td>GABA_A^α_6</td>
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<tr>
<td><em>IL10</em></td>
<td>interleukin 10</td>
<td>IL-10</td>
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<tr>
<td><em>IL1B</em></td>
<td>interleukin 1, beta</td>
<td>IL-1β</td>
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<td><em>IL1RN</em></td>
<td>interleukin 1 receptor antagonist</td>
<td>IL-1ra</td>
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<tr>
<td><em>LY96</em></td>
<td>lymphocyte antigen 96</td>
<td>MD-2</td>
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<tr>
<td><em>NFKB1</em></td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
<td>NFκB</td>
</tr>
<tr>
<td><em>OPRM1</em></td>
<td>opioid receptor, mu 1</td>
<td>MOR</td>
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<tr>
<td><em>TLR2</em></td>
<td>toll-like receptor 2</td>
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<tr>
<td><em>TLR4</em></td>
<td>toll-like receptor 4</td>
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Chapter I Introduction

With its consumption believed to have begun more than ten thousand years ago, alcohol (ethanol) continues to feature prominently in many societies and cultures worldwide. Unfortunately amongst the two billion annual consumers, alcohol abuse poses a significant issue, with over seventy six million people diagnosed with an alcohol abuse disorder (WHO, 2004). Related to more than sixty types of disease and injury, alcohol consumption is the third biggest risk factor for disease burden in developed countries, such as the United States, Canada, Germany, France, the United Kingdom, Australia, and Japan, and is the largest risk factor in developing countries, such as China, the Philippines, Vietnam, Indonesia, Thailand, Brazil, and Mexico (WHO, 2004). Furthermore, alcohol is commonly abused together with opioids, as morphine is rarely the only drug detected at heroin-related deaths with the presence of alcohol ranging from 29 to 74% (Warner-Smith et al., 2001). The abuse of alcohol increases the risk of heroin-related deaths (Hickman et al., 2008; Levine et al., 1995). As alcohol dependence and abuse remain continuing and important health problems, strategies to overcome these addictions are urgently required to reduce the burden of their related diseases in society.

Understanding the mechanisms underpinning the initial effects of alcohol, alcohol dependence, and alcohol-opioid interaction would have wide reaching implications in developing targeted pharmacological therapies for alcohol abuse-related conditions, such as alcohol dependence and alcohol overdose-induced brain damage. Despite several decades of research, the mechanism of alcohol’s effects has not been fully understood. Much research has focused on how the nervous system adapts to chronic alcohol exposure, however, to understand how alcohol influences the
central nervous system (CNS) in the short term, it is critical to characterise the initial effects of alcohol. Apart from the neuronal consequences of alcohol exposure, recent studies have implicated alcohol-induced glial toll-like receptor (TLR)-dependent proinflammatory signalling in the CNS in the chronic neurotoxic effects of alcohol, although little work has examined if these non-neuronal effects contribute to the acute behavioural responses of alcohol.

Based on the above research gaps, in this project, the proinflammatory effects of acute alcohol or alcohol-morphine exposure have been investigated using mouse behavioural models. The role of brain immunocompetent cells (microglia), innate immune receptors (TLR2 and TLR4) and their related signalling cascades, and proinflammatory cytokine (interleukin-1, IL-1) receptor signalling in acute alcohol-induced sedation and motor dysfunction will be demonstrated in this thesis. The involvement of TLR2, TLR4, and IL-1 signalling in the sedative interaction between alcohol and morphine is assessed by sedation. Alcohol-induced sedation and motor dysfunction are measured by the duration of loss of righting reflex (LORR, sleep time) and rotarod performance, respectively. Furthermore, the association between TLR4 single nucleotide polymorphisms (SNPs) and alcohol or opioid dependence will be investigated with a human genetic study. With the results from these studies, we will gain global knowledge of the proinflammatory mechanism of alcohol’s effects, including the involved cell types, receptors, intracellular signalling cascades, and cytokines, in both mouse and human models.

This introduction will cover the background of these research topics listed above. Prior to outlining the aims and hypotheses of the thesis, firstly, the behavioural effects of alcohol and the animal models to assess these effects will be introduced. Secondly, the site of action of alcohol in
the brain will be discussed. Thirdly, the current understanding of neuronal mechanisms of alcohol’s effects and alcohol-opioid interaction will be reviewed. Fourthly, a background on how alcohol, as well as other drugs of abuse, activates glial TLR signalling cascades, and why this glial activation is important to the neuronal consequences of alcohol exposure will be given. The potential treatment of alcohol dependence by blocking glial cells, TLRs, and IL-1 receptor signalling activation will be summarised. Fifthly, the pharmacokinetics of alcohol will be summarised, as any pharmacodynamic alteration of alcohol effects by medications or genetic modifications may be mediated by changes in alcohol pharmacokinetics. Finally, it will be discussed why human TLR4 genetic variability is a factor worth evaluating for an association with alcohol and opioid dependence.

1.1. Behavioural effects of alcohol

1.1.1. Methods to investigate the behavioural effects of alcohol

The behaviourally relevant mechanism of alcohol’s effect is informative for developing new therapeutic drugs. A variety of animal models have been used in researching the behavioural effects of alcohol. To understand the acute effects of alcohol, the sleep time test by measuring duration of alcohol-induced LORR is used to investigate the sedative and motor effects of alcohol (Hatch et al., 1988); the rotarod test is utilised to assess alcohol-induced motor impairment (Goodlett et al., 1991); open-field test is used to analyse locomotor activity; and elevated plus maze test is used to investigate the anxiety-like behaviour of alcohol (Kiefer et al., 2003). On the other hand, two-bottle choice test is used to assess alcohol-induced reward and patterns of drinking behaviour (Blednov et al., 2011b), and conditioned place preference is used to
investigate positive and negative reward (Boyce-Rustay et al., 2006). Notably, these acute behavioural tests as listed above can also be used to investigate the chronic effects of alcohol. In this introduction, alcohol-induced sedation assessed by sleep time test and motor dysfunction tested by rotarod test will be discussed, as these two behavioural tests were used in this project.

1.1.2. Sedation (duration of LORR, sleep time test)

To assess alcohol-induced sedative or hypnotic effects, a high dose of alcohol is administered to animals, and the length of LORR is measured. As different terms have been used to refer this effect in the literature, in this thesis, I will use “sleep time” as well as “duration of LORR” to describe the latency, and “sleep time test” as well as “LORR test” to indicate the test.

Neuronal gamma-aminobutyric acid (GABA) receptors, which include class A (GABA\textsubscript{A}) and B (GABA\textsubscript{B}) receptors, N-methyl-D-aspartate (NMDA) receptor, and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signalling are demonstrated to be modulated by alcohol and subsequently induce a sedative effect. As ligand-gated ion-channels, six α, three β, three γ, one δ, three ρ, one ε, one π and one θ subunits of the GABA\textsubscript{A} receptor and NR1, NR2A, NR2B, NR2C, NR2D, NMDA-R3A, and NMDA-R3B subunits of the NMDA receptor have been reported in mammals (Alexander et al., 2009). In contrast, the GABA\textsubscript{B} receptor is a G protein-coupled receptor with two subunits termed GABA\textsubscript{B1} and GABA\textsubscript{B2} (Alexander et al., 2009). Alcohol-induced sedation was attenuated by GABA\textsubscript{A} receptor antagonists in wild-type mice (Boyce-Rustay et al., 2005; Linden et al., 2011). Specifically, mice deficient in the α1, α2, or β2 subunits of the GABA\textsubscript{A} receptor had decreased sedation in response to alcohol compared to their wild-type counterparts; however, mice deficient in the α5, α6, β3, γ2L, or δ subunits of the
GABA_\textsubscript{A} receptor did not show this reduced effect of alcohol (Boehm et al., 2004). Furthermore, a GABA_\textsubscript{B} receptor agonist enhanced alcohol-induced sedation in mice (Besheer et al., 2004). In addition, attenuation of the NR2B subunit of the NMDA receptor pharmacologically, but not the NR2A subunit genetically, significantly potentiated the acute sedative effect of alcohol (Boyce-Rustay et al., 2005). The NMDA receptor system was also shown to be associated with chronic behavioural tolerance to the acute sedative effects of alcohol (Wu et al., 2010). Moreover, genetic reduction of cAMP–PKA signalling increased the sensitivity to the sedative effects of alcohol in mice (Wand et al., 2001) (Table 1-1). Thus, acute alcohol administration activates GABA receptors, inhibits the NMDA receptor’s functions, and reduces cAMP–PKA signalling. These neuronal actions of alcohol subsequently cause a sedative effect.

Apart from neuronal receptors and signalling, a recent study demonstrated that mice deficient in chemokine (C-C) motif ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1) or CCL3 showed 60-90% longer duration of LORR induced by alcohol administration than their wild-type counterparts (Blednov et al., 2005) (Table 1-1). This evidence implicated, for the first time, the role of immune components in the sedative effect of alcohol. As such, it is possible that other proinflammatory cytokine and chemokine signalling (will be discussed in section 1.4.2) is also involved in the acute sedative effect of alcohol.

1.1.3. Motor performance (rotarod test)

The rotarod test is used to assess the motor performance (ataxic affect) and balance of animals, which can also be tested by other similar experiments, such as balance beam and stationary dowel. In the rotarod apparatus, there is a rotating rod set at a fixed or accelerating speed. The latency is
recorded as the duration over which the animals remain on the rod. The experiment usually includes a training phase before the testing phase, so as to make sure that all the animals have the same baseline performance (Shiotsuki et al., 2010).

Similar to the neuronal mechanisms of the sedative effect of alcohol, the NMDA and GABA receptors are responsible for alcohol-induced motor impairment, and the evidence is discussed below. The receptor subunits involved may be different in sedative and motor effects of alcohol. Mice deficient in the NR2A subunit of the NMDA receptor showed impaired motor coordination at baseline and following alcohol treatment in an accelerating rotarod test (Boyce-Rustay et al., 2006). Both competitive and non-competitive antagonists of the NMDA receptor inhibited the locomotor stimulation of alcohol in mice (Liljequist, 1991). In contrast, mice with reduced affinity of the glycine binding site on the NR1 subunit of the NMDA receptor displayed an attenuated alcohol-induced motor dysfunction (Kiefer et al., 2003). On the other hand, alcohol-induced motor impairment was suggested to be the consequence of increased extra-synaptic activity of the GABA\textsubscript{A} receptor (Hanchar et al., 2005). It has been demonstrated that there is an association between allelic variation in the gene encoding the γ2 subunit of the GABA\textsubscript{A} receptor and acute alcohol-induced motor incoordination in mice (Hood et al., 2000). However, via genetic deficient mouse models, it was found that the GABA\textsubscript{A} receptor α1 or γ2L subunit-deficient or wild-type mice did not differ in motor impairment induced by alcohol (Boehm et al., 2004). Furthermore, by direct cerebellar microinfusion of baclofen, an agonist, and phaclofen, an antagonist of the GABA\textsubscript{B} receptor in mice, activation and inhibition of the GABA\textsubscript{B} receptor’s function produced a dose-dependent accentuation and attenuation of alcohol-induced motor impairment, respectively (Dar, 1996) (Table 1-1).
In addition to the mechanisms involving neuronal receptors, it has been demonstrated that the systemic administration of lipopolysaccharide (LPS), as an immune stressor, enhanced alcohol-induced motor impairment 24 h later via rotarod test (Drugan et al., 2007) (Table 1-1). This finding implicated that the immune signaling activation may alter the motor effect of alcohol. In fact, it has been demonstrated that environmental stress, such as inescapable shock (Drugan et al., 1996) and intermittent cold water swim (Drugan et al., 2007), increases the hypnotic and ataxic properties of alcohol, and the mechanism of this effect has been hypothesised to be the modulated common neurochemical systems, such as the serotonin, dopamine (DA), and opiate peptide systems, as well as the hypothalamic-pituitary-adrenal axis (Brady et al., 1999). However, recent evidence suggests that environmental stress primes or sensitises the neuroinflammatory response (Frank et al., 2011). Thus, the environmental stress-induced enhancement of the motor effect of alcohol may be due to potentiated immune signalling, which further implies that immunological mechanisms play a role in alcohol-induced motor incoordination. It will be worthwhile to assess the levels of immune activation in such stress-alcohol models. As the involvement of immune factors in the motor effects of alcohol is only beginning to be understood, the specific immune signalling pathways, receptors, and proteins in alcohol-induced motor dysfunction are worthy of further investigation.

In summary, amongst the acute behavioural effects of alcohol, the sedation and motor incoordination are likely responsible for traffic accident-related deaths, and accompany self-administration of alcohol in mice (Chuck et al., 2006). These two behavioural effects have related but different mechanisms. Both NMDA and GABA receptors are important for acute alcohol-induced sedation and motor impairment, with different receptor subunits involved. Other
than neuronal receptors, immune components also play a role in the mechanisms of acute alcohol-induced sedation and motor impairment. However, little work has been done in this area.

**Table 1-1.** The effects of neuronal or immune modification on sedative and motor effects of alcohol in rodents

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Treatment</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohol-induced sedation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
<td>pharmacological blockade</td>
<td>↓</td>
<td>1, 2</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor α1, α2, or β2 subunits</td>
<td>genetic deficiency</td>
<td>↓</td>
<td>3</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor α5, α6, β3, γ2L, or δ subunits</td>
<td>genetic deficiency</td>
<td>no effect</td>
<td>3</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; receptor</td>
<td>pharmacological activation</td>
<td>↑</td>
<td>4</td>
</tr>
<tr>
<td>NMDA receptor NR2B subunit</td>
<td>pharmacological blockade</td>
<td>↑</td>
<td>2</td>
</tr>
<tr>
<td>NMDA receptor NR2A subunit</td>
<td>genetic deficiency</td>
<td>no effect</td>
<td>2, 5</td>
</tr>
<tr>
<td>NMDA receptor NR2B subunit</td>
<td>chronic alcohol induces decrease level of protein</td>
<td>tolerance</td>
<td>6</td>
</tr>
<tr>
<td>cAMP–PKA</td>
<td>genetic reduction</td>
<td>↑</td>
<td>7</td>
</tr>
<tr>
<td>CCL2</td>
<td>genetic deficiency</td>
<td>↑</td>
<td>8</td>
</tr>
<tr>
<td>CCL3</td>
<td>genetic deficiency</td>
<td>↑</td>
<td>9</td>
</tr>
</tbody>
</table>

| **Alcohol-induced motor impairment** | | | |
| GABA<sub>A</sub> receptor α6, β3, and δ subunits | natural polymorphism, increased tonic current induced by alcohol | ↑ | 9 |
| GABA<sub>A</sub> receptor γ2 subunit | natural polymorphism, unknown function | ↓ | 10 |
| GABA<sub>B</sub> receptor | pharmacological activation | ↑ | 11 |
| GABA<sub>B</sub> receptor | pharmacological blockade | ↓ | 11 |
| NMDA receptor NR2A subunit | genetic deficiency | ↑ | 5 |
| NMDA receptor NR1 subunit | genetic reduction | ↓ | 12 |
| immune activation | LPS treatment | ↑ | 13 |

1.2. Sites of effects of alcohol in the brain

Alcohol influences a variety of brain regions (Vilpoux et al., 2009). It has been demonstrated that alcohol produces brain region specific changes in neuronal activities, and the changes are dependent on alcohol dose and the duration of alcohol exposure (Vilpoux et al., 2009). For example, alcohol-drinking mice, that achieved a blood alcohol concentration of 1.0 mg/mL, had increased expression of inducible transcription factors (c-Fos and FosB) at medial posterior ventral portion of the central nucleus of amygdala, core of nucleus accumbens (NAc), and Edinger-Westphal nucleus (the region processing sensory information) in comparison to water or sucrose-consuming animals (Bachtell et al., 1999). The Fos proteins were used as indirect markers of neuronal activity because they are often expressed when neurons fire action potentials (VanElzakker et al., 2008). On the other hand, acute alcohol exposure (single dose of alcohol administration at 1.5 g/kg) led to decreased c-Fos expression in hippocampus and increased expression of this protein in the extended amygdala, the regions processing sensory information, and various stress-related areas (Ryabinin et al., 1997). Moreover, a lower dose of alcohol at 0.5 g/kg reduced basal c-Fos expression in several areas, including neocortex, hippocampus, and hypothalamus (Ryabinin et al., 1997). Thus, it is difficult to choose the brain regions in which alcohol acts to do further analysis, due to different alcohol concentrations and length of alcohol exposure used.

It is more important and difficult to determine the relationship between alcohol-induced sedation or motor impairment and the brain regions which are responsible for such effects. It had been shown that the transgenic mice with decreased cAMP–PKA signalling had increased alcohol-
induced sedation (Wand et al., 2001). However, in these transgenic mice, expression of the transgenes was limited to the forebrain, striatum, and hippocampus, and the transgenes were not expressed in the cerebellum (or any other brain regions). This suggests that changes of cAMP–PKA signalling are not needed in the cerebellum for alcohol-induced sedation. In contrast, another study demonstrated that G protein expression and adenylylcyclase activity in the cerebellum, as well as the frontal cortex, hippocampus, and NAc, were correlated with sensitivity to the sedative properties of alcohol (Froehlich et al., 1997). As such, these data suggest that nearly all the brain regions are involved in alcohol-induced sedation. Regarding the signalling cascades involved in the sedative effects of alcohol, they could be activated or depressed in different brain regions involved.

On the other hand, the cerebellum is considered to be responsible for the motor effect of alcohol, as the cerebellum controls the motor activity (Valenzuela et al., 2010), and the effect of alcohol on cerebellum is generally considered to be responsible for a significant number of traffic accident-related deaths. Furthermore, the enhanced transmission to cerebellar granule cells (Carta et al., 2004) and Purkinje neurons (Hirono et al., 2009) via GABA release in the cerebellum has been considered to be the mechanism of impaired motor skills by alcohol. As such, the cerebellum is important for investigating alcohol-induced motor impairment.

The hippocampus is an important brain region to be investigated in alcohol behavioural studies according to the new neuroimmune mechanisms of alcohol (discussed in section 1.4). A recent study demonstrated that adolescent binge alcohol exposure in rats induced activation of hippocampal microglia (central immunocompetent cells) that lasted up to 28 days (McClain et al.,
2011). Although surprisingly little is known about the subpopulation of microglia within different brain regions in both normal and pathological conditions (Hanisch et al., 2007), hippocampal microglia are able to express higher levels of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α), than microglia in other brain regions (Ren et al., 1999). This evidence suggests that the immune sensitivity of hippocampus is higher than other brain regions. Therefore, the hippocampus could be a potential site to assess alcohol-induced microglial activation and glial-neuronal crosstalk.

1.3. Neuronal mechanisms of alcohol’s effects

1.3.1. Global reward pathway: neuronal circuitry

Alcohol exposure causes neuroadaptation in the reward pathways in the brain, which, in turn, results in the physiological motivation to drink more alcohol. In fact, the responses to addictive drugs, including alcohol, and natural rewards share many commonalities, which are hedonic responses, desire, and rapid learning of both predictive cues and efficient behavioural sequences aimed at obtaining the reward (Hyman et al., 2006). Reward influences behaviours by increasing synaptic DA in the NAc (Bromberg-Martin et al., 2010), which is the major component of the ventral striatum, the reward centre in the brain. All addictive drugs increase levels of synaptic DA within the NAc directly or indirectly. The source of DA is the ventral tegmental area (VTA) of the midbrain. Other than the NAc, the amygdala and the prefrontal cortex are considered to play important roles in the evaluation of reward and the establishment of reward-associated learning (Kalivas et al., 2005). In addition, to obtain reward, DA released from the substantia
nigra (SN) to dorsal striatum consolidates the efficient action repertoires (Vanderschuren et al., 2005) (Figure 1-1).

Figure 1-1. DA projections in the reward pathways in the brain (Hyman et al., 2006). Illustrated are projections from the VTA to the NAc and prefrontal cortex, and projections from the SN to the dorsal striatum.

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Specifically, it is generally considered that the DA release induced by alcohol is an indirect effect in response to an increased firing rate of DA neurons in the VTA (Tambour et al., 2007). However, it remains debatable how alcohol stimulates the neurons (Theile et al., 2011).

1.3.2. Acute effects of alcohol on neuronal systems

The widely accepted neuronal mechanisms of alcohol’s effects are that acute alcohol exposure mainly acts by activating the function of GABA receptors and inhibiting the function of the NMDA receptor (Ikonomidou et al., 2000; Vengeliene et al., 2008). However, nearly all of the major neurotransmitters and receptors are involved, modulated directly or indirectly by alcohol exposure (Schuckit et al., 2004). The behaviourally relevant functions of GABA and NMDA receptors have been reviewed in section 1.1, the following paragraphs will discuss the role of these receptors in mechanisms of alcohol’s effects.

The GABA_A receptor is a chloride ion-channel that predominately mediates rapid inhibitory neurotransmission in the CNS. GABA, the inhibitory neurotransmitter that binds to the GABA_A receptor, changes the conformation state of this receptor and therefore opens the pore to allow chloride ion to pass down an electrochemical gradient. Differently, the GABA_B receptor, as a G protein-coupled receptor for GABA, regulates synaptic transmission and signal propagation by controlling the activity of voltage-gated calcium ion-channel and inward-rectifier potassium ion-channel (Schwenk et al., 2010). Notably, GABAergic mechanisms underlie many of the behavioural effects of alcohol, including anxiolytic, anticonvulsant, cognitive-impairing, sedative hypnotic, and motor incoordination (Blednov et al., 2003; Boehm et al., 2006; June et al., 2007; Kumar et al., 2009). With regard to alcohol drinking behaviours, enhanced alcohol consumption
was found in mice deficient in the GABA_A receptor compared to their wild-type counterparts (Blednov et al., 2003; June et al., 2007). Activation of the GABA_B receptor signalling suppressed alcohol drinking behaviour and reinforcement in rats (Maccioni et al., 2008). In contrast, lower function of the GABA_B receptor has been found in limbic areas of alcohol-preferring than –non-preferring rats (Castelli et al., 2005).

Although various sources of evidence have suggested that alcohol enhances the potentiation of GABA receptor signalling as discussed in section 1.1, the exact mechanism of alcohol’s effects on such receptors remains unclear. Apparently, different GABA receptor subtypes respond differentially to varying alcohol concentrations. It is documented that lower concentrations of alcohol directly act on the GABA_A receptor by enhancing tonic current, while higher amounts of alcohol may associate with modulating phasic inhibition of the GABA_A receptor (Kumar et al., 2009). Alcohol enhanced the function of G protein-coupled inwardly rectifying potassium channels which coupled to the GABA_B receptors in cerebellar granule cells (Lewohl et al., 1999). Further, alcohol could enhance presynaptic GABA release, which is indirectly responsible for many GABAergic effects of alcohol (Ariwodola et al., 2004). In addition, alcohol-induced elevation of neuroactive steroids is capable of enhancing GABAergic transmission (Kumar et al., 2009). Alcohol could also modify the phosphorylation of proteins which have consensus sites on GABA_A receptor subunits, such as protein kinase C (PKC) (Choi et al., 2008) and PKA (Maas et al., 2005).

As another important neuronal receptor involved in the mechanism of alcohol’s effect, NMDA receptor signalling is known to be attenuated by acute alcohol application (Lovinger et al., 1990;
Proctor et al., 2006). The NMDA receptor is an ionotropic glutamate receptor, and its channel commonly has a high relative permeability to calcium ion and is blocked, in a voltage-dependent manner, by magnesium ions such that at resting potentials the response is substantially inhibited (Alexander et al., 2009). The blockade of the NMDA receptor’s function by alcohol is likely to contribute to alcohol-induced motor dysfunction (Allgaier, 2002). Inhibition of the activities of the NMDA receptor appears to be time- and dose-dependent (Grover et al., 1994; Lovinger et al., 1989). An elevated concentration of alcohol produced greater reduction of the NMDA-activated current, with up to 61% of the reduction in the range of 5-50 mM alcohol (Lovinger et al., 1989). This effect diminished after 15 min of alcohol exposure (60 mM) in brain slices (Grover et al., 1994), which suggested the development of acute tolerance of the NMDA receptor’s function by alcohol treatment.

1.3.3. Chronic effects of alcohol on neuronal systems

In contrast to the acute effects of alcohol, the neuroadaptation developed in chronic alcohol consumption acts to neutralise the acute influences of alcohol by normalising the function of neuronal cells (Tambour et al., 2007), which leads to alcohol tolerance. It has been demonstrated that the chronic consumption of alcohol is able to induce a GABAergic down-regulation and a glutamatergic up-regulation (Ikonomidou et al., 2000). Chronic alcohol consumption leads to many adaptations of GABA receptors’ function, which is attributed to altered and non-uniform expression of GABA<sub>A</sub> receptor subunits (Cagetti et al., 2003; Grobin et al., 2000), synaptic localisation (Liang et al., 2006), and trafficking of the GABA<sub>A</sub> receptor on the cell surface (Kumar et al., 2003). On the other hand, chronic alcohol treatment has been reported to up-
regulate the activities of the NMDA receptor (Roberto et al., 2004), and to increase the expression of the NMDA receptor (Lack et al., 2007; Roberto et al., 2006). Taken together, while the exact molecular mechanisms attributable to each chronic behavioural effect of alcohol, such as increased anxiety, hyperalgesia, and disruptions in sleep states, have not been fully understood, an overwhelming amount of evidence is pointing to the involvement of GABA and NMDA receptors (Grobin et al., 1998; Kumar et al., 2009). Despite several decades of investigation on the neuronal mechanisms of alcohol’s effects, the number of “unknowns” in this research area has increased significantly. As the importance of central glial cells has been realised by neuroimmunologists, a new direction in understanding the mechanism of alcohol’s effects is opened.

1.4. Alcohol exposure and glial activation

1.4.1. Glia - the immunocompetent cells in the CNS

Other than neurons, glial cells and immune components are considered to be involved in the molecular effects of alcohol since the 1980s (Alling et al., 1986; Hansson et al., 1987; Ronnback et al., 1988). The defining characteristic of neurons is their ability to fire action potentials. All the other cells in the brain that lack this property are classified into a broad class termed glia (Allen et al., 2009). Glia, making up approximately 90% of the human brain and roughly 65% of the mouse brain, were thought by many that their purpose was to solely provide physical support and housekeeping for neurons (Miller, 2005). Being called “brain glue” until the last two decades, glial cells are in fact not all the same, and have different subtypes. They are pivotal to maintaining a healthy brain, yet are also related to a variety of diseases.
Glia are classified into microglia, astrocytes, and oligodendrocytes on the basis of morphology, function, and location in the CNS. Microglia are recognised as the prime component of the immune system in the brain (Streit et al., 2004). They are quickly activated in acute pathological states such as those observed during infection, inflammation, and neurodegeneration, and contribute to the altered CNS function (Kreutzberg, 1996). In contrast, astrocytes play an important role in the CNS through the “tripartite synapse” (Araque et al., 1999) or the “neural threesome” hypothesis (Smith, 2010), which is a bidirectional communication system between astrocytes, presynaptic neurons, and postsynaptic neurons (Perea et al., 2002) (Figure 1-2). Oligodendrocytes produce a lipid-rich membrane called myelin, which enwraps axons, thereby speeding up the conduction of electrical impulses (Allen et al., 2009; Fields et al., 2002). Generally, as immunocompetent cells in the CNS, glial cells can be activated by different stimuli, including viruses and bacteria, and subsequently release various active substances, such as proinflammatory cytokines, which could in turn activate other glial cells to enhance this signal (Watkins et al., 2005).
Figure 1-2. A tripartite synapse (Allen et al., 2009). Neurons release neurotransmitters from synaptic nerve terminals into the synaptic cleft to communicate with other neurons. The neurotransmitters released from the synapse can also reach neurotransmitter receptors in astrocytes, eliciting the increase of intracellular calcium ion concentrations in astrocytes. Subsequently, astrocytes release glutamate, which feeds back to the presynaptic nerve terminal to modulate synaptic neurotransmission. (Araque et al., 1999).

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1.4.2. Alcohol and glial activation

It has been demonstrated that alcohol exposure could activate glial cells in the brain. Rodents chronically treated with alcohol display increased levels of glial fibrillary acidic protein (GFAP, proinflammatory astrocyte marker) in the VTA and cerebral cortex (Ortiz et al., 1995; Udomuksorn et al., 2011), as well as cluster of differentiation 11b (CD11b, proinflammatory microglial marker) within the cerebral cortex (Alfonso-Loeches et al., 2010). Furthermore, the activation of microglia in the rat hippocampus post-binge alcohol exposure lasted up to 28 days (McClain et al., 2011). However, the mechanisms, by which alcohol acts on glial cells, remain unclear.

Recently, it was demonstrated by Blanco et al. (2008) that alcohol directly acts on glia by interacting with lipid rafts caveolae. Rafts/caveolae are the plasma membrane micordomains. They have been identified as platforms for receptor signalling, constitute an important integrator of protein sorting, membrane trafficking, and signal-transduction events. The role of lipid rafts in immune cell activation has been documented (Manes et al., 2003). Triantafilou et al. (2002) have demonstrated the recruitment of TLR4 and CD14 into lipid rafts upon LPS stimulation. With regard to the mechanism of alcohol’s effects, the regulation of lipid rafts by alcohol promotes interleukin-1β receptor type I (IL-1RI) and TLR4 recruitment, triggering their endocytosis via caveosomes and downstream signalling stimulation (Blanco et al., 2008).

Interestingly, Lee et al. (2011) demonstrated that astrocytes and microglia were capable of expressing GABA_A and GABA_B receptors, and GABA could in turn suppress the reactive response of both astrocytes and microglia to LPS and interferon-γ (IFN-γ) by inhibiting induction
of inflammatory pathways mediated by nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) and p38/mitogen-activated protein kinase (MAPK). Thus, the suppressed GABA receptor signalling by acute alcohol exposure may lead to glial activation. Furthermore, since the pathogen TLR agonists, such as LPS, are not able to pass the blood-brain barrier (BBB) to activate TLR signalling in the brain, the endogenous TLR agonists, such as high-mobility group box proteins (Yanai et al., 2009), may mediate the activation of TLRs by alcohol exposure. Although it is still debatable if alcohol directly or indirectly acts on glial cells, many researchers have observed alcohol-activated brain immunity using a variety of experiments (Alfonso-Loeches et al., 2010; McClain et al., 2011; Zou et al., 2010).

Although both astrocytes and microglia can be activated by alcohol, microglial cells will be the focus in this thesis. It has been demonstrated that human microglia express TLRs 1-9 messenger ribonucleic acid (mRNA), whereas human astrocytes express robust level of TLR3, low-level TLR1, 4, 5, and 9, and rare TLR2, 6, 7, 8, and 10 mRNA (Jack et al., 2005). In mice, microglia have been demonstrated to be the major non-neuronal cell type in the CNS for expression of TLR4 (Lehnardt et al., 2002). Considering the pivotal role of TLR4 in alcohol-induced neuroinflammation (see section 1.4.2.1), microglia are possibly the initial cells where this neuroinflammation occurs.

1.4.2.1. Alcohol and TLR4 signalling

As many ex vivo and in vitro studies have indicated, TLR4 is the key receptor in the mechanisms of alcohol’s action on glial cells (microglia and astrocytes) (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009). The activation of TLR4 signalling enhances
inflammatory mediators through NFκB (Blanco et al., 2004; Valles et al., 2004). The well understood function of TLR4 is to recognise LPS from gram-negative bacteria, cooperating with myeloid differentiation factor 2 (MD-2) and CD14 (Park et al., 2009) (Figure 1-3). TLR4 signalling cascades are classified into myeloid differentiation primary response gene 88 (MyD88)-dependent and independent pathways. For the MyD88-dependent pathway, myelin and lymphocyte protein (MAL) and MyD88 lead to the activation of the serine/threonine kinase interleukin-1 receptor-associated kinase 4 (IRAK4), which engages with MAPK cascades [c-Jun N-terminal kinase (JNK), extracellular regulated kinase (ERK), and p38] (Figure 1-4) and leads to NFκB activation and the induction of proinflammatory cytokines such as IL-1β. Alternatively, downstream of TLR4, phosphoinositide 3 kinase (PI3K)/AKT pathways could also induce NFκB activation (Hua et al., 2007). For the MyD88-independent pathway, TLR4 recruits toll/IL-1R domain containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) in endosomes, which leads to the activation of the transcription factor IFN regulatory factor 3 (IRF3) and the induction of genes such as those encoding Type I IFNs (Akira et al., 2004; Hennessy et al., 2010).

The effect of alcohol on the activation of glial TLR4 signalling depends on the dose and time-course of alcohol exposure, as well as the specific pathways involved (Suk, 2007). It has been demonstrated that alcohol activates glial MAPK and NFκB pathways within minutes of stimulation in vitro, which is TLR4-dependent (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009). In wild-type astrocyte culture, 10–50 mM of alcohol increased ERK, IRAK, p38, and JNK phosphorylation and MyD88 protein levels, with peak levels at 10 min post-alcohol exposure. Moreover, the enhanced phosphorylation of NFκB inhibitor alpha
(IkBα), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) were also observed, with peak concentrations at 30 min post-alcohol treatment (Alfonso-Loeches et al., 2010; Blanco et al., 2005). The increase of both COX-2 and iNOS in astrocytes by 75 mM alcohol even lasted for 7 days (Valles et al., 2004). However, in the presence of TLR4 or IL-1RI antibodies, neither wild-type (Blanco et al., 2005) nor TLR4-deficient astrocytes (Alfonso-Loeches et al., 2010) were activated by alcohol treatment, which implies the involvement of TLR4 and IL-1 receptor pathways. Similarly, in wild-type cortical microglial cells, 50 mM of alcohol-induced phosphorylation of ERK, JNK, p38, and IRAK, which peaked at 0.5–1 h post-alcohol exposure then decreased to baseline after exposure to alcohol for 24 h (Fernandez-Lizarbe et al., 2009). Furthermore, mRNA or protein expression of TNF-α, IFN-β, and IRF-1 were increased after wild-type microglial cells were treated with alcohol for 3 h (Fernandez-Lizarbe et al., 2009). This result suggested the involvement of microglial MyD88-dependent and -independent pathways in the effect of alcohol. However, this quick proinflammatory activation by alcohol was not found in TLR4-deficient microglia, which indicated the key role of TLR4 in the microglial activation induced by acute alcohol exposure. As such, the above evidence indicates that alcohol exposure is capable of rapidly activating TLR4-dependent MAPK and NFκB pathways in both astrocytes and microglia in vitro. The receptors, intracellular signalling proteins, and cytokines which are influenced by alcohol exposure have been summarised in Table 1-2.

Interestingly, glia respond differently to a range of alcohol concentrations. It was found that 10–100 mM of alcohol enhanced ERK, JNK, and p38 phosphorylation in mouse neonatal glia, and this elevated phosphorylation was increased with increasing alcohol concentrations (Fernandez-
Lizarbe et al., 2009). In contrast, 200 mM of alcohol did not activate MAPK cascades (Fernandez-Lizarbe et al., 2009). These results provided evidence that alcohol-induced TLR4-dependent glial activation was alcohol dose-dependent. However, 200 mM of alcohol is higher than mouse blood and brain alcohol concentrations following alcohol administration at up to 6 g/kg (blood alcohol concentration: ~ 150 mM; brain alcohol concentration: ~ 185 mM) (Smolen et al., 1989). Comparatively, 100 mM of alcohol is equivalent to blood and brain alcohol concentrations post-alcohol administration (3.5 g/kg) which has been considered as a high dose of alcohol and could induce LORR in mice (Smolen et al., 1989). Physiologically relevant alcohol concentrations (lower than 100 mM) need to be carefully chosen in future studies. As such, the MAPK signalling could be activated at moderate to high concentrations of alcohol in vitro.

Utilising a chronic ex vivo animal model, 5 months of alcohol treatment elevated CD14, MyD88, phosphorylated ERK, NFκB-p65, TNF-α, IL-1β, IL-6, iNOS, and COX-2 in the brains of wild-type mice, however, these changes were lacking in the brains of Tlr4 null mutant mice (Alfonso-Loeches et al., 2010). Notably, the above ex vivo study used female animals, whilst other studies utilised male animals. Since a sex difference has been reported in both alcohol drinking behaviours (Clark et al., 2007) and alcohol-induced changes of active substances in the CNS (Sun et al., 2005) in rats, the results from female animals may not be comparable with those from male animals. In male animals, on the other hand, four days of alcohol treatment triggered microglial activation, induction of NFκB-DNA binding, and enhancement of COX-2 expression in rat brain (Crews et al., 2006) (Table 1-2).
Figure 1-3. TLR4 signalling cascade. TLR4 is a type of trans-membrane receptor, which cooperates with MD-2 and CD14, mediating the innate immune response. TLR4 signalling leads to NFκB activation, cytokine release, and inflammatory responses via MyD88-dependent or –independent pathways. LBP, LPS binding protein; TIR, toll/interleukin-1 receptor.

Adapted by permission from Macmillan Publisher Ltd: Nature (Park et al., 2009), copyright 2009.
Importantly, the role of alternative non-MAPK pathways has been highlighted. It has been demonstrated that alcohol challenge induces a robust AKT phosphorylation in mouse striatum *ex vivo* (Bjork *et al.*, 2010) (Table 1-2), which highlighted the involvement of AKT pathways (Figure 1-4). This AKT signalling activation was blocked by (-)-naltrexone (Bjork *et al.*, 2010), a TLR4 signalling inhibitor and µ opioid receptor (MOR) antagonist (Hutchinson *et al.*, 2008b), which implies that such activation may be TLR4- or MOR-dependent. The activation of AKT signalling occurred at 15 min post-alcohol administration in the nucleus accumbens of rat, and inhibition of the AKT signalling within the nucleus accumbens attenuated binge drinking of alcohol (Neasta *et al.*, 2011). Chronic alcohol exposure also induced AKT signalling activation in neurons (Liu *et al.*, 2010), however, the glial AKT signalling post-alcohol exposure has not been examined to date.

Collectively, alcohol activated glial TLR4 signalling cascades in 10 min post-alcohol exposure *in vitro*, and in a long period or even 5 months of alcohol treatment *ex vivo*. As such, there is a lack of evidence at present showing glial activation by acute alcohol exposure *ex vivo*. Moreover, the behavioural consequences of TLR4-dependent glial activation induced by acute alcohol exposure are still unknown. Finally, TLR4 downstream MAPK, AKT, and NFκB signalling pathways are worthy of investigation.
1.4.2.2. Behavioural effects of alcohol and central immune activation

Emerging data indicate that the functions of neuroimmune molecules go beyond their established roles in neuroinflammation and that glial cells and various immune skewing factors have been
considered to also be involved in the behavioural responses observed following alcohol exposure (Alling et al., 1986; Hansson et al., 1987; Ronnback et al., 1988). Although the mechanisms of alcohol-induced sedation and motor impairment have been discussed in section 1.1, evidence of immune activation was limited with these two behavioural models. In this section, the neuroimmune studies using behavioural models of alcohol will be discussed together.

A recent study showed that minocycline (a microglial attenuator, see section 1.4.4.1.) reduced alcohol drinking in mice, and this is independent of minocycline-induced changes of water intake or body weight (Agrawal et al., 2011). This finding suggests that microglial activation may be related to the development of alcohol dependence. However, as minocycline has a variety of activities, to confirm this result is microglia-related, the degree of microglial activation in the brain needs to be assessed.

It has been demonstrated that mice lacking TLR4 were protected against inflammatory damage and the behavioural associated effects induced by chronic alcohol consumption (Pascual et al., 2011). Moreover, selective blockade of TLR4 in central nucleus of the amygdala of alcohol-preferring rats inhibited binge drinking (Liu et al., 2011). These results provide solid evidence that TLR4 signalling activation by alcohol administration is related to behaviours.

With regards to intracellular signalling cascades, activation of MAPK pathways by the glial cell line-derived neurotrophic factor administration reduced the motivation to both consume and seek alcohol (Carnicella et al., 2008). In addition, several genes involved in the MAPK pathways, including Mapk3, p38, Map2k1, and Map2k2, were found to be up-regulated in the NAc of the
high alcohol consuming rat strain (Arlinde et al., 2004). These results provide evidence that MAPK cascade activation may be related to alcohol consumption.

Multiple cytokines, including chemokines, are to be discussed below in both animal alcohol models and human alcoholic experiments. Increases in glial activation markers such as these can be indicative of a proinflammatory response, and is further supported by work demonstrating elevated CCL2 expression in human brains of alcoholics (He et al., 2008; Liu et al., 2006). Animal evidence also showed that deletion of chemokine (C-C motif) ligand receptor 2 (CCR2), CCL2 (females), or CCL3 in mice resulted in lower preference for alcohol, and CCL2 or CCL3-deficient mice showed longer duration of alcohol-induced LORR than wild-type mice (Blednov et al., 2005), which together suggested the involvement of these chemokines and their receptors. Mice deficient in β-2-microglobulin (B2M), cathepsin S (CTSS), cathepsin F (CTSF), IL-1 receptor antagonist (IL-1ra, encoded by Il1rn gene), CD14, or IL-6 had reduced alcohol consumption in the 24 h two-bottle choice test (Blednov et al., 2011b), suggesting a role for proinflammatory cytokines, the TLR4 signalling pathway, and major histocompatibility complex (MHC) molecules (B2M, CTSS, and CTSF) in alcohol drinking behaviour. Furthermore, human genetic studies have demonstrated associations between IL1B (Liu et al., 2009; Pastor et al., 2005), IL1RN (Pastor et al., 2000; Pastor et al., 2005), IL10 (Marcos et al., 2008), and NFKB1 (Edenberg et al., 2008) genetic polymorphisms and alcohol dependence - indirect evidence of a proinflammatory contribution to alcohol addiction or dependence (Table 1-2).
### Table 1-2. Evidence of alcohol-induced neuroimmune activation

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Description</th>
<th>Material</th>
<th>Treatment</th>
<th>Animal/human</th>
<th>Effect</th>
<th>TLR4-dependent</th>
<th>Reference</th>
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<td>mice</td>
<td>↑</td>
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<td>mice</td>
<td>↑ phosphorylation</td>
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<td>1, 2, 3</td>
</tr>
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<td>mice</td>
<td>↑ phosphorylation</td>
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<td>MAPK signalling</td>
<td>astrocytes, microglia</td>
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<td>mice</td>
<td>↑ phosphorylation</td>
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</tr>
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<td>mice</td>
<td>↑</td>
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<td>acute alcohol</td>
<td>mice</td>
<td>↑</td>
<td>yes</td>
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<td>mice</td>
<td>↑</td>
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<td></td>
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<td>mice</td>
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Table 1-2. Evidence of alcohol-induced neuroimmune activation (continued)

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<th>Material</th>
<th>Treatment</th>
<th>Animal/human</th>
<th>Effect</th>
<th>TLR4-dependent</th>
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**Ex vivo study**

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Table 1-2. Evidence of alcohol-induced neuroimmune activation (continued)

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<td>-</td>
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* acute alcohol, less than 24 h.
Importantly, it has been demonstrated that the systemic administration of LPS enhanced alcohol-induced motor impairment 24 h later via rotarod test (Drugan et al., 2007). A prolonged increase of voluntary alcohol intake in wild-type mice induced by LPS administration was lacking in **Cd14** (a key component of TLR4 signalling, see the discussion in section 1.4.2.1) null mutant mice (Blednov et al., 2011a). Although LPS could not pass the BBB to activate brain TLR4 signalling directly, the above evidence still highlights the fact that immune activation facilitated the behavioural effects of alcohol.

In conclusion, even though various *ex vivo* and *in vitro* studies have demonstrated the potential role of brain TLR4 signalling in the effects of alcohol as discussed above, it is still unclear as to whether TLR4 signalling contributes to alcohol-induced sedation and motor dysfunction particularly within an acute setting.

### 1.4.3. Glial-neuronal communications

Generally, activated glial cells release a variety of active substances, including proinflammatory cytokines, prostaglandins, brain-derived neurotrophic factor, adenosine triphosphate (ATP), nitric oxide (NO), D-serine, and glutamate (Watkins et al., 2005), which in turn modulate neuronal activity, although the mechanisms by which such modulation occurs are only beginning to be understood (Ren et al., 2008). The possible mechanisms of glial-neuronal interaction are summarised in Figure 1-5.

Among the mechanisms of glial-neuronal crosstalk, cytokine signalling is generally considered to be the mechanism underpinning alcohol-induced neuronal death caused as a result of neuroinflammatory damage (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009). As summarised
in section 1.3.2 and 1.3.3, NMDA and GABA receptors are responsible for the neuronal activities of alcohol. Interestingly, IL-1β signalling, which can be activated by alcohol exposure (Blanco et al., 2005), also reduces astrocytic glutamate transporter-1 expression, and subsequently drives excitotoxic motor neuron injury (Prow et al., 2008). In addition, binding of glutamate to the NMDA receptor modulates LPS-induced innate immune activation, including TNF-α signalling, and this is TLR4-dependent (Glezer et al., 2003). Furthermore, chemokine (C-X-C motif) ligand 12 (CXCL12) could enhance GABA synaptic activity at serotonin neurons in rats (Heinisch et al., 2010). This evidence suggests that chemokines may facilitate the activation of GABA receptors by acute alcohol exposure. Thus, apart from directly acting on neurons, alcohol could also modify neuronal receptor functions via cytokine signalling induced by alcohol exposure.

There is also a profound relationship between the activation of cell signalling proteins involved in innate immunity and altered neuronal receptor functions post-alcohol exposure. A recent study has indicated that the NMDA receptor was resistant to acute alcohol inhibition, following chronic alcohol consumption (Wu et al., 2010). This resistance occurred with increased striatal enriched protein tyrosine phosphatase 33, enhanced phosphorylated p38 levels, and acquisition of tolerance to alcohol-induced sedation (Wu et al., 2010), suggesting the role of MAPK pathways in glial-neuronal crosstalk induced by alcohol exposure. In neurons, it has been demonstrated that chronic alcohol exposure activated ERK/p44 and JNK/p46 (Singh et al., 2007). This chronic alcohol treatment also up-regulated the effects of LPS on ERK, p38, and JNK phosphorylation, enhanced LPS-induced activation of NFκB/RelA-p50, but blocked NFκB/p50-p50 activation by LPS (Singh et al., 2007). These changes in signalling proteins in MAPK and NFκB pathways may be possible consequences of glial-neuronal communication induced by alcohol exposure.
Figure 1-5. Schematic summary of glial-neuronal interactions. Pain-related signalling is used in this graph as an example to demonstrated the signalling transduction between glia and neurons. (A) Signals leading to central glial activation. (B) Activation of microglia. (C) Role of astrocytes. BK, bradykinin; CX43, connexin 43; D-ser, D-serine; DYN, dynorphin; EP2, prostaglandin E receptor subtype; gln, glutamine, glu, glutamate; GS, glutamine synthetase; KOR, κ opioid receptor; P, phosphorylation.

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To summarise, alcohol directly acts on neuronal NMDA and GABA receptors in the CNS. On the other hand, alcohol activates glial cells (microglia and astrocytes) via TLR4, leading to activation of downstream signalling cascades, including MAPK and NFκB pathways, and release of cytokines and other active substances. The cytokines released by activated glial cells could in turn modify NMDA and GABA$_A$ receptors’ functions.

1.4.4. Potential role of immune signalling inhibitors in alcohol abuse-related diseases

The pharmacotherapy of alcohol dependence has significantly changed during the past few years. Disulfiram and lithium have been studied and used in alcohol dependence since more than two decades ago (Garbutt et al., 1999; Lejoyeux et al., 1993). More recently, the pharmacotherapy for treating alcohol dependence has been switched to opioid receptor antagonists [(-)-isomers of naltrexone, naloxone, and nalmefene], acamprosate, and various serotonergic agents. Among these pharmaceutical agents, as several system reviews indicated (Garbutt et al., 1999; Srisurapanont et al., 2005), naltrexone and acamprosate improved therapeutic outcomes by decreasing alcohol consumption. However, most of the subjects did resume drinking alcohol during such treatments (Garbutt et al., 1999; Srisurapanont et al., 2005) which indicates the low efficacy of these drugs.

Considering the emerging evidence of glia-mediated modulation of alcohol’s effects by TLR4 and IL-1β, and of the pivotal neuroinflammatory effects of alcohol, central immune signalling inhibitors could potentially be an additional treatment for alcohol abuse-related conditions. Alcohol and other drugs of abuse, such as morphine (Hutchinson et al., 2008a) and methamphetamine (Zhang et al., 2006), activate glial TLR4 signalling, and share the same
reward pathways (Hyman et al., 2006). Therefore, central immune signalling inhibitors which have been studied to alter the effect of other drugs of abuse may modulate the effect of alcohol. These central immune signalling inhibitors and their effects on other drugs of abuse are discussed below.

1.4.4.1. Glial attenuators

Glial attenuators, including minocycline, ibudilast (AV411, MN166), and propentofylline (HWA 285), have been reported to modulate the effects of drugs of abuse in animal studies. Minocycline, a putative microglial attenuator, was found to suppress morphine-induced respiratory depression and reward, but enhance systemic morphine-induced analgesia (Hutchinson et al., 2008a). Moreover, minocycline inhibited the development of cocaine-induced locomotor sensitisation (Chen et al., 2011; Chen et al., 2009) and methamphetamine-induced hyperlocomotion, behavioural sensitisation, and impairment of recognition memory (Mizoguchi et al., 2008; Zhang et al., 2006). Similar to minocycline, administration of ibudilast attenuated several morphine effects, including its activation of brain microglia and astrocytes and its induction of elevated DA levels (Bland et al., 2009), reward, tolerance, and withdrawal (Hutchinson et al., 2009; Ledeboer et al., 2007). Furthermore, ibudilast inhibited prime- and stress-induced methamphetamine relapse (Beardsley et al., 2010). Propentofylline, another glial attenuator, diminished the rewarding effects induced by morphine and methamphetamine (Narita et al., 2006), morphine tolerance, and withdrawal-induced hyperalgesia (Raghavendra et al., 2004). Considering all these facts together, these glial attenuators may modulate the behavioural effects of alcohol, since
alcohol, similar to other drugs of abuse, activates central glial cells (Alfonso-Loeches et al., 2010; Watkins et al., 2009).

As a semisynthetic tetracycline that has been used for more than 30 years, minocycline has an overall good safety record for its clinical use (Kircik, 2010). Minocycline is a small (495 kDa) and highly lipophilic molecule at physiological pH [Apparent Partition Coefficients with Octanol/Aqueous Buffer at pH 6.6: tetracycline, 0.052; doxycycline, 0.92; minocycline, 1.48; and at pH 8.0: tetracycline, 0.010; doxycycline, 0.32; minocycline, 0.36 (Colaizzi et al., 1969)], which is capable of crossing the BBB better than other tetracyclines, such as doxycycline (Carney et al., 1974; Saivin et al., 1988). As such, the neuroprotective effect of minocycline has been studied and proven in numerous disease studies using animal models, such as ischaemic and haemorrhagic stroke, multiple sclerosis, spinal-cord injury, Parkinson’s disease, Huntington’s disease, and amyotrophic lateral sclerosis models (Yong et al., 2004). Although the mechanisms of minocycline’s effects are still unclear, minocycline is considered to be a potent inhibitor of microglial activation and of apoptotic pathways, because microglial activation and the attendant neurotoxic products generated by persistently activated microglia are common features of these diseases summarised above (Yong et al., 2004).

1.4.4.2. TLR4 signalling inhibitors

As TLR4 signalling has only been considered to be involved in the abuse mechanisms of drugs since the 2000s, only a few of these signalling inhibitors, including (+)-naloxone and (+)-naltrexone, have been examined in this area (Hutchinson et al., 2010a). Other TLR4 signalling inhibitors, such as eritoran (E5564), resatorvid (TAK-242), and anti-TLR4 antibodies (NI-0101...
and 1A6), have been tested in clinical studies for treating severe sepsis and inflammation (Kim et al., 2007; Rossignol et al., 2008; Yamada et al., 2005). It is well established that (+)-isomers of naloxone and naltrexone blocked morphine-induced hyperalgesia in rodents (Hutchinson et al., 2010a; Hutchinson et al., 2010b). Unlike their (-)-isomers, (+)-naloxone and (+)-naltrexone are inactive at classical neuronal opioid receptors (Hutchinson et al., 2008b), and the result of in silico docking analysis indicated that they were human MD-2 (signalling protein facilitating TLR4 responses) sensitive (Hutchinson et al., 2010a). Since the TLR4 signalling activities of (+)-naloxone and (+)-naltrexone were discovered only three years ago (Hutchinson et al., 2008b), human clinical data of these two drugs are still not available. Other TLR4 signalling inhibitors that have been investigated in clinical studies could potentially become the treatment of drugs of abuse-related conditions.

1.4.4.3. Other anti-inflammatory therapies

Numerous drugs of abuse, including alcohol, have been demonstrated to increase the production of proinflammatory cytokines in the CNS (Alfonso-Loeches et al., 2010; Watkins et al., 2009). Treatments blocking these cytokine signalling pathways could potentially be therapies for drugs of abuse-related conditions. Among immunosuppressants, human recombinant IL-1ra is capable of preventing the development of morphine tolerance (Shavit et al., 2005) in rodents via systemic injection.

Human recombinant IL-1ra (Anakinra), consisting of 153 amino acids, blocks the biological activity of IL-1 by competitively inhibiting IL-1 binding to IL-1RI. It is indicated for the
treatment of active adult rheumatoid arthritis, and has shown modest benefits in clinical trials (Cohen et al., 2002; Gabay et al., 2010).

To sum up, the various drugs summarised above, all acting on glial or TLR4 signalling cascades, have shown their effectiveness on altering the behavioural effects of drugs of abuse in animals. In addition, some of them have been demonstrated to be well tolerated in human clinical studies (Hennessy et al., 2010). Thus, these medications may provide new targeted therapies to drug dependence, including alcohol dependence.

1.5. Alcohol-opioid interactions

As one of the oldest drug classes known in the world, opioids produce analgesic effects by binding to opioid receptors. As has been discussed at the beginning of this Introduction, there is a clear synergistic interaction between alcohol and opioids. Alcohol consumption increases the risk of opioid-related deaths (Hickman et al., 2008; Levine et al., 1995), however, levels of opioids detected at autopsy from heroin addicts were below the predicted fatal range for highly tolerant individuals (Tagliaro et al., 1998), although levels of alcohol found were not particularly high (Darke et al., 2003). In fact, multiple studies of opioid overdose-induced deaths have reported a significant inverse relationship between blood alcohol and blood opioid post mortem concentrations (Darke et al., 1997; Fugelstad et al., 2003), suggesting that this alcohol-opioid interaction is unlikely to account for the modification of pharmacokinetic profiles (increased blood alcohol and opioid concentrations) of alcohol and opioids (Johnson et al., 2008).
1.5.1 Animal studies

A variety of alcohol-morphine interactions have been examined by animal behavioural models, such as the potentiated duration of alcohol-induced LORR by morphine (Forney et al., 1962), cross tolerance (Fidecka et al., 1986; Malec et al., 1987), and altered drinking behaviours (Gelfand et al., 1976; Sinclair, 1974), motor effects (Lessov et al., 2003), and withdrawal syndromes (Jones et al., 1977) of one of the drugs by the other. However, the interaction between alcohol and morphine in the CNS is not a simple symmetric relationship – but instead the effect of alcohol on the action of morphine and the effect of the latter on the action of the former may be via different mechanisms. For example, using rodent drinking preference models, morphine administration depressed alcohol consumption (Sinclair, 1974); in contrast, alcohol administration was not able to alter morphine consumption (Gelfand et al., 1976). However, a morphine-induced reduction in alcohol intake was established in hamsters (Ross et al., 1976). In a cross-sensitisation study, morphine pre-exposure induced an enhanced activation in locomotor activities in response to an alcohol challenge; however, alcohol-sensitised mice did not show cross-sensitisation to morphine (Lessov et al., 2003). In the case of withdrawal syndrome, alcohol suppressed morphine withdrawal (Ho et al., 1979), and similarly alcohol-induced convulsions were reduced by morphine in mice (Blum et al., 1976). Interestingly, increased alcohol consumption was found during opioid withdrawal in rats, while increased morphine self-administration after alcohol withdrawal in monkeys was also reported (Satinder, 1982). As such, alcohol’s effect on the action of morphine may not be the same as the effect of morphine on the action of alcohol, and studies in both directions need to be done and interpreted separately.
1.5.2. Neuronal mechanisms of alcohol-opioid interactions

1.5.2.1 Neuronal mechanisms of respiratory depression

Although a number of animal studies on alcohol-opioid interactions have been done, seldom have the studies focused on the mechanism of such interactions (White et al., 1999). Among alcohol-opioid interactions, respiratory depression has been researched most. Its mechanism has been hypothesised, as the prevailing interpretation of alcohol-induced heroin-related death, that opioids and alcohol have pharmacological interaction on respiratory depression (Hickman et al., 2008; White et al., 1999).

Respiration is controlled principally through medullary respiratory centres with peripheral input from chemoreceptors and other sources. There are a number of neurotransmitters mediating the control of respiration, and glutamate and GABA are the major excitatory and inhibitory neurotransmitters, respectively. On one hand, opioids induce respiratory depressant effects by activation of MORs (Olofsen et al., 2010; Romberg et al., 2003). On the other hand, alcohol facilitates the inhibitory effects of GABA at GABA receptors, and reduces the excitatory effects of glutamate at the NMDA receptor (see section 1.3.2.). Collectively, alcohol may enhance the respiratory depression as induced by opioids. Nonetheless, data supporting this hypothesis are extremely limited.

1.5.2.2 Other neuronal mechanisms

Amongst various alcohol-opioid interactions, the acute sedative interaction (the increased sedative effects of alcohol by morphine) is the focus in this Introduction, as this effect is assessed
in this thesis (see chapter IV). The neuronal mechanisms of the sedative effect of alcohol were suggested to be the modulation of GABA receptors, the NMDA receptor, and cAMP-PKA signalling by alcohol (Boyce-Rustay et al., 2005; Linden et al., 2011; Wand et al., 2001). Although alcohol exposure is capable of activating opioid receptors indirectly by enhancing β-endorphin and enkephalin levels (Mendez et al., 2008), this activation occurred 2 h post-alcohol administration (Mendez et al., 2001), which suggests that such activation was unlikely to be related to the rapid interactions between alcohol and opioids.

Regarding the effects of morphine on GABA receptors, acute morphine administration at 25 mg/kg decreased the binding affinity of GABA to GABA receptors in the cerebellum, cortex, and striatum, but not in the midbrain, diencephalon, or pons medulla, at 10 min post-morphine administration in rats (Ticku et al., 1980). In another study, increased maximal GABA receptor binding affinity in the cortex was observed at 60 min post-morphine administration at 20 mg/kg in mice (Sivam et al., 1982). Hence, morphine exposure is capable of rapidly modifying GABA receptor signalling, and the changes are likely to be a decrease, followed by an increase, in binding affinity of GABA to GABA receptors (Martin et al., 1999). Moreover, the mechanism of the sedative effect of morphine was suggested to be the activation of opioid receptors (Young-McCaughan et al., 2001). Collectively, the enhanced activities of GABA receptors and the activated opioid receptors following acute morphine exposure may serve as part of the mechanisms of the acute sedative interaction induced by alcohol and morphine.
1.5.3. Opioids and glial TLRs activation

Similar to alcohol, opioids have central immune signalling activities (Miguel-Hidalgo, 2009). Since both alcohol and opioids activate central glia, this glial activation could serve as an important background of alcohol-opioid interactions. A brief summary of the recent findings of opioid-induced immune signalling in the CNS follows.

As a widely used clinical opioid, morphine has been demonstrated to have microglial (Horvath et al.) and astrocyte activities (Alonso et al., 2007; Stiene-Martin et al., 1993) with brain region specificity (Lazriev et al., 2001). Furthermore, the glial activation attenuators minocycline (Hutchinson et al., 2008a) and ibudilast (Hutchinson et al., 2009) were able to attenuate such glial activation by morphine. The downstream consequences of opioid exposure in the CNS can be collectively considered as proinflammatory, with opioids able to:

1) increase expression or release of proinflammatory cytokines or their receptors, including IL-1β (Hook et al., 2011; Shavit et al., 2005), TNF-α (Niwa et al., 2007; Sawaya et al., 2009), CCL2 (El-Hage et al., 2005; El-Hage et al., 2006; Turchan-Cholewo et al., 2009), CCL5 (Avdoshina et al., 2010), CCR3, and CCR5 (Mahajan et al., 2005);

2) enhance NO release (Magazine et al., 1996) and COX-1 expression (Hutchinson et al., 2008a); and

3) activate MAPK (Ikeda et al., 2010; Miyatake et al., 2009; Xie et al., 2010b), NFκB (Sawaya et al., 2009), and AKT (Takayama et al., 2005; Xie et al., 2010b) signalling.
Recently, Hutchinson et al. (2010b) demonstrated the key role of TLR4 and MD-2 in opioid-induced immune activation, as both (+)-isomers (TLR4 signalling active) and (-)-isomers (clinically used, active at both TLR4 and opioid receptors) of methadone and morphine led to microglial activation in the spinal cord through TLR4 and MD-2. Interestingly, these responses could be non-stereoselectively blocked by naloxone and naltrexone via inhibiting TLR4-MD-2 signalling, while only (-)-isomers of naloxone and naltrexone are opioid receptor antagonists (Hutchinson et al., 2010a; Hutchinson et al., 2010b).

Furthermore, Li et al. (2009) showed that morphine also had TLR2 activities. In HEK293 cells, TLR2-MyD88 signalling activation induced a significant increase in morphine-induced apoptosis (Li et al., 2009). Utilising genetically deficient mouse models, mice deficient in TLR2 had 3- to 5-fold lower morphine-induced IL-6 and TNF-α expressions, and less microglial activation (quantified by CD11b levels) and morphine withdrawal, when compared to their wild-type counterparts (Zhang et al., 2011). These findings are different from what has been known about alcohol-induced central immune activation - no evidence has yet demonstrated a role for TLR2 activity of alcohol with in vitro, ex vivo, or behavioural evidence (Table 1-2).

In summary, opioids activate glial cells in the CNS, and this contributes to the unwanted rewarding property, withdrawal, tolerance, and respiratory depression (Hutchinson et al., 2009; Hutchinson et al., 2008a; Watkins et al., 2009). The activation of TLR2 and TLR4 has been established to be responsible for the glial activation induced by opioids.
1.5.4. Potential role of glial TLRs in alcohol-opioid drug interactions

As discussed previously in this introduction, both alcohol and opioids act on neuronal receptors, and they both activate glial cells through TLR4 signalling. Opioids can also activate TLR2 signalling. As such, other than the neuronal mechanisms, the possible mechanisms of alcohol-opioid interactions could include:

1) the activation of glia by alcohol and opioids;

2) the crosstalk between TLR2 and TLR4 cascades; and

3) the modification of neuronal receptors by a variety of proinflammatory cytokines produced by these activated glia.

Although it has been established that alcohol and opioids act on certain now known neuronal receptors and glial receptors, these may be different in the case of alcohol-opioid drug interactions. Firstly, because of the drug interactions, side effects of opioids, such as sedation, occur at a lower dose of opioids in the presence of alcohol. Thus, research should focus on the effects of alcohol and opioids at lower doses, and this may be different from the effects at higher doses. Secondly, due to the complexity of neuronal-neuronal, neuronal-glial, and glial-glial communications, some receptors or active substances may not be as important as others when using alcohol or opioids alone or together. Thirdly, receptors and active substances react to alcohol and opioids at various times of exposure, and therefore the sequence of their reactions is critical to the outcomes.
To summarise, although it is clear that the MOR, the NMDA receptor, GABA receptors, TLR2, TLR4, and proinflammatory cytokines such as IL-1β play pivotal roles in the mechanisms of alcohol and opioids individually, it may be different in the case of alcohol-opioid drug interactions. Therefore, it is worthy to investigate if all these factors are involved in the interaction between alcohol and opioids, which will improve our knowledge of the mechanisms of alcohol-opioid drug interactions.

1.6. Alcohol pharmacokinetics

Alcohol is principally metabolised in the liver with small amounts (about 1%) excreted in the breath, urine, and sweat (Holford, 1987). In addition, a variety of drugs and food interact pharmacokinetically with alcohol (Lennernas, 2009; Weathermon et al., 1999). As such, the concentrations of alcohol in humans, which can be externally validated by breath and blood samples, vary between individuals under different conditions. Understanding alcohol pharmacokinetics is important for investigating the mechanisms of alcohol’s effect and distinguishing the pharmacokinetic interactions between alcohol and other drugs from the pharmacodynamic interactions.

Alcohol permeates all tissues of the body and affects most vital functions, as it is a small molecule soluble in both water and lipids (Lieber, 1995). As such, the distribution of alcohol throughout the body is largely governed by the water content of various organs and tissues at equilibrium (Holford, 1987). No plasma protein binding to alcohol has been reported (Ramchandani et al., 2001).
At present, three enzymatic pathways of alcohol elimination have been recognised: alcohol dehydrogenase (ADH)-aldehyde dehydrogenase (ALDH), cytochrome P450 (CYP) enzymes, and catalase. The involvement of these enzymes in alcohol metabolism varies and this is influenced by the genotype, sex, body composition and lean body mass, liver volume, food composition, and ethnicity of individuals (Ramchandani et al., 2001).

1.6.1. ADH and ALDH

In the liver, ADH and ALDH are primarily responsible for alcohol metabolism. In ADH-mediated oxidation of alcohol, hydrogen is transferred from alcohol to the cofactor nicotinamide adenine dinucleotide (NAD), subsequently converting it to its reduced form NADH, while acetaldehyde is produced. This reaction has been developed into an enzyme-based method to measure alcohol concentrations in vitro (Smolen et al., 1989).

\[
\text{Alcohol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+
\]

Following the above reaction, acetaldehyde is oxidised into acetate in the presence of NAD, and this reaction is mediated by ALDH.

\[
\text{Acetaldehyde} + \text{H}_2\text{O} + \text{NAD}^+ \xrightarrow{\text{ALDH}} \text{Acetate} + \text{NADH} + \text{H}^+
\]

1.6.2. CYPs

CYP2E1, CYP1A2, and CYP3A4 are capable of oxidising alcohol in both the liver and brain (Hipolito et al., 2007; Lands, 1998; Salmela et al., 1998). Although the participation of these enzymes in alcohol oxidation is clearly lower than that of ADH, the CYP metabolic pathways are
responsible for most of the pharmacokinetic interactions between alcohol and drugs (Lieber, 1997). This is because many other drugs may increase or decrease the activities of CYPs and subsequently influence alcohol metabolism (Takahashi et al., 1993). Hence, it is important to make sure that the methods used to investigate the pharmacodynamic effects of alcohol, such as studies with animals of different genetic deficiencies or co-administration of pharmacological inhibitors of alcohol’s effects, do not alter alcohol metabolism. Otherwise the observed effects may be due to the modification of alcohol pharmacokinetics by those methods.

1.6.3. Catalase

Under physiological conditions, catalase does not appear to play a major role in alcohol oxidation in the liver (Lieber, 2004), however, catalase accounts for approximately 60% of the alcohol metabolism in the brain (Zimatkin et al., 2006). In the brain, physiologically active ADH is at low levels, and CYPs have a lower alcohol catalytic efficiency than does catalase. The locally produced acetaldehyde in the CNS could mediate some of the behavioural and neurotoxic effects of alcohol (Hipolito et al., 2007).

Blood and brain alcohol concentrations are related to the behavioural effects of alcohol, as alcohol is lipid permeable and easily passes the BBB. Blood alcohol concentrations are used to test the drink driving limits, as the samples can be easily accessed. Nevertheless, due to the differences in distribution of these enzymes in the liver and brain (Hipolito et al., 2007), peripheral alcohol pharmacokinetics may not exactly mirror brain pharmacokinetics in some circumstances. For example, in pharmacological inhibition and genetic deficiency models, the effects on alcohol pharmacokinetics may vary in the brain due to differences in the expression the
enzymes that metabolise alcohol. Thus, it is important to examine both blood and brain alcohol pharmacokinetics when examining the effects of alcohol in these models.

1.7. Alcohol and opioid pharmacogenetics

Pharmacogenetics is the discipline of researching the impact of genetic variability on individual responses to drugs and their metabolism. With a humble beginning in drug metabolising enzyme CYPs, molecular studies in pharmacogenetics have now been extended to numerous drug receptors and transporters (Lotsch et al., 2005; Ma et al., 2011). It has been demonstrated that up to 60% of opioid and alcohol dependence is heritable (Dick et al., 2006; Lachman, 2006). Investigation of genetic variants in metabolism pathways, transporters, and receptors has been conducted to explain a large percentage of this heritability.

1.7.1. Opioid pharmacogenetics

It was found that genetic polymorphisms could alter both pharmacokinetics and pharmacodynamics of opioids, and subsequently influence opioid dependence (Somogyi et al., 2007). Several genes associated with opioid dependence or the methadone dose requirement for treating opioid dependence have been identified, such as the ATP-binding cassette sub-family B member 1 (ABCB1) gene coding for P-glycoprotein (Coller et al., 2006; Hoffmeyer et al., 2000; Levran et al., 2008), CYP3A4 and CYP2B6 (Crettol et al., 2006), and the OPRM1 gene encoding the MOR (Deb et al., 2010). However, the association between these genetic polymorphisms and opioid dependence is still debatable. For example, meta-analyses have shown the lack of association between OPRM1 Asn40Asp SNP and opioid dependence (Arias et al., 2006; Coller et al., 2009).
Except for the genes related to drug metabolism and classical opioid receptor genes, immune genes are beginning to be investigated with regard to possible association with opioid dependence. As discussed in section 1.5.2, opioids are capable of activating central glia via TLRs, and subsequently increase the expression or release of proinflammatory cytokines, such as IL-1β (Hook et al., 2011; Shavit et al., 2005). The genetic variability of \( IL1B \) gene has been identified as being associated with opioid dependence (Liu et al., 2009). The frequencies of \( IL1B \) alleles at position -31 and -511 differed between opioid dependent patients and healthy controls (Liu et al., 2009), as the variants of \( IL1B \) -31 (Chakravorty et al., 2006) and -511 (Hall et al., 2004) could increase IL-1β secretion \textit{ex vivo}. Since opioids also have TLR4 signalling activity, the genetic variability of \( TLR4 \) may also alter opioid dependency. Hutchinson et al. (2010b) have shown with \textit{in silico} docking results that opioids dock to the LPS binding cleft of MD-2. Thus, it is possible that the genetic variability of \( TLR4 \) and \( LY96 \), encoding TLR4 and MD-2, respectively, alters the binding sites of opioids on TLR4/MD-2 complex, and therefore influences the binding affinity.

\textbf{1.7.2. Alcohol pharmacogenetics}

Alcohol pharmacogenetic studies have focused on alcohol metabolic enzymes (Gemma et al., 2006), including ADH, ALDH, and CYP2E1. The functions and importance of these enzymes have been summarised in section 1.6, and the key genes on which research has been done are as follows.

ADH is a polygenic family, and \( ADH4 \) is considered to be one of several alcohol-dependence-related loci in this cluster (Edenberg et al., 2006). It has been demonstrated that, at a promoter polymorphic site, individuals with the \( ADH4 \) -75A allele have 2-fold higher promoter activity
than those with the -75C allele do (Edenberg et al., 1999). In addition, possession of the 
*ADH1B*2 allele has been found to be protective against alcohol dependence in Asian populations (Luczak et al., 2006).

Another very important enzyme in alcohol metabolism is ALDH. Genes encoding ALDH are classified into 9 major families, in which only *ALDH1A1* and *ALDH2* are considered to be involved in acetaldehyde oxidation (Gemma et al., 2006). In Asian populations, a variant in *ALDH2*, that abolishes ALDH activity and therefore impedes the clearance of acetaldehyde, has been demonstrated to be protective against alcohol dependence (Eng et al., 2007). A meta-analysis showed that subjects heterozygous for the *ALDH2*2 allele had only one-fourth the risk of alcohol dependence as those with two functional alleles in the *ADH1B* and *ALDH2* genes (Luczak et al., 2006). This variant form of the allele is hypothesised to alter conversion rates during alcohol metabolism and lead to an excess build-up of acetaldehyde (Eriksson, 2001). The excess acetaldehyde leads to the unpleasant effects of facial flushing, tachycardia, nausea, and vomiting and thereby reduces heavy alcohol use, associated problems, and the development of alcohol use disorders (Wall et al., 2005).

CYP2E1 metabolises less than 10% of alcohol to acetaldehyde and subsequently to acetate, after ingestion of low amount of alcohol (Howard et al., 2003) (see section 1.6.2). It has been demonstrated that the *CYP2E1*1D allele with a minor allele frequency of 1% in Caucasians and 23% amongst Chinese may disrupt negative regulatory elements of this gene (Hu et al., 1999). As a result, homozygotes and heterozygotes of *CYP2E1*1D were found to have higher CYP2E1 activity after consuming alcohol than wild-type individuals (Hu et al., 1999). The association
between the *CYP2E1*\textsuperscript{1D} polymorphism and alcohol dependence has been reported (Howard *et al.*, 2003).

Other than genes encoding alcohol metabolic enzymes, the GABA\textsubscript{A} receptor, as the major neuronal receptor that alcohol acts on, has been studied in alcohol pharmacogenetics (Ducci *et al.*, 2008). A human variant of *GABRA6* Pro385Ser has been demonstrated to be associated with low alcohol response and alcoholism (Hu *et al.*, 2005).

Similar to opioid pharmacogenetics, a range of immune genetic polymorphisms, including *IL1B* \textsuperscript{−511} [genotype: odds ratio (OR) = 2.08 (Pastor *et al.*, 2005), allele: OR = 1.95 (Pastor *et al.*, 2005) and 1.91 (Liu *et al.*, 2009)], *IL1RN* VNTR [genotype: OR = 4.81, allele: OR = 3.69 (Pastor *et al.*, 2005)], *IL10* \textsuperscript{−592} [genotype: OR = 1.25 (Marcos *et al.*, 2008)], and eight SNPs of *NFkB1* (Edenberg *et al.*, 2008), have been demonstrated to be associated with alcohol dependence. Considering the importance of TLR4 in the mechanisms of alcohol’s effects (as discussed in section 1.4), the *TLR4* gene may be an important candidate gene in the pharmacogenetic research of alcohol and opioids. The candidate SNPs of *TLR4* in alcohol and opioid dependence research are discussed as follows.

**1.7.3. TLR4 polymorphisms**

Among the SNPs of TLR4, both Asp299Gly (D299G, A896G, rs4986790) and Thr399Ile (T399I, C1196T, rs4986791) variants have been demonstrated to lead to diminished TLR4-NF\textsuperscript{κ}B signalling (Arbour *et al.*, 2000; Rallabhandi *et al.*, 2006). Asp299Gly is an A/G transition at SNP rs4986790, which induces an Asp/Gly polymorphism at amino acid position 299. Thr399Ile is a C/T transition at SNP rs4986791, which causes a Thr/Ile polymorphism at amino acid position.
399. The Asp299Gly mutant showed decreased response to LPS and two structurally unrelated TLR4 agonists (the respiratory syncytial virus fusion protein and chlamydial heat shock protein 60) in vitro, while the Thr399Ile mutant had reduced response to LPS and the chlamydial heat shock protein 60, but not the respiratory syncytial virus fusion protein (Rallabhandi et al., 2006). Therefore, it is unlikely that the Asp299Gly and Thr399Ile polymorphisms alter these specific ligand binding sites of TLR4 to alter the response. However, these polymorphisms did not alter TLR4 protein expression compared to the wild-type in HEK293T (human embryonic kidney cell line) cells (Rallabhandi et al., 2006). It has been hypothesised that Asp299Gly and Thr399Ile mutants result in a disrupted interaction between such mutants and a serum component (e.g., CD14, LBP, or MD-2), as the reduction of serum concentrations in the culture medium led to a profound effect on LPS responsiveness (Rallabhandi et al., 2006). However, another study showed that the THP-1 (human acute monocytic leukemia cell line) cells transfected with the Asp299Gly allele had significantly reduced response (NFκB activity) to LPS compared to wild-type cells, whereas those transfected with the Thr399Ile allele had an intermediate response to LPS without significant differences compared to wild-type cells (Arbour et al., 2000). Even though the mechanism of these two SNPs’ effects on the function of TLR4 is still debatable, the reduction of response to TLR4 ligands in the Asp299Gly mutant is likely to be greater than that in the Thr399Ile mutant.

Intriguingly, the Asp299Gly and Thr399Ile SNPs were found to be absent in Han Chinese (Wu et al., 2006; Yan et al., 2008), Japanese (Tahara et al., 2007), Korean (Yoon et al., 2006), and Papuan populations (Ferwerda et al., 2007). However, the minor allele frequency of Asp299Gly was extremely high in Iranian, with 20.7% in healthy controls (Rezazadeh et al., 2006).
Asp299Gly and Thr399Ile were found to be in high linkage disequilibrium in Caucasians [Utah residents with ancestry from northern and western Europe, \( r^2 = 1 \) (Molvarec et al., 2008)], with minor allele frequencies of 3.5% (Asp299Gly) and 3.1% (Thr399Ile) in Caucasians in HapMap (http://hapmap.ncbi.nlm.nih.gov/). In contrast, no Thr399Ile mutant was found in West African populations, with only Asp299Gly allele (the minor allele frequency: 11.4%) (Newport et al., 2004). Therefore, the linkage disequilibrium between Asp299Gly and Thr399Ile SNPs varies in different ethnicities. These various distributions of TLR4 polymorphisms worldwide might be one of the reasons for the varying disease susceptibility, including alcohol and opioid dependence, in different ethnic populations.

Since Asp299Gly and Thr399Ile polymorphisms have been shown to alter TLR4-mediated signalling (Montes et al., 2006; Rallabhandi et al., 2006), a variety of related disease conditions have been studied. Increased susceptibility to infection diseases has been found in the mutants. For example:

1) *Helicobacter pylori* infection was associated with the Asp299Gly polymorphism, although the Thr399Ile SNP was not studied (Moura et al., 2008);

2) *Candida* bloodstream infection (Van der Graaf et al., 2006), tonsillar disease due to *Streptococcus pyogenes* and *Haemophilus influenza* (Liadaki et al., 2011), and *Streptococcus pneumonia* (Yuan et al., 2008) were associated with both Asp299Gly and Thr399Ile polymorphisms, which were found to be in high linkage disequilibrium in these studies; and
3) Gram-negative infections and haematogenous osteomyelitis (Montes et al., 2006) and respiratory syncytial virus infection (Puthothu et al., 2006) were associated with Asp299Gly only, but not the Thr399Ile polymorphism.

Other than infection diseases:

1) The association between the Asp299Gly polymorphism and cardiovascular diseases (Hernesniemi et al., 2008), graft failure after transplantation (Dhillon et al., 2010), methotrexate toxicity (Kooloos et al., 2010), metabolic syndrome (Cuda et al., 2011; Steinhardt et al., 2010), endometriosis (Latha et al., 2011), giant cell arteritis (Palomino-Morales et al., 2009), or Alzheimer's disease (Balistreri et al., 2008) has been established, although the Thr399Ile SNP was not studied;

2) The Thr399Ile polymorphism was associated with gallbladder cancer, although the Asp299Gly SNP was not studied (Srivastava et al., 2010);

3) Endotoxin hypo-responsiveness (Pacheco et al., 2008), gastric carcinogenesis (Rigoli et al., 2010), Crohn's disease and ulcerative colitis (Shen et al., 2010), body and liver fat (Weyrich et al., 2010), early-onset preeclampsia (Xie et al., 2010a), neurocysticercosis (Verma et al., 2010), Guillain-Barré syndrome (Nyati et al., 2010), and leprosy (Bochud et al., 2009) were associated with both Asp299Gly and Thr399Ile polymorphisms;

4) Invasive aspergillosis after stem cell transplantation was associated with only the Asp299Gly polymorphism, and not the Thr399Ile polymorphism (de Boer et al., 2011); and
5) in contrast, gastric cancer (Santini et al., 2008) and chronic obstructive pulmonary disease (COPD) (Speletas et al., 2009) were associated with only the Thr399Ile, but not the Asp299Gly polymorphism.

Therefore, both the Asp299Gly and Thr399Ile polymorphisms were correlated to disease susceptibilities as summarised in Table 1-3(A).

Other TLR4 SNPs, such as rs1927911 (Enquobahrie et al., 2008; Kastelijn et al., 2010; Kerkhof et al., 2010), rs10759932 (Cheng et al., 2007; Kerkhof et al., 2010), rs2770150 (Banus et al., 2007; Kerkhof et al., 2010), rs11536889 (Duan et al., 2009; Fukusaki et al., 2007; Wang et al., 2011; Zhou et al., 2010), rs10759931, rs2737190 (Kerkhof et al., 2010), rs7037117, rs10759930, rs1927914, rs12377632, and rs2149356 (Shibuya et al., 2008), have been suggested to be correlated with diseases. The positions and frequencies of these SNPs have been summarised in Table 1-3(A). These TLR4 SNPs could be important candidate SNPs in alcohol and opioid pharmacogenetic research since TLR4 is the key receptor in the effects of alcohol and opioid on glial cells (as discussed in section 1.4).

The TLR4 SNPs which are close to the MD-2 and LPS binding sites (Park et al., 2009) may alter TLR4 binding affinity. The SNPs, which encode amino acids around (before or after five amino acids) the MD-2 and LPS binding sites of TLR4, are listed in Table 1-3(B). Amongst these SNPs, only TLR4 Asp299Gly is worthy of investigating, considering minor allele frequencies of the other SNPs are less than 1% in Caucasians. The inner core of LPS interacts with the D294, Y296 of TLR4 (Park et al., 2009), which are close to Asp299Gly. However, as discussed above, the functional consequence of the Asp299Gly mutant is likely to be a disrupted interaction with a
serum component (e.g., CD14, LBP, or MD-2), instead of LPS (Rallabhandi et al., 2006). Further studies need to be done to evaluate the structural and functional consequences of the Asp299Gly mutant.

The SNPs near the TLR4 phosphorylation and glycosylation sites may also alter the functions of TLR4. The TLR4 SNPs are compared with the published phosphorylation and glycosylation sites of TLR4 (www.uniprot.org, www.phosphosite.com) and summarised in Table 1-3(C). However, these SNPs have minor allele frequencies less than 1% in Caucasians and Han Chinese from Hapmap.

Taken together, investigation of genetic variants in metabolism pathways, transporters, and receptors has failed to explain a large percentage of heritability in alcohol and opioid dependence research (Gelernter et al., 2009; Kreek et al., 2005). Given the expanding role of glial TLR4 in mediating the effects of opioids and alcohol, genes within this glial activation and signalling cascade are likely candidates. To date, amongst the TLR4 SNPs, most disease association studies have focussed on the Asp299Gly and Thr399Ile, in which Asp299Gly is located near the LPS binding sites of TLR4 (Park et al., 2009), suggesting the possibility of altered substrate binding to TLR4. In addition, the reduction of response to TLR4 ligands in the Asp299Gly mutant is likely to be greater than that in the Thr399Ile mutant (Arbour et al., 2000; Rallabhandi et al., 2006). Furthermore, as these two SNPs were found to be in high linkage disequilibrium in Caucasians (discussed in this section), in theory only one of them need to be studied in pharmacogenetic studies with Caucasian populations, although this linkage disequilibrium observed in healthy
populations could be altered in disease states. Hence, TLR4 Asp299Gly and Thr399Ile, especially Asp299Gly, could be candidate SNPs in alcohol and opioid pharmacogenetic research.

**Table 1-3.** Summary of the TLR4 SNPs associated to diseases and potentially related to TLR4 function

(A) TLR4 SNPs related to diseases

<table>
<thead>
<tr>
<th>refSNP ID</th>
<th>Location</th>
<th>AA position</th>
<th>dbSNP allele</th>
<th>MAF in Caucasians</th>
<th>MAF in Han Chinese</th>
<th>Related disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10759930</td>
<td>promoter</td>
<td>C/T</td>
<td>38.4%</td>
<td>60.3%</td>
<td>glaucoma</td>
<td></td>
</tr>
<tr>
<td>rs2770150</td>
<td>promoter</td>
<td>C/T</td>
<td>28.8%</td>
<td>0.4%</td>
<td>whole-cell pertussis vaccination, asthma</td>
<td></td>
</tr>
<tr>
<td>rs10759931</td>
<td>promoter</td>
<td>G/A</td>
<td>N/A</td>
<td>N/A</td>
<td>asthma</td>
<td></td>
</tr>
<tr>
<td>rs2737190</td>
<td>promoter</td>
<td>A/G</td>
<td>32.3%</td>
<td>39.1%</td>
<td>asthma</td>
<td></td>
</tr>
<tr>
<td>rs1927914</td>
<td>5' near gene</td>
<td>C/T</td>
<td>32.3%</td>
<td>39.3%</td>
<td>glaucoma</td>
<td></td>
</tr>
<tr>
<td>rs10759932</td>
<td>5' near gene</td>
<td>C/T</td>
<td>13.5%</td>
<td>26.1%</td>
<td>prostate cancer, asthma</td>
<td></td>
</tr>
<tr>
<td>rs1927911</td>
<td>intron</td>
<td>C/T</td>
<td>26.5%</td>
<td>39.1%</td>
<td>nonfatal myocardial infarction, asthma, bronchiolitis obliterans syndrome after lung transplantation</td>
<td></td>
</tr>
<tr>
<td>rs12377632</td>
<td>intron</td>
<td>C/T</td>
<td>39.5%</td>
<td>61.6%</td>
<td>glaucoma</td>
<td></td>
</tr>
<tr>
<td>rs2149356</td>
<td>intron</td>
<td>A/C</td>
<td>27.7%</td>
<td>36.4%</td>
<td>glaucoma</td>
<td></td>
</tr>
<tr>
<td>rs11536889</td>
<td>3' UTR</td>
<td>G/C</td>
<td>13.8%</td>
<td>21.6%</td>
<td>chronic periodontitis, late-onset Alzheimer's disease, HBV recurrence after liver transplantation, major trauma</td>
<td></td>
</tr>
<tr>
<td>rs7037117</td>
<td>downstream</td>
<td>A/G</td>
<td>20.8%</td>
<td>23.3%</td>
<td>glaucoma</td>
<td></td>
</tr>
</tbody>
</table>
(A) TLR4 SNPs related to diseases (continued)

| refSNP ID  | Location | AA position | dbSNP allele | MAF in Caucasians # | MAF in Han Chinese # | Related disease *
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4986790</td>
<td>exon 3</td>
<td>Asp299Gly</td>
<td>A896G</td>
<td>3.5%</td>
<td>0%</td>
<td>infection, endotoxin hyporesponsiveness, cardiovascular diseases, cancers, graft failure after transplantation, gastric carcinogenesis, Crohn's disease, methotrexate toxicity, COPD, metabolic syndrome, endometriosis, early-onset preeclampsia, Guillain-Barré syndrome, giant cell arteritis, neurocysticercosis, Alzheimer's disease</td>
</tr>
<tr>
<td>rs4986791</td>
<td>exon 3</td>
<td>Thr399Ile</td>
<td>C1196T</td>
<td>3.1%</td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>

(B) TLR4 SNPs near* MD-2 and LPS binding sites

<table>
<thead>
<tr>
<th>refSNP ID</th>
<th>AA position of binding site</th>
<th>AA position of SNP</th>
<th>dbSNP allele</th>
<th>MAF in Caucasians #</th>
<th>MAF in Han Chinese #</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs55799839</td>
<td>R264</td>
<td>L260P</td>
<td>T779C</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>rs4986790</td>
<td>D294, Y296</td>
<td>D299G</td>
<td>A896G</td>
<td>3.5%</td>
<td>0%</td>
</tr>
<tr>
<td>rs5031050</td>
<td>K341</td>
<td>F342Y</td>
<td>T1025A</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>rs11536884</td>
<td>S386, S388</td>
<td>L385F</td>
<td>G1155T</td>
<td>0%</td>
<td>0.4%</td>
</tr>
<tr>
<td>rs55715411</td>
<td>F440, L444</td>
<td>V442V</td>
<td>G1326A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>rs5030716</td>
<td>F440, L444</td>
<td>F443L</td>
<td>C1329A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F443F</td>
<td>C1329T</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>rs5030719</td>
<td>Q507</td>
<td>Q510H</td>
<td>G1530T</td>
<td>0.9%</td>
<td>1.9%</td>
</tr>
<tr>
<td>rs55751510</td>
<td>L815</td>
<td>A814T</td>
<td>G2240A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
(C) *TLR4* SNPs near* the phosphorylation and glycosylation sites

<table>
<thead>
<tr>
<th>refSNP ID</th>
<th>AA position of phosphorylation sites</th>
<th>AA position of SNP</th>
<th>dbSNP allele</th>
<th>MAF in Caucasians</th>
<th>MAF in Han Chinese</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs16906079</td>
<td>T175</td>
<td>T175A</td>
<td>A523G</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>rs5030713</td>
<td>S189</td>
<td>Q188R</td>
<td>A563G</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>rs11536884</td>
<td>S381</td>
<td>L385F</td>
<td>G1155T</td>
<td>0%</td>
<td>0.4%</td>
</tr>
<tr>
<td>rs55905951</td>
<td>Y674, Y680</td>
<td>A676G</td>
<td>C2027G</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>refSNP ID</th>
<th>AA position of glycosylation sites</th>
<th>AA position of SNP</th>
<th>dbSNP allele</th>
<th>MAF in Caucasians</th>
<th>MAF in Han Chinese</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs16906079</td>
<td>N173</td>
<td>T175A</td>
<td>A523G</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>rs2770144</td>
<td>N309</td>
<td>V310G</td>
<td>T929G</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

AA, amino acid; MAF, minor allele frequency; N/A, not available on HapMap; UTR, untranslated region; HBV, hepatitis B virus.


* before or after five AAs of sites.
1.8. Aims and hypotheses

Understanding the mechanism of alcohol’s effects is the key to future strategies to prevent alcohol abuse. However, the neuronal mechanisms of alcohol’s effects, via the NMDA and GABA receptors (Mukherjee et al., 2008), may not account for all the behavioural effects of alcohol (Blednov et al., 2005). Recent findings demonstrated that four days of alcohol exposure causes proinflammatory responses through glial cells within the CNS (Crews et al., 2006). Furthermore, brain TLR-MyD88-NFκB signalling is important for alcohol-induced glial activation (Fernandez-Lizarbe et al., 2009), and up-regulates cytokine levels, such as IL-1β (Alfonso-Loeches et al., 2010). In addition, microglia are recognised as the main component of the brain’s immune system (Streit et al., 2004) and the principal cell type which expresses TLR2 and TLR4 (Jack et al., 2005) in the CNS.

Therefore, considering the emerging evidence of microglial mediated modulation of the:

1) effect of alcohol by proinflammatory cytokines;

2) pivotal neuroinflammatory effects of alcohol; and

3) TLR activities of alcohol in CNS, the main aims of this thesis are:

Aim A. To determine:

(a) the role of microglia in acute sedation and motor dysfunction following alcohol administration utilising the microglial attenuator, minocycline (Figure 1-6);
(b) the alteration of acute behavioural effects of alcohol following pharmacological TLR4 and IL-1 receptor signalling blockade with (+)-naloxone and IL-1ra, respectively (Figure 1-6); and

c) the involvement of innate immune receptors and intracellular signalling proteins, including TLR2, TLR4, and MyD88, in the acute behavioural effects of alcohol using genetically deficient mice (Figure 1-6).

**Aim B.** To characterise:

(a) the effects of alcohol exposure on MAPK signalling (p38, JNK, and ERK) and NFκB activation (IkBα); and

(b) the modulation of these signalling cascades by pharmacological or genetic attenuation of microglial and TLR4 signalling activation.

**Aim C.** To examine the relationship between the observed effects and changes in alcohol pharmacokinetics.

**Aim D.** To assess the effect of morphine (Figure 1-7) on alcohol dose-induced sedation, and the alteration of morphine’s effects by:

(a) pharmacological attenuation of MOR and TLR4 by (-)-naloxone;

(b) pharmacological inhibition of TLR4 and IL-1 receptor signalling following (+)-naloxone and IL-1ra administration, respectively; and

(c) blockade of receptors and intracellular signalling proteins in TLR signalling cascades via genetic deletion in TLR2, TLR4, TLR2 and TLR4 combination, and MyD88.
**Aim E.** To investigate the association between the *TLR4* Asp299Gly SNP and alcohol and opioid dependence.

On the basis of the existing literature, the major hypotheses to be examined are:

**Hypothesis A.** Alcohol-induced microglial activation and TLR2- and TLR4-MyD88-IL-1 receptor signalling contribute to its acute behavioural effects (Figure 1-6).

**Hypothesis B.** Genetic deficiency or pharmacological attenuation of microglial activation, IL-1 receptor signalling, and TLR2- and TLR4-MyD88 signalling will significantly alter the effect of alcohol *ex vivo* and *in vitro* (Figure 1-6).

**Hypothesis C.** Alcohol and morphine administration activate both the MOR and TLR2- and TLR4-MyD88 signalling cascades (Figure 1-7).

**Hypothesis D.** Blockade of MOR, IL-1 receptor, TLR2, TLR4, TLR2 and TLR4 combination, and MyD88 pharmacologically or genetically will attenuate the sedative effects following alcohol and morphine co-administration (Figure 1-7).

**Hypothesis E.** People with variants of the *TLR4* A896G SNP, which decreases TLR4 activation, are less likely to be dependent to opioids and alcohol.
Figure 1-6. Diagram of hypotheses of alcohol’s effects on neurons and microglia. Alcohol acts on both neurons and microglia in the CNS. Microglia could be activated by alcohol via TLR2- and TLR4-MyD88-NFκB-dependent signalling. Such activation in turn induces the signalling of cytokines and their receptors, including IL-1 receptor, which could further modify the neuronal receptor functions. As such, both the neuronal and non-neuronal activities of alcohol are responsible for its acute behavioural actions (sedation and motor dysfunction). Attenuation of microglial activation (minocycline), TLR2 (genetic deficiency), TLR4 [(+)-naloxone, or genetic deficiency], both TLR2 and TLR4 (genetic double deficiency), MyD88 (genetic deficiency), and IL-1 receptor signalling (IL-1ra) decreases alcohol-induced sedation and motor dysfunction.
Figure 1-7. Diagram of hypotheses of alcohol-morphine drug interactions. Both alcohol and morphine act on neurons and microglia in the CNS. Morphine modifies MOR signalling and subsequently induces neuronal effects. Glia could be activated by both alcohol and morphine via TLR2- and TLR4-MyD88-NFκB-dependent signalling. Such activation subsequently induces the signalling of cytokines and their receptors, including IL-1 receptor, which could in turn alter the neuronal receptor functions. As such, both neuronal and non-neuronal activities of these two drugs are responsible for the sedative interaction between alcohol and morphine. Attenuating MOR [(-)-naloxone], TLR2 (genetic deficiency), TLR4 [(+)-naloxone or genetic deficiency], both TLR2 and TLR4 (genetic double deficiency), MyD88 (genetic deficiency), and IL-1 receptor signalling (IL-1ra) results in decreased sedation induced by morphine and alcohol co-administration.
Chapter II Microglial and IL-1 receptor signalling mediates acute behavioural effects of alcohol


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This study aimed firstly to determine the effect of administration of a microglial attenuator, minocycline, or the commercially available human IL-1ra on alcohol-induced sedation assessed by the sleep time test (LORR) and motor impairment using the rotarod test. Secondly, it intended to characterise whether any observed behavioural effects were related to changes in alcohol peripheral and central pharmacokinetics. Thirdly, it aimed to investigate the effects of alcohol exposure on MAPK signalling by measuring the phosphorylation of p38, JNK, and ERK as well as NFκB by determining IκBα protein levels, and the modulation of such signalling by inhibition of microglial activation with minocycline or blockade of IL-1 receptor signalling utilising IL-1ra.

The present study showed that, in mice, the sedative effect of acute alcohol dose was inhibited by attenuation of microglial activation or IL-1 receptor signalling. This finding suggested that alcohol was capable of rapidly modifying proinflammatory immune signalling in the brain. In
addition, I demonstrated that mice treated with IL-1ra recovered faster from acute alcohol dose-induced motor impairment than control animals. In contrast, minocycline caused dose-dependent enhancement of alcohol-induced motor impairment, which implied differential mechanisms in the effects of minocycline on alcohol-induced sedation and motor impairment.

Furthermore, the pharmacokinetic study revealed that the dosing regimens of minocycline or IL-1ra did not change peripheral or central pharmacokinetic profiles of alcohol following a high dose of alcohol. This result suggested that the alteration in the behavioural effects of alcohol by minocycline or IL-1ra administration was not due to changes in alcohol pharmacokinetics.

In addition, I showed that, at the cellular level, IκBα protein levels in mixed hippocampal cells were rapidly elevated in response to alcohol exposure in a time-dependent manner. Both minocycline and IL-1ra attenuated the elevation of IκBα protein levels induced by acute alcohol exposure. This *in vitro* result further supported the behavioural findings.

Collectively, these data suggest that alcohol is capable of rapid modification of proinflammatory immune signalling in the brain and this contributes significantly to the pharmacology of alcohol. Whilst these results have implied the possible role of microglial and IL-1 receptor signalling in acute effects of alcohol, the mechanism by which alcohol induces molecular events that subsequently lead to profound behavioural effects remains unclear. Due to emerging evidence implicating a role for TLR4 in the CNS effects of alcohol (section 1.4.2), studies focused on the involvement of TLR4 signalling in the acute behavioural effects of alcohol are needed (Chapter III).
Attenuation of microglial and IL-1 signaling protects mice from acute alcohol-induced sedation and/or motor impairment

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Abstract

Alcohol-induced proinflammatory central immune signaling has been implicated in the chronic neurotoxic actions of alcohol, although little work has examined if these non-neuronal actions contribute to the acute behavioral responses elicited by alcohol administration. The present study examined if acute alcohol-induced sedation (loss of righting reflex, sleep time test) and motor impairment (rotarod test) were influenced by acute alcohol-induced microglial-dependent central immune signaling. Inhibition of acute alcohol-induced central immune signaling, through the reduction of proinflammatory microglial activation with minocycline, or by blocking interleukin-1 (IL-1) receptor signaling using IL-1 receptor antagonist (IL-1ra), reduced acute alcohol-induced sedation in mice. Mice treated with IL-1ra recovered faster from acute alcohol-induced motor impairment than control animals. However, minocycline led to greater motor impairment induced by alcohol, implicating different mechanisms in alcohol-induced sedation and motor impairment. At a cellular level, iNOS protein levels in mixed hippocampal cells responded rapidly to alcohol in a time-dependent manner, and both minocycline and IL-1ra attenuated the elevated levels of iNOS protein by alcohol. Collectively these data suggest that alcohol is capable of rapid modification of proinflammatory immune signaling in the brain and this contributes significantly to the pharmacology of alcohol.

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1. Introduction

Alcohol abuse results in approximately 1.8 million deaths globally (3.2% of all deaths) each year and accounts for 4.0% of disease burden (WHO, 2007). In high income countries, the weighted average societal cost of alcohol abuse is 1.6% of gross domestic product (GDP) purchasing power parity (Mehapatra et al., 2010). As alcohol dependence and abuse remains a continuing and important health problem, strategies to overcome these addictions are urgently required to reduce the burden of such diseases on society. With a thorough understanding of the mechanisms underpinning the actions of alcohol, it may become possible to develop novel targeted therapies to help combat this worldwide problem.

The neuronal mechanisms of alcohol's effects that have been widely accepted (Mukherjee et al., 2008) are that alcohol mainly acts by inhibiting N-methyl-D-aspartate (NMDA) receptor function and activating gamma-aminobutyric acid alpha (GABA\textsubscript{A}) receptors (Bonomi et al., 2000; Mukherjee et al., 2008). There is also increasing evidence supporting the concept that alcohol can alter neuronal lipid profiles (Saito et al., 2005) and therefore alter the response of channel proteins to alcohol, which can subsequently lead to the development of tolerance (Yuan et al., 2008).

Apart from these neuronal mechanisms, glial cells (microglia and astrocytes), the immunocompetent cells in the central nervous systems (CNS), contribute to alcohol-induced neurodegeneration (Blanco and Guerri, 2007; Blanco et al., 2005; Crews et al., 2006; Fernandez-Lizarbe et al., 2009; Zou and Crews, 2010). Microglia are recognized as the principal component of the brain's immune system (Streit et al., 2004). In acute pathological states such as those observed during infection, inflammation, and neurodegeneration, microglia are quickly activated, and contribute to altered CNS function (Kreutzberg, 1996). The effect of alcohol on the activation of microglial cell signaling pathways depends on the dose and time-course of alcohol exposure, as well as the specific pathways involved (Suk, 2007). Acute alcohol exposure leads to...
activation of the microglial nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway in vivo (Crews et al., 2006), and in vitro (Fernandez-Lizarbe et al., 2009). Astrocytes also play an important role in CNS function through the "tripartite synapse" (Aracue et al., 1999) or the "neural threesome" hypothesis (Smith, 2010), a bidirectional communication system between astrocytes, presynaptic neurons and postsynaptic neurons (Perea and Araque, 2002). Alcohol exposure also increases cytokine expression by astrocytes via NFκB activation (Davis and Syapin, 2004). Since NFκB is integral to induction of glial proinflammatory cytokines, activation of both microglia and astrocytes by alcohol triggers an increase in the CNS concentrations of several cytokines such as interleukin-1β (IL-1β) (Alfonso-Loeches et al., 2010).

Glia-neuronal cross-talk can occur via several mechanisms including cytokine signaling, and this is generally considered to be the mechanism underpinning alcohol-induced neuronal death caused as a result of neuroinflammatory damage (Blanco et al., 2005; Crews et al., 2006; Fernandez-Lizarbe et al., 2009; Zou and Crews, 2010). Human genetic studies have demonstrated that IL-1β polymorphisms are associated with alcohol dependence (Liu et al., 2009; Pastor et al., 2005), providing indirect evidence of a proinflammatory contribution to alcohol addiction/dependence. However, it is unclear if glial activation plays a role in alcohol-induced behavioral abnormalities. This possibility is raised based on the growing evidence that glial cells are critical in a number of other animal behaviors altered by xenobiotics, such as the development of opioid tolerance (Song and Zhao, 2001), respiratory depression (Ballanyi et al., 2010), analgesia (Hutchinson et al., 2008), dependence, and reward (Hutchinson et al., 2007; Watkins et al., 2009).

Therefore, considering the emerging evidence of microglial mediated modulation of drug actions by proinflammatory cytokines, and of the pivotal neuroinflammatory effects of alcohol, we hypothesized that pharmacological inhibition of microglial activation, or blockade of the IL-1β receptor would alter the behavioral actions of alcohol. This study aimed to determine if administration of minocycline (a microglial attenuator (Romero-Sandoval et al., 2008), or recombinant IL-1β would influence the outcome of an alcohol-induced sleep time test (sedation, loss of righting reflex) and/or performance in an alcohol rotated test (motor co-ordination) as motor and sedative effects of alcohol are responses that frequently accompany self-administration of alcohol (Chuck et al., 2006), and finally whether any observed effect was related to changes in alcohol pharmacokinetics. Furthermore, we aimed to characterize the effects of alcohol exposure on signaling via p38, JNK, ERK, and IκBα (NFκB inhibitor alpha, the main inhibitor protein of NFκB) which are all involved in NFκB activation and whether this signaling was modulated by minocycline or IL-1β.

2. Materials and methods

2.1. Animals

Pathogen-free male Balb/c wildtype mice (10–14 weeks old; n=4–5 mice/group for the pharmacokinetics study, n=6–17 mice/group for behavioral studies) were used in the experiments. Mice were housed in temperature (23 ± 3°C) and light/dark cycle (12h/12h) controlled rooms with standard rodent food and water available ad libitum. All animal studies were approved by the University of Adelaide Animal Ethics Committee.

2.2. Drugs

Human IL-1α (Kineret®, Amgen Inc, Thousand Oaks, CA, USA) was purchased from the Queen Elizabeth Hospital Pharmacy (Woodville, SA, Australia). Clinical grade endotoxin-free minocycline (microglial attenuator) was the gift of Alphapharm Pty Ltd., Australia (Glebe, NSW, Australia). Lipopolysaccharide (LPS) from Escherichia coli was purchased from Sigma (St. Louis, MO, USA). Alcohol (ethanol) was obtained from Chem-Supply (Gillman, SA, Australia). All other reagents and chemicals were of analytical grade quality.

For animal behavioral studies, minocycline and IL-1α were freshly diluted in endotoxin-free saline (0.9% sodium chloride) and injected intraperitoneally (i.p.) at 0.1 ml/10 g. The volume for injection of alcohol (i.p.) varied, based on weight of animals and dose of alcohol. Alcohol was diluted in endotoxin-free saline into 20% (v/v). The average weight of mice was 25 g (22–30 g). Thus, the volume for injection of alcohol was 0.32 ml (0.28–0.38 ml) at 2.0 g/kg, 0.40 ml (0.35–0.48 ml) at 2.5 g/kg, 0.55 ml (0.49–0.67 ml) at 3.5 g/kg, and 0.71 ml (0.63–0.86 ml) at 4.5 g/kg of alcohol. Endotoxin-free saline was used as a saline control.

For cell culture studies, minocycline, IL-1α, LPS, and alcohol were diluted in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), and RPMI 1640 was used as a vehicle control.

2.3. Experimental procedures

2.3.1. Alcohol dose-sedation assessment

Following a dose of saline (−30 min), an alcohol dose (2.5, 3.5 or 4.5 g/kg) was administered (0 min) and sleep time recorded from 0 min (see Section 2.4.1). Consequently, ED50 (effective dose, 50%) of alcohol was determined in this experiment. The ED50 was used as supportive data for the selection of the alcohol dose used in subsequent sleep time tests; the dose chosen produced a sub-maximal response, so that either reductions or enhancements of alcohol action could also be reliably quantified.

2.3.2. Effects of minocycline and IL-1α administration on acute alcohol-induced sedation and motor impairment

To examine the effects of minocycline and IL-1α on alcohol-induced sedation, mice were treated with one dose of minocycline (0.5, 5, 25, 46, or 60 mg/kg, −30 min), IL-1α (100 mg/kg, −30 min), or saline, followed by alcohol exposure (3.5 g/kg, 0 min). To further assess the effect of the minocycline dosing regimen, either of three doses of minocycline (0.05, 0.5, or 46 mg/kg) or saline were each administered repeatedly three times (−48 h, −24 h, and −30 min) prior to alcohol exposure (3.5 g/kg).

To assess differences in motor co-ordination using a rotated method (see Section 2.4.2), one dose of minocycline (46 mg/kg, −30 min) or IL-1α (100 mg/kg, −30 min), or three doses of minocycline (0.5 or 46 mg/kg, −48 h, −24 h, and −30 min) were administered prior to alcohol exposure (2.0 g/kg, 0 min). Mice were weighed daily during the 3-day dosing regimen.

2.3.3. Effect of repeated minocycline administration on modified response to alcohol-induced sedation upon alcohol re-exposure

This experiment was designed to investigate if the pharmacological effects of minocycline on alcohol’s action would last for 5 days. The modified response upon alcohol re-exposure was assessed by the administration of a single dose of alcohol (2.0 g/kg) 5 days prior to the sleep time test (alcohol dose 3.5 g/kg). Sleep time was assessed to confirm the development of the modified response as indicated by a reduction in overall sleep time in mice not administered minocycline or IL-1α compared to control animals that did not receive pretreatment.

To test the effect of minocycline on the modified response by alcohol re-administration, mice were pretreated with three doses of minocycline (0.5 or 46 mg/kg) or saline, each administered once per day on days 7, 6 and 5 prior to the sleep time test (alcohol dose 3.5 g/kg). Thirty minutes after the third daily injection of...
minocycline or saline, mice were administered a single dose of alcohol (2.0 g/kg) to induce modified response of alcohol. Five days later, the sleep time test was performed following alcohol exposure (3.5 g/kg).

2.3.4. Minocycline and IL-1ra effects on regulation of p38, JNK, and ERK phosphorylation by alcohol and LPS in splenocyte cultures

LPS-stimulated splenocytes were used as a positive control, as it is known that LPS causes mitogen-activated protein kinase (MAPK) signaling activation in mouse splenocytes (Kadoki et al., 2010). Splenocytes (isolated as described in Section 2.5) from each mouse were mixed and separated into fresh tubes. Cells were stimulated with 1 μg/mL LPS, 50 mM alcohol (based on previous studies (Alfonso-Loeches et al., 2010)), or vehicle at 37°C, 5% CO₂ for 15 min, in the presence or absence of 50 μg/mL minocycline or 50 μg/mL IL-1ra. Phosphorylated (phospho) ERK, phospho JNK, phospho p38, and total p38 (phosphorylated plus unphosphorylated) levels were quantified by a Cytometric Bead Array (CBA) assay as described in Section 2.6.

2.3.5. Determination of p38, JNK, and ERK phosphorylation and IkBα stimulation by alcohol in mixed hippocampal cell culture

Hippocampal cells (isolated as described in Section 2.5) from each mouse were mixed well and separated into fresh tubes. To test the effect of alcohol on p38, JNK, and ERK phosphorylation, cells were treated with 50 mM alcohol or RPMI 1640 for 15 min (37°C, 5% CO₂). Phospho ERK, phospho JNK, phospho p38, and total p38 levels were quantified by a CBA assay as per Section 2.6.

For Western blotting, the IkBα relative level was investigated in a first study to assess the responses of cells at various time points post-alcohol exposure. Cells were stimulated with alcohol (50 mM) at 37°C, 5% CO₂ for 0, 15, 30, 45, or 60 min. A suitable exposure time was chosen from this result, and used in the following Western blotting experiments.

To analyze the effect of alcohol on IkBα levels and the influence of minocycline and IL-1ra, cells were treated with minocycline (50 μg/mL), IL-1ra (50 μg/mL), or RPMI 1640 at 37°C, 5% CO₂ for 30 min, prior to stimulation with alcohol (50 mM) or RPMI 1640 for a further 30 min. The experiments were repeated three times with cells obtained from different mice.

2.3.6. Effect of minocycline and IL-1ra on peripheral and brain alcohol pharmacokinetics

To examine the influence of minocycline and IL-1ra on alcohol blood and brain pharmacokinetics, three doses of high dose minocycline (46 mg/kg) or the equivalent dose of IL-1ra (100 mg/kg as used in the sleep time and rotarod studies) were administered with the high dose of alcohol (3.5 g/kg) used in most of the sleep time tests.

One dose of IL-1ra or saline was administered (−30 min) to mice, followed by alcohol treatment (0 min). In the case of minocycline, three doses of drug were injected (−48 h, −24 h, and −30 min) prior to alcohol exposure (0 min). Mice were anaesthetized by an overdose of sodium pentobarbitone (300 mg/kg) 4 min before the blood and tissue collection. Blood samples were taken via cardiac puncture at 15, 60, 120, or 180 min following alcohol treatment, or 4 min after the mice awoke (as described in Section 2.4). Following blood collection, mice were perfused transcardially with saline and the brain was then removed. Blood and brain samples were immediately placed on ice. The alcohol concentration in each sample was measured with a nicotinamide adenine dinucleotide (NAD)–alcohol dehydrogenase (ADH) assay, as per Section 2.8. In this experiment, the blood and brain samples that were collected at various time points were from different animals.

The areas under the alcohol concentration–time curves (AUCs) from 15 min to 180 min post-alcohol administration were calculated using the linear trapezoidal rule. Slopes of the alcohol concentration–time curves were calculated by linear regression.

2.4. Behavioral testing

2.4.1. Sleep time (sedation, loss of righting reflex)

After mice were injected with alcohol, they were placed into separate cages with bedding. The length of sleep time was measured as the time from loss of righting reflex to the time of righting themselves three times in 30 s (waking up).

2.4.2. Rotarod (motor co-ordination)

The rotarod apparatus (Orchid Scientifics, Nashik, India) with a 3-cm-diameter dowel was set at a fixed speed of 16 rpm. The latency to fall was recorded as the duration that the mice remained on the rod, with a maximum cutoff latency of 180 s.

Each mouse underwent a training phase one day prior to experimental testing. Training involved the mouse remaining on the rotarod for 180 s in three sequential trials. On the experimental testing day, mice underwent a baseline trial before any dosing to ensure they performed at the training standard time of 180 s; this was repeated if they fell off the rod before the 180 s cutoff. The mice were then dosed with drug (minocycline or IL-1ra) or saline and returned to the cage for 30 min prior to alcohol administration (2.0 g/kg, 0 min). Another baseline test was conducted before alcohol administration. Mice were tested at 2, 5, 7, 13, and 20 min, and every 10 min thereafter, until they could remain on the rod for 180 s cutoff in two sequential trials. The duration of the mice remaining on the rod was recorded.

2.5. Cell isolation

Naïve adult BALB/c mice that had not received any alcohol or drugs were anaesthetized with an overdose of sodium pentobarbitone, followed by transcardial perfusion with saline. The spleen and brain were collected using aseptic techniques, and immediately placed on ice prior to cell isolation.

2.5.1. Spleenocyte isolation

Spleenocytes were isolated with OptiPrep (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions. The cells were diluted to 2 × 10⁷ cells/mL with RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), and allowed to rest at 37°C, 5% CO₂ for 2 h after isolation.

2.5.2. Mixed hippocampal cell preparation

Following brain removal, both sides of the hippocampus were immediately and carefully dissected out. The hippocampal samples were each subsequently homogenized in 4 mL of RPMI 1640 containing 10% heat-inactivated FBS, separately, and allowed to rest at 37°C, 5% CO₂ for 2.5 h.

2.6. Cytometric Bead Array

Cellular enzymes were denatured by boiling at 100°C for 5 min with Denaturation Buffer from BD CBA Cell Signaling Master Buffer Kit (BD Biosciences, San Diego, CA, USA), and samples subsequently stored at −80°C until analysis.

Phospho ERK 1/2 (T202/Y204), phospho JNK 1/2 (T183/Y185), phospho p38 (T180/Y182), and total p38 were quantified with BD CBA Flex Set (BD Biosciences, San Diego, CA, USA) and Cell Signaling Master Buffer Kit, according to the manufacturer’s instructions. Data were acquired with a FACSCanto flow cytometer (BD).
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Biosciences, San Diego, CA, USA) and analyzed with BD CBA Software according to the manufacturer’s instructions.

2.7. Western blotting

The preparation of cell lysates was performed as described previously (Lousberg et al., 2010). Briefly, cells were incubated on ice for 10 min, collected by centrifugation (4500 rpm, 4 °C, 5 min), and washed with ice-cold Dulbecco’s Phosphate Buffered Saline (DPBS, Invitrogen, Carlsbad, CA, USA). Cell pellets were resuspended in modified radiouimmunoprecipitation assay (RIPA) buffer (10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM sodium orthovanadate, 20 mM Na3PO4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, and complete EDTA-free protease inhibitor cocktail) for 15 min on ice. Following cell lysis, lysates were clarified via centrifugation (13,000 rpm, 4 °C, 5 min) and stored until analysis at −80 °C. Protein concentration was determined by a bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA, USA).

For Western blotting, samples were heated in SDS loading buffer at 97 °C for 5 min, fractionated by polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (GE Healthcare Biosciences, Pittsburgh, PA, USA). The membranes were subsequently blocked with 5% ECL Blocking Agent (GE Healthcare Biosciences, Pittsburgh, PA, USA) in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature, incubated with primary antibodies anti-IL1Ra (L35AS) (1:1000); Cell Signaling Technology, Danvers, MA, USA) or anti-β-actin (3:5000; Rockland Immunochemicals, Gilbertsville, PA, USA) overnight at 4 °C. Blots were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (1:2000) or anti-rabbit (1:10,000) IgG antibodies (GE Healthcare Biosciences, Pittsburgh, PA, USA) at room temperature for 1 h. The immuno-reactive signal was visualized by a chemiluminescence method (ECL Western Blotting Detection Reagents, GE Healthcare Biosciences, Pittsburgh, PA, USA) followed by exposure to X-ray films. ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used for quantifying the intensity of Western blot bands allowing comparison to the relevant actin controls.

2.8. NAD–ADH assay (alcohol quantification)

A NAD–ADH assay was used to quantify alcohol concentrations in blood and brain samples as previously described (Smolen and Smolen, 1989). The assay accuracy was expressed as the relative error (RE) according to the equation: RE (%) = 100 × (measured concentration – spiked concentration)/spiked concentration, and the precision was evaluated by the coefficient of variation (CV). The intra-assay precision and accuracy were estimated by analyzing five replicates at three different quality control levels (700, 500, and 200 mg/100 mL). Intra-assay precision and accuracy ranged from 2.8% to 9.8%, and −2.2% to 8.8%, respectively.

To test if minocycline, IL-1ra, or acetadylede would interfere with this assay, 0.001–100 μM of each drug was added to serum (containing 500 mg alcohol per 100 mL serum) or an equal volume of brain homogenized solution (containing the equal concentration of alcohol), and assayed together in the absence of drugs. The results showed that the presence of any of the vehicles did not influence the results obtained from this assay.

2.9. Statistical analysis

Graphpad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis. Unpaired t-test, one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test, or two-way ANOVA followed by Bonferroni’s post hoc test were performed. Data were presented as mean ± standard error of the mean (SEM). p values of 0.05 or less were considered significant.

3. Results

3.1. Alcohol’s sedative effect increased with alcohol dose

Alcohol-induced sleep time was dose-dependent as an increase in dose led to an increase in the length of sleep time (p < 0.0001, one-way ANOVA, Fig. 1a). The ED50 (effective dose, 50%) of alcohol was 3.4 ± 0.1 g/kg.

3.2. Minocycline had differential effects on acute alcohol-induced sedation and motor dysfunction

3.2.1. Alcohol-induced sleep time is reduced by minocycline treatment

A single dose of minocycline did not significantly reduce the sedative effect of alcohol (p = 0.087, one-way ANOVA, Fig. 1b), however, the administration of three doses of minocycline prior to alcohol exposure elicited a profound effect, decreasing sleep time significantly (p < 0.0001, one-way ANOVA, Fig. 1c).

3.2.2. Minocycline treatment increased alcohol-induced motor impairment

As shown in Fig. 2, all mice were trained and could perform equally well prior to alcohol treatment. Their latency to fall-off decreased from 180 s to less than 3 s in all treatment groups after alcohol administration (2.0 g/kg), with a gradual improvement over time.

A single dose of minocycline (46 mg/kg, Fig. 2a) or three low doses of minocycline (0.5 mg/kg, Fig. 2b) did not alter alcohol-induced motor impairment (p = 0.62 single dose, p = 0.76 for three doses, two-way ANOVA, not significant at any time point with Bonferroni’s post hoc test). However, three high doses (46 mg/kg) of minocycline showed a significantly greater loss of performance than three doses of saline following treatment with alcohol (p = 0.046, two-way ANOVA, p < 0.05 at 40 and 50 min with Bonferroni’s post hoc test). Repeated administration of minocycline at 46 mg/kg for 3 days did not change the bodyweight of mice (p = 0.62, repeated one-way ANOVA, minocycline treated mice bodyweight compared between days, p = 0.78, repeated two-way ANOVA, minocycline treated mice bodyweight compared with saline controls).

3.2.3. Repeated minocycline administration has no effect on the reduction of alcohol sedation by alcohol re-administration

In this experiment, a single alcohol administration (2.0 g/kg) on 5 days prior to the sedation test significantly reduced the alcohol-induced (3.5 g/kg) sleep time in the sedation test (p = 0.0048, one-way ANOVA). However, once daily minocycline days 5–7 prior to the sedation test did not alter the reduction of alcohol-induced sleep time (p = 0.16, one-way ANOVA, Fig. 1d).

3.3. IL-1ra treatment protected mice from alcohol-induced behavioral changes

3.3.1. Alcohol-induced sedation was reduced by IL-1ra treatment

Systemic injection of human IL-1ra significantly reduced the sedative effects of alcohol (p = 0.0014, unpaired t-test, Fig. 1e).

3.3.2. IL-1ra treatment resulted in shorter recovery time from alcohol-induced motor impairment

IL-1ra-treated mice displayed a shorter recovery time from alcohol-induced motor impairment (p = 0.0063, two-way ANOVA;...
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**Fig. 1.** Alcohol-induced sleep time is reduced by minocycline and IL-1ra treatment; (a) sleep time was significantly increased as a direct result of increased alcohol dose \((N=6)\); (b) a single dose of minocycline did not decrease alcohol-induced sleep time \((N=8)\); (c) three doses of minocycline significantly decreased alcohol-induced sedation \((N=6-11)\); (d) three doses of minocycline pretreatment 5 days before sleep test did not alter the modified response to alcohol-induced sedation upon alcohol re-exposure \((N=9-17)\); and (e) IL-1ra \((100\text{mg/kg})\) treatment reduced alcohol-induced sleep time \((N=10)\). Data are presented as mean ± SEM. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).

\(p < 0.001\) at 20 min, and \(p < 0.05\) at 30, 40 and 50 min with Bonferroni’s post hoc test, (Fig. 2a).

**3.4 Induction of p38 phosphorylation by LPS in splenocytes inhibited by minocycline and IL-1ra treatment**

**Fig. 3** shows the ratio of phospho p38 and total p38 under various treatment conditions. LPS caused a significant elevation p38 phosphorylation when compared to alcohol-stimulated and vehicle-treated cells \((p = 0.035\), two-way ANOVA). In the absence of minocycline or IL-1ra (control groups open bars in Fig. 3), \(p = 0.021\), one-way ANOVA), alcohol-stimulated cells \((83\% \pm 22\%\) of baseline) had a lower ratio of phosphorylated and total p38 than LPS-stimulated cells \((146\% \pm 19\%\) of baseline, \(p < 0.01\), Bonferroni’s multiple comparison test). In cells cultured with the combination of LPS and alcohol there was no statistically significant difference in p38 phosphorylation compared to vehicle-treated controls \((107\% \pm 12\%\) of baseline). While minocycline \((p = 0.332\), one-way ANOVA) and IL-1ra \((p = 0.678\), one-way ANOVA) did not change p38 phosphorylation by vehicle or alcohol, they did block induction of p38 phosphorylation by LPS.

Phospho JNK and phospho ERK levels in splenocytes were very low, and could not be detected by CBA after restting the cells at 37 °C for 2 h and remained undetectable following LPS and ethanol stimulation.
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3.5. Cell signaling proteins were differentially regulated by alcohol in mixed hippocampal cell samples

3.5.1. The degree of p38, JNK, and ERK phosphorylation did not change following alcohol stimulation

No significant difference in phosphorylated cell signaling proteins was observed between control and alcohol-treated hippocampal cells (phospho JNK, p = 0.65; phospho ERK, p = 0.09; phospho p38 was too low to be detected by CBA. Data are not shown by graph).

3.5.2. Regulation of cellular IkBα levels by alcohol was inhibited by minocycline and IL-1ra

Band densities of IkBα are represented as a percentage of the density of the loading control actin (Fig. 4). In the first study, cell pellets were harvested at various time points following alcohol stimulation (Fig. 4a and c). An increase in IkBα levels was observed at 15 min (157% of baseline) and 30 min (138% of baseline) post-alcohol exposure, and a decrease observed at 45 (22% of baseline) and 60 min (58% of baseline) post-exposure.

When cells were cultured in the presence of minocycline or IL-1ra, a significant main effect of alcohol stimulation was observed at 30 min of alcohol exposure using a repeated two-way ANOVA (p = 0.013; subject matching, p = 0.031, Fig. 4b and d). A significant interaction between alcohol and drug treatments was also observed (p = 0.030). In the control groups (absence of minocycline and IL-1ra), IkBα was significantly increased by alcohol exposure (p < 0.05, Bonferroni's post hoc test). In contrast, in the presence of minocycline or IL-1ra, IkBα levels were not changed by alcohol stimulation (p > 0.05, Bonferroni's post hoc test). Minocycline increased IkBα levels alone (362 ± 135% of baseline), while IL-1ra did not influence IkBα levels (173 ± 91% of baseline).

3.6. Minocycline and IL-1ra did not influence peripheral and brain alcohol pharmacokinetics

No significant differences in serum alcohol concentrations were found between the minocycline (p = 0.034) or IL-1ra (p = 0.02) treated mice compared to saline controls (Fig. 5a). However, brain alcohol concentrations in IL-1ra treated mice (p = 0.0003; p < 0.01 at 60 min), but not minocycline treated mice (p = 0.38, Fig. 5b), were transiently higher than the saline controls (two-way ANOVA with Bonferroni's post hoc test).

The serum alcohol AUC values of saline, minocycline treated and IL-1ra treated group were 9.3, 10.4, and 9.9 mg/mL respectively, and the slopes of the concentration–time curves were 0.67 ± 0.06, 0.36 ± 0.05, and 0.65 ± 0.05 h⁻¹ respectively. The brain alcohol AUCs of saline, minocycline treated and IL-1ra treated group were 345, 379, and 475 mg/100 mg h, respectively, and the slopes of the concentration–time curve were 0.18 ± 0.05, 0.24 ± 0.06, and 0.29 ± 0.10 h⁻¹ respectively.

Serum and brain samples were also collected when the mice awoke after each treatment. No significant difference in alcohol concentration was found with either serum samples (p = 0.11, Fig. 5c) or brain samples (p = 0.25, Fig. 5d) tested with one-way ANOVA.

4. Discussion

The present study shows that inhibition of acute alcohol-induced proinflammation, through a minocycline-sensitive mechanism possibly implicating microglia, or by blocking IL-1 receptor signaling using IL-1ra, was successful in protecting mice from acute alcohol-induced sedation. Together, this suggests that alcohol is capable of rapid modification of proinflammatory immune signaling in the brain. In addition, we demonstrated that mice treated with IL-1ra recovered faster from acute alcohol-induced motor impairment than control animals. On the contrary, minocycline lead to dose-dependent enhancement of alcohol-induced motor impairment, implicating differential mechanisms in the actions of minocycline on alcohol-induced sedation and motor impairment. Furthermore, we showed at a cellular level, that IkBα protein levels in mixed hippocampal cells were rapidly elevated in response to alcohol in a time-dependent manner, and both minocycline and IL-1ra attenuated this response. These findings are discussed below.

4.1. Minocycline’s differential effects on alcohol-induced behavioral changes

Although the activation of brain inflammation by alcohol has been demonstrated for microglia in vitro (Fernandez-Lizarbe et al., 2009) and in vivo (Crews et al., 2006), it is still unknown if
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the activation of microglia by alcohol could contribute the acute behavioral effects of alcohol. We have for the first time shown that minocycline, a drug broadly accepted to block microglial activation (Romero-Sandoval et al., 2008), is sufficient to protect mice from the sedative effects of acute alcohol treatment. Importantly, we documented that these effects cannot be accounted for by changes in alcohol pharmacokinetics in blood or brain. Thus a different mechanism must underlie the observed reduction in alcohol sedation by minocycline. In previous studies, it has been demonstrated that alcohol induces sedation by activating GABA$_A$ receptors and blocking NMDA receptors (Beleslin et al., 1997; Lu et al., 2008) in neuronal systems. However, the mechanism of action here remains unclear considering that proinflammatory cytokines such as IL-1β and TNF-α are sleep-promoting substances (Kapas and Krueger, 1992) that also mediate loss of glutamate transport (Prow and Irani, 2008; Zou and Crews, 2005).
Interestingly, we found three doses of minocycline significantly reduced the alcohol-elicited sleep time, but not a single dose 30 min prior to alcohol administration. The differential effects could be due to several reasons. Firstly, minocycline achieves its peak concentration in the brain at 4 h after administration to rodents (Colovic and Caccia, 2003), and therefore a single dose of minocycline 30 min before alcohol exposure is unlikely to have provided peak minocycline concentrations in the brain at the time that alcohol achieved its peak effect site concentrations. However, effect site concentration may not be the only explanation as repeated administrations of both high dose (46 mg/kg) and low dose (0.5 mg/kg) minocycline reduced alcohol-induced sedation. Secondly, timing of drug administration could be critical as 30 min may not have been sufficient for minocycline to have its pharmacodynamic action to protect against pronflamatory immune activation within the brain. This is supported by other studies where similar multiple dose minocycline regimens were employed (Chen et al., 2006; Homsi et al., 2010). As such, further work is required to fully understand the molecular events that contribute to the slow onset of the pharmacodynamic action of minocycline.

Due to our finding that three doses of minocycline were more effective than a single dose, we proposed that this repeated dosing regimen of minocycline was adequate to achieve pharmacodynamic modification of brain immunity. Thus, we designed an experiment investigating if the pharmacodynamic action of minocycline would last for 5 days using modified response to alcohol-induced sedation upon alcohol re-exposure. The alcohol re-exposure resulted in a reduction in the sedative effects of alcohol. As opposed to exposure to alcohol 30 min after minocycline treatment, three doses of minocycline did not influence this modified response by alcohol 5 days post minocycline administration. This experiment is similar to alcohol tolerance models with a longer time between the two doses of alcohol administration than previous acute alcohol tolerance models (Erwin et al., 1986; Nowak et al., 2006); it is interesting that we could still observe the modified response by a single dose of alcohol 5 days prior to sleep time tests. Although the biology of alcohol tolerance is unclear, it is thought to involve several types of adaptation, ranging from alteration in membrane lipid composition (Yuan et al., 2008) and neuroadaptive changes in target proteins (Martin et al., 2008) to modification in alcohol metabolism (Bennett et al., 1993). By comparing acute alcohol tolerance (initial tolerance) and chronic alcohol tolerance (diminished effect after longer alcohol exposure), previous studies suggest that increased tolerance appears with additional days of alcohol (Radow, 2006), suggesting the role of immune components may be better evaluated with a chronic alcohol tolerance model.

Finally, analysis of the effects of minocycline on motor dysfunction induced by alcohol, as tested by the rotarod apparatus, showed a dose-dependent effect wherein three daily high doses of minocycline increased the degree of alcohol-induced motor impairment. As the animals’ bodyweight was not changed by this minocycline dosing regimen, the enhancement of motor dysfunction was unlikely to be a consequence of minocycline-induced sickness. This supports previous studies utilizing motor impairment models which indicate that minocycline is detrimental in Parkinson’s and Huntington’s disease models with treatment resulting in a worsening of the mean motor score and a slower recovery slope (Digu et al., 2004a). Minocycline may have variable and even opposite (beneficial or detrimental) effects in different species and models of neurological disorders (Digu et al., 2004b). It has been shown that minocycline treatment reduced microglial activation in axotomized precerebellar nuclei, but failed to mitigate either astrocytic response or neuronal loss after focal cerebellar lesion (Visconi et al., 2008). Our animal behavioral findings also imply that minocycline exerts both positive and negative effects on the behavioral actions elicited by alcohol treatment, depending on behavior type.

4.2. IL-1α treatment protects against alcohol-induced behavioral changes

We identified that systemic IL-1α protected against acute alcohol-induced sedation and motor impairment. Notably, a prior study has documented that in mice, systemic IL-1α (at comparable doses as used here) can effectively penetrate into the CNS to significantly impact behavior (Shavit et al., 2005). This suggests that systemic IL-1α in the present study may alter alcohol-induced effects via blockade of IL-1 signaling in the CNS. If true, this in turn suggests that alcohol activated, IL-1 mediated signaling in the CNS occurs within a relatively rapid time-frame.

The IL-1 pathway has been linked to alcohol dependence (Liu et al., 2009), as well as alcohol-induced gial activation (Blanco et al., 2005). Recent human immunogenic studies conducted by our group revealed lower variant SNP (IL-1β SNPs –511CT and –31T/C) frequencies in alcohol-dependent populations (Liu et al., 2009), and as these variant SNPs cause low IL-1β expression, this provides indirect evidence to support our hypothesis that increased IL-1β expression is related to alcohol dependence. Furthermore, alcohol is capable of inducing phosphorylation of IL-1 receptor-associated kinase within 10 min in cultured gial cells (Blanco and Guerri, 2007; Blanco et al., 2005).

4.3. No change in alcohol pharmacokinetic profiles by minocycline and IL-1α

To confirm that the behavioral changes induced by minocycline and IL-1α treatment were not simply a result of modifying the peripheral or central pharmacokinetics of alcohol, we measured alcohol concentrations following the dosing regimens used in sleep time tests. Serum alcohol concentrations obtained were similar to those obtained in previous studies (Smolen and Smolen, 1980). However, concentrations of alcohol in the brain were lower than those previously observed, most likely as a result of the transcerebral perfusion performed prior to brain tissue collection. Overall minocycline treatment did not change the concentration of alcohol in either the serum or brain samples as the AUC values were similar between minocycline and IL-1α treated and, untreated groups. To our surprise, IL-1α treatment transiently increased brain alcohol concentrations. Since IL-1α attenuated alcohol-induced behavioral effects, we would have expected to observe a decreased alcohol concentration in IL-1α treated group if the effect was pharmacokinetic in nature. In contrast, IL-1α treated animals had shorter sleep time and improved recovery from motor impairment, albeit while displaying a transiently higher brain alcohol concentration. This result further supports our hypothesis that the behavioral effects observed following alcohol treatment do not depend directly on alcohol concentrations. As we did not observe a reduction in alcohol concentration following minocycline or IL-1α treatment, we expected that mice in the minocycline and IL-1α treated groups, which woke up earlier, to have higher peripheral and brain alcohol concentrations following their awakening. However, there was no significant difference in alcohol concentrations between minocycline and IL-1α treated groups and controls, which may be a result of the small slope of the alcohol concentration-time curves.

4.4. Differential effects of alcohol on cell signaling proteins

The concentrations of minocycline and IL-1α in our in vitro experiments were equivalent to plasma or brain tissue concentrations in previous rodent studies (Colovic and Caccia, 2003; Shavit Yue Wu, PhD Thesis 2011
et al., 2005), and the alcohol concentration was based on the maximal brain alcohol concentration observed in our pharmacokinetics study as well as previous publications (Alfonso-Loeches et al., 2010).

Alcohol has different effects on peripheral and CNS immune cells due to the distinct functional differences of these cells. Acute alcohol treatment inhibits LPS-induced NFκB (Mandrekar et al., 2007) and MAPK (Oak et al., 2006) activation in peripheral monocytes, which supports the results we observed in our spleenocyte preparations. In contrast, alcohol activates immune cells in the brain in situ (Zou and Crews, 2010) and in vivo (Crews et al., 2006) by NFκB and MAPK pathways. We next examined the adult mouse primary mixed hippocampal cells which comprise a significant number of microglia (Inno et al., 2007). In treating adult mouse primary mixed hippocampus cells with alcohol, we did not observe any significant evidence of alcohol-induced p38, JNK, and ERK phosphorylation in the MAPK pathway at physiologically relevant concentrations, which is contrary to previous findings with cultured neonatal microglia (Fernandez-Lizarbe et al., 2009) and astrocytes (Blanco et al., 2005) and this could be related to different phenotypes between adult and neonatal glial cells (Beaulieu et al., 2008).

The main effects we observed related to IkBα protein levels, which were enhanced by acute alcohol stimulation (15–60 min) as observed in our first experiment, highlighting the rapid response of NFκB signaling to alcohol treatment. A negative association has been observed between the relative concentration of nuclear NFκB and the overall cellular IkBα protein levels, indicating a major role of IkBα in controlling NFκB localization (Pogson et al., 2008). Real-time binding kinetic experiments have also demonstrated that IkBα increases the dissociation rate of NFκB from DNA in a highly efficient kinetic process (Bergqvist et al., 2009). The decrease in IkBα protein levels following 45 and 60 min of alcohol stimulation in the present study indicates its rapid degradation and associated NFκB activation (Alkalay et al., 1995; Sun et al., 1993). This is supported by findings utilizing human astrocytes that showed that in the presence of alcohol, nuclear p65 levels and NFκB DNA binding activity increased at 3 h (Davis and Sayin, 2004), and studies with neonatal rodent microglia (Fernandez-Lizarbe et al., 2009) and astrocytes (Blanco et al., 2005) that reported increased nuclear p65 levels following treatment with alcohol for 30 min. In contrast, the observed increase in IkBα protein after 15 or 30 min of alcohol stimulation was unexpected. The synthesis and degradation of IkBα protein is influenced by multiple factors (Kanarek et al., 2004). Thus, it is possible that the elevated IkBα protein level we observed could be a result of NFκB activation leading to IkBα protein stabilization, released free IkBα from nucleNFκB, and increased transcription of IkBα mRNA (Ferreiro and Komives, 2010; Scott et al., 1993). In an attempt to maintain relevance with the acute behavioral responses, as sedation and motor impairment occurred within minutes of alcohol administration and the substantial behavioral differences observed at 30 min after alcohol, 30 min of alcohol stimulation was chosen for subsequent analyses to test the effect of alcohol, and the influence of microglial and IkBα-1α on IkBα protein levels. It is interesting to note that we found alcohol modified NFκB pathways but had no effects on MAPK pathways in mouse hippocampal cells. There are several signaling pathways that could lead to NFκB activation, including MAPK pathways and the phosphoinosride 3 kinase (PI3K)/Akt pathways (Hua et al., 2007). A recent paper demonstrated that acute alcohol challenge induced a robust Akt phosphorylation in mouse striatum (Bjork et al., 2010), further highlighted the involvement of the non-MAPK pathways. Thus, our results suggest alcohol could activate NFκB through non-MAPK pathways.

Minocycline has been shown to attenuate the up-regulation of phospho p38 in activated microglia (Li et al., 2010), and NFκB DNA binding activity (Orio et al., 2010). In our studies, both minocycline and IL-1α attenuated LPS-induced p38 phosphorylation in adult mouse primary splenocytes, as well as elevations in IkBα protein levels in mixed hippocampal cells induced by alcohol. These results demonstrate that alcohol rapidly activates proinflammatory cascades within the brain, which appear to be critical to alcohol-induced sedation and motor impairment, possibly through the activation of microglia and IL-1 signaling using NFκB. Minocycline and IL-1α treatment suppresses alcohol-induced proinflammatory cytokine-mediated immune activation in brain cells, which appears to protect mice from some of the behavioral effects induced by alcohol. Interestingly, minocycline induced an increase in IkBα protein levels by itself, implicating the mechanism of microglial attenuation by minocycline may be related to interference of IkBα protein degradation. In an acute time course, this may be related to a greater loss of motor function by minocycline; however, future work needs to be done to confirm this. Indeed, as we chose 30 min of alcohol exposure, which induced elevated IkBα levels, to evaluate the influence of minocycline and alcohol to IkBα levels, there is the possibility that the increased IkBα levels by minocycline may mask the effect of alcohol.

The current study demonstrates for the first time a key role of microglia and the IL-1 signaling cascade in the acute actions of alcohol on sleep time and rotorod test performance. Therefore, novel pharmacological strategies that target alcohol-induced microglial and immune signaling may have clinical utility in treating various alcohol-related conditions, such as alcohol overdose and dependence. While these studies have implicated the possible role of microglial and IL-1 signaling in acute actions of alcohol, the mechanism by which alcohol induces these molecular events, leading to these profound behavioral actions, is currently unclear. Our ongoing investigations are currently focused on the major immune signaling cascades activated in the brain by alcohol.

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References


Chapter II Microglial and IL-1 receptor signalling mediates acute behavioural effects of alcohol


Lousberg, E.L., Fraser, C.K., Tovey, M.G., Diener, K.R., Hayball, J.D., 2010. Type I interferons mediate the innate cytokine response to recombinant fowlpox virus by inhibiting the induction of proinflammatory dendritic cell-dependent adaptive immunity. J. Virol. 84, 6549–6563.


Chapter III TLR4-MyD88 signalling mediates acute behavioural effects of alcohol


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This study represented a logical progression from Chapter II. Due to the critical role of microglial and IL-1 receptor signalling in alcohol dose-induced sedation and motor impairment, this study focused on characterising the innate immune receptor and intracellular signalling cascades.

The first aim of this particular study was to determine the involvement of the TLR4-MyD88-dependent signalling cascade in alcohol dose-induced sedation and motor impairment using mice deficient in TLR4 or MyD88 and those treated with (+)-naloxone [the MOR-inactive isomer, a TLR4 signalling inhibitor (Hutchinson et al., 2010a; Hutchinson et al., 2008b)]. The LORR (sleep time) test and rotarod test were utilised to assess the acute behavioural effects of alcohol. Secondly, it aimed to characterise if any of the observed effects were related to any differences in the central and peripheral pharmacokinetics of alcohol between the mouse strains. Finally, it intended to determine if the alcohol-induced alteration of MAPK (JNK, ERK, and p38) and IκBα
(the main inhibitor protein of NFκB), which were all involved in NFκB signalling cascades, was TLR4-MyD88-dependent.

The current study showed that inhibition of TLR4-MyD88 signalling activation through genetic and pharmacologic manipulations reduced the duration of LORR and the level of motor dysfunction induced by acute alcohol dose in mice. The mechanistic hypothesis underlying these behavioural observations was postulated by the investigation in intracellular signalling proteins. I demonstrated that IκBα protein levels were elevated in response to 30 min of alcohol exposure in mixed hippocampal cells from wild-type mice, but not in cells from mice deficient in TLR4 or MyD88. Collectively, these results indicated that alcohol was capable of rapidly modifying innate immune signalling in the brain through the TLR4-MyD88-IκBα cascade, and such alterations were related to acute behavioural effects of alcohol.

These data provide new evidence linking the contribution of TLR4-MyD88-dependent signalling to the behavioural response induced by acute alcohol administration. The various findings in this chapter, together with the data presented in Chapter II, have demonstrated the role of microglia, TLR4, MyD88, and IL-1 receptor in sedation and motor impairment following acute alcohol dose.
Inhibiting the TLR4-MyD88 signalling cascade by genetic or pharmacologic strategies reduces acute alcohol dose-induced sedation and motor impairment in mice

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Chapter III TLR4-MyD88 signalling mediates acute behavioural effects of alcohol

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Chapter III TLR4-MyD88 signalling mediates acute behavioural effects of alcohol

Summary

BACKGROUND AND PURPOSE

Emerging evidence implicates a role for toll-like receptor 4 (TLR4) in the central nervous system effects of alcohol. The current study aimed to determine whether TLR4-MyD88-dependent signalling was involved in the acute behavioural actions of alcohol and if alcohol could activate TLR4-downstream MAPK and NFκB pathways.

EXPERIMENTAL APPROACH

The TLR4 pathway was evaluated using the TLR4 antagonist (+)-naloxone (µ-opioid receptor-inactive isomer) and mice with null mutations in the TLR4 and MyD88 genes. Sedation and motor impairment induced by a single dose of alcohol were assessed by loss of righting reflex (LORR) and rotarod tests, separately. The phosphorylation of JNK, ERK, and p38, and levels of IκBα were measured to determine the effects of acute alcohol exposure on MAPK and NFκB signalling.

KEY RESULTS

After a single dose of alcohol, both pharmacological inhibition of TLR4 signalling with (+)-naloxone and genetic deficiency of TLR4 or MyD88 significantly (p < 0.0001) reduced the duration of LORR by 45-78%, and significantly (p < 0.05) decreased motor impairment recovery time to 62-88% of controls. These behavioural actions were not due to changes in the peripheral or central alcohol pharmacokinetics. IκBα levels responded to alcohol by 30 min in mixed hippocampal cell samples, from wild-type mice, but not in cells from TLR4 or MyD88 deficient mice.
CONCLUSIONS AND IMPLICATIONS

These data provide new evidence that TLR4-MyD88 signalling is involved in the acute behavioural actions of alcohol in mice.

Keywords

Alcohol, TLR4, MyD88, IκBα, Loss of Righting Reflex, Sedation, Motor Impairment, Mice

Abbreviations

ADH, alcohol dehydrogenase; AUC, area under the alcohol concentration-time curves; BCA, bicinchoninic acid; CBA, Cytometric Bead Array; ED$_{50}$, median effective dose; ERK, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; IL, interleukin; IκBα, NFκB inhibitor α; JNK, c-Jun N-terminal kinase; LORR, loss of righting reflex; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NAD, nicotinamide adenine dinucleotide; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; TLR4, toll-like receptor 4; WT, wild-type
Introduction

Alcohol is consumed annually by two billion people world-wide with its abuse posing a significant health and social problem, with over 76 million people diagnosed with an alcohol abuse disorder (WHO, 2004). Among acute alcohol-induced behavioural actions, sedation and motor incoordination are responsible for a significant number of traffic accident-related deaths (Lin et al., 2009). The mechanisms causing impaired motor skills by alcohol were considered to be the enhanced gamma-aminobutyric acid (GABA) transmission to cerebellar granule cells (Carta et al., 2004) and Purkinje neurons (Hirono et al., 2009) in the cerebellum. Furthermore, mice with reduced affinity of the glycine binding site on N-Methyl-D-aspartate (NMDA) receptor NR1 subunit displayed an attenuated alcohol-induced motor dysfunction (Kiefer et al., 2003), implicating this system in alcohol action as well. Moreover, the GABA receptor (Linden et al., 2011), NMDA receptor (Boyce-Rustay et al., 2005), and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signalling (Wand et al., 2001) were demonstrated to be related to the sedative effects of alcohol. A variety of genes encoding second-messenger systems, neurotransmitters or opioid receptors, and alcohol metabolic enzymes has been demonstrated to be related to alcoholism (Schuckit et al., 2004). However, such purely neuronal and pharmacokinetic mechanisms of alcohol actions, which are still being elucidated, may not account for all of the behavioural actions induced by alcohol (Hyman et al., 2006), and an pro-inflammatory response induced by alcohol within the central nervous system may also play a role (He et al., 2008; Wu et al., 2011).

Glial cells and various immune modifying factors are activated following alcohol exposure in vitro (Alling et al., 1986; Hansson et al., 1987; Ronnback et al., 1988). Furthermore, rodents
Chronically treated with alcohol have increased levels of glial fibrillary acidic protein (GFAP, a pro-inflammatory astrocyte marker) in the ventral tegmental area (Ortiz et al., 1995), as well as CD11b (pro-inflammatory microglial marker) within cerebral cortex (Alfonso-Loeches et al., 2010). In addition, several genes involved in the mitogen-activated protein kinase (MAPK) pathway are found to be up-regulated in the nucleus accumbens of a high alcohol-consuming rat line (Arlinde et al., 2004). Recently, toll-like receptor 4 (TLR4) has been demonstrated to be a key receptor in the activation of glial cells (microglia and astrocytes) following acute alcohol exposure in vitro, and in chronic alcohol exposure ex vivo (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009). This is hypothesized to occur via the interaction between alcohol and the lipid rafts that trigger TLR4 signalling (Blanco et al., 2008), thus leading to an enhanced release of pro-inflammatory mediators following nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) up-regulation (Blanco et al., 2004; Valles et al., 2004). However, there still remains a lack of direct evidence that acute alcohol administration triggers TLR4 signalling to modify its behavioural effects.

Emerging evidence indicates that the functions of certain neuroimmune molecules may contribute to the behavioural changes induced by alcohol exposure. At the cell signalling level, activation of the MAPK pathway reduced the motivation of rats to consume and seek alcohol (Carnicella et al., 2008). Moreover, null mutation of genes encoding chemokine (C-C motif) ligand 2 (CCL2, females), CCL3, or CCL receptor 2 (CCR2) resulted in a lower preference for alcohol in mice, and mice with genetic deficiency of CCL2 or CCL3 showed longer duration of alcohol-induced loss of righting reflex (LORR) than wild-type (WT) mice (Blednov et al., 2005). In addition, the systemic administration of lipopolysaccharide (LPS, a TLR4 ligand) in mice
enhanced alcohol-induced motor impairment (Drugan et al., 2007) and alcohol consumption (Blednov et al., 2011). Furthermore, deletion of TLR4 protected mice against conditional learning and memory recognition dysfunctions as elicited by chronic alcohol consumption (Pascual et al., 2011). However, in the acute behavioural effects of alcohol, the role of TLR4 signalling has not been investigated.

Therefore, considering this new evidence for the role of TLR4 in the effects of alcohol within the brain, and the pivotal neuroinflammatory influence on the behavioural responses induced by alcohol, we hypothesized that inhibition of TLR4 signalling, by either genetic or pharmacologic means, would reduce behavioural effects following acute alcohol administration in mice. Two behavioural tests, the LORR and rotarod tests, were chosen to assess acute alcohol dose-induced sedation and motor incoordination, respectively. Our aim was to determine whether the TLR4-myeloid differentiation primary response gene 88 (MyD88)-dependent signalling cascade was involved in alcohol-induced sedation and motor impairment. Both genetic strategies (Tlr4 null mutant and Myd88 null mutant mice) and treatment with the TLR4 signalling inhibitor (+)-naloxone [the µ-opioid receptor-inactive isomer of naloxone (Hutchinson et al., 2010a; Hutchinson et al., 2008)] were used to assess the role of the TLR4 pathway. Furthermore, we examined whether any of the observed effects were related to changes in blood or brain pharmacokinetics of alcohol. Finally, we determined if the activation by alcohol of MAPK [c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38], and NFκB inhibitor α (IκBα, the main inhibitor protein of NFκB), which are all involved in NFκB signalling cascades, was TLR4-dependent.
Chapter III TLR4-MyD88 signalling mediates acute behavioural effects of alcohol

Methods

Animals

Pathogen-free male Balb/c WT mice, and mice with null mutations in the Tlr4 gene (Tlr4-/− mice) and Myd88 gene (Myd88-/− mice) (all 10–14 weeks old; n = 6-17 mice per group for behavioural studies, n = 4–5 mice per group for the pharmacokinetic study) were used in the experiments. Both Tlr4-/− and Myd88-/− mice, back-crossed onto Balb/c for more than 10 generations, were sourced from Prof. Akira (Osaka University, Osaka, Japan), and purchased from Dr. Simon Phipps (University of Queensland, Queensland, Australia) and Prof. Paul Foster (University of Newcastle, New South Wales, Australia). Mice were housed in temperature (23 ± 3 °C) and light/dark cycle (12/12 h) controlled rooms with standard rodent food and water available ad libitum. All animal studies were approved by the University of Adelaide Animal Ethics Committee.

Drugs, Doses and Solutions

Endotoxin-free (+) naloxone was kindly provided by Dr. Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland, USA). Alcohol was obtained from Chem-Supply (99.5%, Gillman, South Australia, Australia). All other reagents and chemicals were of analytical grade quality. The receptor and channel nomenclature used in the paper follows Alexander et al. (2009).
For animal behavioural studies, (+)-naloxone was injected intraperitoneally (i.p.) to the mice at 0.01 mL·g⁻¹. The volume for injection of alcohol (20%, v/v, i.p.) varied and was based on animal weight and dose of alcohol. The weight of mice was 25 g on average, and ranged from 22 to 30 g. Thus, the volume for injection of alcohol was 0.32 mL (range: 0.28-0.38 mL) at 2.0 g·kg⁻¹, 0.40 mL (range: 0.35-0.48 mL) at 2.5 g·kg⁻¹, 0.55 mL (range: 0.49-0.67 mL) at 3.5 g·kg⁻¹, and 0.71 mL (range: 0.63-0.86 mL) at 4.5 g·kg⁻¹ of alcohol. Endotoxin-free saline (0.9% sodium chloride) was used as the vehicle control.

For cell culture studies, (+)-naloxone and alcohol were diluted in endotoxin-free RPMI 1640 (Invitrogen, Carlsbad, CA, USA), which was used as the vehicle control.

*Alcohol-induced sedation and motor impairment*

Effect of alcohol in WT, *Tlr4*⁻/⁻, and *Myd88*⁻/⁻ mice

Following a dose of saline (-30 min), single alcohol doses of 2.5, 3.5 or 4.5 g·kg⁻¹ were administered (0 min) once only to groups of WT, *Tlr4*⁻/⁻, and *Myd88*⁻/⁻ mice and alcohol-induced sedation, as measured by the duration of LORR, was recorded from 0 min. Subsequently, an ED₅₀ (median effective dose) value of alcohol was estimated (see Statistical analysis).

To assess alcohol-induced motor dysfunction with the rotarod test, saline (-30 min) was administered prior to a single dose of alcohol (2.0 g·kg⁻¹) to these three groups of mice.

Effect of (+)-naloxone treatment
To examine the effects of (+)-naloxone on alcohol-induced sedation, WT mice were treated with (+)-naloxone (10 or 60 mg·kg\(^{-1}\)) or saline (each at -30 min), prior to a single 3.5 g·kg\(^{-1}\) alcohol dose (0 min) and duration of LORR recorded. To further assess any effect of (+)-naloxone on alcohol-induced sedation in null mutant mice, (+)-naloxone (60 mg·kg\(^{-1}\)) or saline (each at -30 min) were administered to Tlr4\(^{-/-}\) or Myd88\(^{-/-}\) mice prior to a single 4.5 g·kg\(^{-1}\) alcohol dose (0 min). These alcohol doses were chosen based on the ED\(_{50}\) in WT and null mutant mice, so that either reductions or enhancements of alcohol action could be reliably quantified. Alcohol-induced sedation was subsequently evaluated via LORR test after alcohol administration.

To assess differences in motor co-ordination using the rotarod method, (+)-naloxone (60 mg·kg\(^{-1}\); -30 min) was administered prior to a single 2.0 g·kg\(^{-1}\) alcohol dose (0 min) in WT and the two null mutant groups of mice.

\textit{In vitro and ex vivo molecular studies}

Effects of alcohol on IκBα protein levels in mixed hippocampal cell cultures

Mixed hippocampal cells were isolated as previously described (Wu et al., 2011) from naïve drug-free WT, Tlr4\(^{-/-}\), and Myd88\(^{-/-}\) mice (n = 3 each). To analyze the effect of alcohol on IκBα levels, and the influence of (+)-naloxone, cells were treated with (+)-naloxone (153 µM, 50 µg·mL\(^{-1}\)), or RPMI 1640 at 37°C, 5% CO\(_2\) for 30 min, prior to stimulation with alcohol (50 mM) or RPMI 1640 for a further 30 min and relative IκBα protein levels were investigated by western blotting.

Regulation of brain p38, JNK, and ERK phosphorylation by alcohol
To examine the effects of alcohol in vitro, hippocampal cells from each naïve mouse (WT or Tlr4-/-) were prepared as previously described (Wu et al., 2011). Cells were stimulated with 50 mM alcohol [based on previous studies (Alfonso-Loeches et al., 2010)] or vehicle at 37°C, 5% CO₂ for 10 min.

To evaluate these molecular effects of alcohol ex vivo, WT or Tlr4-/- mice were dosed with 3.5 g·kg⁻¹ of alcohol or saline (0 min), and anaesthetized by an overdose of sodium pentobarbitone (300 mg·kg⁻¹, 10 min). The hippocampus and cerebellum were isolated (15 min) with aseptic techniques after transcardial perfusion, and immediately homogenized in 2 mL of Denaturation Buffer from BD CBA Cell Signaling Master Buffer Kit (BD Biosciences, San Diego, CA, USA). Then, cellular enzymes within the samples were denatured by boiling at 100°C for 5 min with Denaturation Buffer, and samples subsequently stored at -80°C until analysis. Phosphorylated ERK, JNK, and p38 and total p38 (phosphorylated plus unphosphorylated) levels were quantified by a Cytometric Bead Array assay (see below for details). Protein concentrations of ex vivo samples were determined by the bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA, USA) to normalize the data.

Alcohol pharmacokinetics

Effect of (+)-naloxone administration and genetic TLR4 or MyD88 deficiency on peripheral and brain alcohol concentrations

To examine the influence of (+)-naloxone treatment and genetic TLR4 or MyD88 deficiency on blood and brain alcohol pharmacokinetics, (+)-naloxone (60 mg·kg⁻¹) or saline (each at -30 min) was administered to WT or Tlr4-/- and Myd88-/- mice with a single dose of alcohol (3.5 g·kg⁻¹, 0
min). Mice were anaesthetized by an overdose of sodium pentobarbitone 4 min before blood and tissue collection. Blood samples were taken via cardiac puncture at 15, 60, 120, or 180 min following the alcohol dose, or 4 min after the mice awoke. Following blood collection, mice were perfused transcardially with saline and the brain then removed. Blood and brain samples were immediately placed on ice. The alcohol concentration in each sample was measured with a nicotinamide adenine dinucleotide (NAD)-alcohol dehydrogenase (ADH) assay (see below). For this experiment, blood and brain samples at each time point were collected from different animals.

**Behavioural testing**

**LORR (sedation)**

Mice were placed into separate cages with bedding, after being injected with alcohol. The duration of LORR was measured from the time of mice losing their righting reflex to the time of righting themselves 3 times in 30 s.

**Rotarod (motor co-ordination)**

The rotarod apparatus (Orchid Scientifics, Nashik, India) with a 3-cm-diameter dowel was set at a fixed speed of $4.3 \times 10^{-3} \times g$ (16 rpm). The latency to fall was recorded as the duration that the mice remained on the rod, with a maximum cutoff latency of 180 s.

Each mouse underwent a training phase one day prior to experimental testing. Training involved the mouse remaining on the rotarod for 180 s in 3 sequential trials. On the experimental testing day, mice underwent a baseline trial before any dosing to ensure they performed at the training standard time of 180 s; this was repeated if they fell off the rod before the 180 s cutoff. The mice
were then dosed with (+)-naloxone or saline and returned to the cage for 30 min prior to alcohol administration (0 min). Another baseline test was conducted before alcohol administration. Mice were tested at 2, 5, 7, 13, and 20 min, and every 10 min thereafter, until they could remain on the rod for 180 s cutoff in 2 sequential trials. The duration of the mice remaining on the rod was recorded.

Molecular and chemical analyses

Western blotting

The preparation of cellular lysates was performed as described previously (Lousberg et al., 2010). Briefly, mixed hippocampal cells were incubated on ice for 10 min, collected by centrifugation (2264 x g, 4 °C, 5 min), and washed with ice-cold Dulbeccos Phosphate Buffered Saline (DPBS, Invitrogen, Carlsbad, CA, USA). Cell pellets were resuspended in modified radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM sodium orthovanadate, 20 mM Na₄P₂O₇, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, and complete EDTA-free protease inhibitor cocktail] for 15 min on ice. Following cell lysis, lysates were clarified via centrifugation (18894 x g, 4°C, 5 min) and stored until analysis at -80°C. Protein concentration was determined by BCA assay prior to western blot.

For western blotting, samples were heated in SDS loading buffer at 97°C for 5 min, fractionated by polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (GE Healthcare Biosciences, Pittsburgh, PA, USA). The membranes were subsequently blocked with 5% ECL Blocking Agent (GE Healthcare Biosciences, Pittsburgh, PA, USA) in Tris-buffered
saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature, and incubated with primary antibodies anti-IκBα (L35A5, 1:1000; Cell Signaling Technology, Danvers, MA, USA) or anti-β-actin (3:5000; Rockland Immunochemicals, Gilbertsville, PA, USA) overnight at 4°C. Blots were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (1:2000) or anti-rabbit (1:10000) IgG antibodies respectively (GE Healthcare Biosciences, Pittsburgh, PA, USA) at room temperature for 1 h. The immunoreactive signal was visualized by a chemiluminescence method (ECL Western Blotting Detection Reagents, GE Healthcare Biosciences, Pittsburgh, PA, USA) followed by exposure to Hyperfilm ECL (GE Healthcare Biosciences, Pittsburgh, PA, USA). ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used for quantifying the intensity of western blot bands allowing comparison to the relevant β-actin controls.

Cytometric Bead Array

Phosphorylated ERK 1/2 (T202/Y204), JNK 1/2 (T183/Y185), and p38 (T180/Y182) and total p38 were quantified in hippocampus and cerebellum tissue and in hippocampal cells with BD CBA Flex Set (BD Biosciences, San Diego, CA, USA) and Cell Signaling Master Buffer Kit, according to the manufacturer’s instructions. Data were acquired with a FACSCanto flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed with BD CBA Software according to the manufacturer’s instructions.

NAD-ADH assay (alcohol quantification)

A NAD-ADH assay was used to quantify alcohol concentrations in blood and brain samples as previously described (Smolen et al., 1989; Wu et al., 2011). The assay accuracy was expressed as
the relative error (RE) according to the equation: RE (%) = 100% \times (\text{measured concentration - spiked concentration})/\text{spiked concentration}, and the precision evaluated by the coefficient of variation (CV). The intra-assay precision and accuracy were estimated by analyzing five replicates at three different quality control levels (700, 500, and 200 mg per 100 mL). Intra-assay precision and inaccuracy ranged from 2.8% to 9.8%, and -2.2% to 8.8%, respectively.

To test if (+)-naloxone or acetaldehyde would interfere with this assay, between 0.001 and 100 µM of each drug was added to serum (containing 500 mg alcohol per 100 mL serum) or an equal volume of brain homogenized solution (containing the equal concentration of alcohol), and assayed together in the absence of drugs. The results demonstrated that the presence of either of the drugs did not influence the results obtained from this assay.

Statistical analysis

Graphpad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis. One-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test, or two-way ANOVA followed by Bonferroni’s post hoc test were performed. Data are presented as mean ± standard error of the mean (SEM). \(p\) values of 0.05 or less were considered significant.

\(ED_{50}\) was calculated from the dose-response curves, which were developed from the data of duration of alcohol-induced LORR. To fit these data, nonlinear regression (Graphpad Prism 5.02) was used, where Minimum was set as 0, and Maximum was the maximum duration of LORR. This resulted in estimate of \(ED_{50}\), Slope, and Maximum.
The areas under the alcohol concentration-time curves (AUCs) from 0.25 to 3 h post-alcohol administration were calculated using the linear trapezoidal rule. Slopes of the alcohol concentration-time curves were calculated by linear regression, which followed zero-order kinetics.

**Results**

*Mice deficient in TLR4 or MyD88 display decreased sedative and motor effects of alcohol*

Both *Tlr4*−/− and *Myd88*−/− mice displayed a shorter duration of alcohol-induced LORR than WT mice (*p* < 0.0001) at both 3.5 g·kg−1 (*Tlr4*−/−, 16 ± 4 min, *p* < 0.001, *n* = 9; *Myd88*−/−, 31 ± 8 min, *p* < 0.001, *n* = 10; WT, 73 ± 3 min, *n* = 13) and 4.5 g·kg−1 (*Tlr4*−/−, 56 ± 6 min, *p* < 0.001, *n* = 8; *Myd88*−/−, 78 ± 19 min, *p* < 0.01, *n* = 8; WT, 123 ± 3 min, *n* = 6) of alcohol (Fig. 1a). The ED50 of alcohol was 3.4 ± 0.1 [95% confidence interval (95% CI), 3.2-3.6] and 4.2 ± 0.2 (95% CI, 3.8-4.6) g·kg−1 for WT and *Myd88*−/− mice, respectively, and more than 4.5 g·kg−1 (the highest dose used) for *Tlr4*−/− mice.

Mice deficient in TLR4 or MyD88 recover more quickly from alcohol-induced motor impairment.
As shown in Fig. 1b, the latency to fall-off decreased from 180 s prior to treatment, to less than 3 s in all treatment groups after alcohol administration (2.0 g·kg\(^{-1}\)), with a gradual improvement over the monitoring time.

Both \textit{Tlr4-/-} and \textit{Myd88-/-} mice displayed a shorter recovery time from alcohol-induced decreases in rotarod performance compared to WT mice (\textit{Tlr4-/-}, \(p = 0.002\), \(n = 9\), two-way ANOVA; \(p < 0.001\) at 20 min, and \(p < 0.05\) at 30, 40 and 50 min with Bonferroni post hoc test; \textit{Myd88-/-}, \(p = 0.030\), \(n = 6\), two-way ANOVA; \(p < 0.001\) at 20 min, and \(p < 0.05\) at 30, 40 and 50 min with Bonferroni post hoc test; WT, \(n = 9\), Fig. 1b).

\textit{Alcohol-induced behavioural changes are reduced by (+)-naloxone treatment}

Shorter duration of alcohol-induced LORR in (+)-naloxone-treated WT, but not in \textit{Tlr4-/-} or \textit{Myd88-/-} mice

Administration of (+)-naloxone (10 or 60 mg·kg\(^{-1}\)) in WT mice significantly reduced the duration of alcohol-induced (3.5 g·kg\(^{-1}\)) LORR when compared to control untreated animals (\(p < 0.0001\), one-way ANOVA; 10 mg·kg\(^{-1}\), 40 ± 8 min, \(p < 0.01\), \(n = 6\), and 60 mg·kg\(^{-1}\), 32 ± 6 min, \(p < 0.001\), \(n = 10\), vs. saline, 73 ± 3 min, \(n = 13\), with Bonferroni’s multiple comparison test, Fig. 2a). Conversely however, (+)-naloxone treatment (60 mg·kg\(^{-1}\)) in \textit{Tlr4-/-} mice (\(n = 8\)) or \textit{Myd88-/-} mice (\(n = 5\)) did not reduce alcohol-induced (4.5 g·kg\(^{-1}\)) sedation (\(p = 0.72\), two-way ANOVA) as compared to untreated null mutant mice (\(n = 8\)), respectively (data not shown).

Shorter recovery time from alcohol-induced motor impairment in (+)-naloxone-treated WT mice
The alcohol-induced (2 g·kg⁻¹) decrease in rotarod performance was reduced by (+)-naloxone treatment (60 mg·kg⁻¹, n = 9) in WT mice when compared to controls (n = 9) that only received alcohol (p = 0.007, two-way ANOVA; p < 0.001 at 20 and 30 min, p < 0.01 at 40 min, and p < 0.05 at 50 min with Bonferroni post hoc test, Fig. 2b).

**Cellular IκBα protein levels are differentially regulated by alcohol and (+)-naloxone in WT, but not in Tlr4-/- or Myd88-/-, mixed hippocampal cells in vitro**

In WT cells (n = 3), IκBα protein levels were significantly increased by 30 min of alcohol exposure (p < 0.05, Bonferroni post hoc test) or (+)-naloxone exposure (p < 0.01, Bonferroni post hoc test), separately. A significant interaction between alcohol and (+)-naloxone treatments was also observed (p = 0.002). In the presence of both (+)-naloxone and alcohol, however, IκBα levels were decreased (p < 0.01, Fig. 3a and b).

In cells from Tlr4-/- mice and Myd88-/- mice (both n = 3), alcohol and/or (+)-naloxone exposure did not change IκBα levels when analyzed by repeated two-way ANOVA followed by Bonferroni post hoc test [Tlr4-/-: alcohol, p = 0.57, (+)-naloxone, p = 0.63, interaction, p = 0.51; Myd88-/-: alcohol, p = 0.80, (+)-naloxone, p = 0.53, interaction, p = 0.091; p > 0.05 by Bonferroni post hoc test, Fig. 3c-f].

**Acute alcohol stimulation does not change brain p38, JNK, or ERK phosphorylation in vitro or ex vivo**

In analyzing mixed hippocampal cell samples treated with alcohol in vitro, we observed no significant difference in the phosphorylation of cell signalling proteins (JNK, ERK, and p38)
between control and alcohol-treated hippocampal cells (phosphorylated JNK, $p = 0.33$; phosphorylated ERK, $p = 0.84$; phosphorylated p38 was below the limit of detection of CBA), or between WT and $Tlr4^{-/-}$ cells (phosphorylated JNK, $p = 0.10$; phosphorylated ERK, $p = 0.73$). However, total p38 was significantly higher in $Tlr4^{-/-}$ than WT cells (genotype, $p = 0.0043$; alcohol treatment, $p = 0.22$; fluorescence intensity: $Tlr4^{-/-}$, 1006 ± 44; WT, 569 ± 33). Data were tested by two-way ANOVA.

In the ex vivo brain samples, lower phosphorylated JNK was observed in hippocampus of $Tlr4^{-/-}$ mice when compared to WT (genotype, $p = 0.023$; alcohol treatment, $p = 0.62$). In contrast, no significant difference was found in phosphorylated p38, phosphorylated ERK, or total p38 in hippocampal samples (genotype, $p = 0.23, 0.061$, and 0.057, respectively; alcohol treatment, $p = 0.60, 0.31$, and 0.42, respectively). No significant effect of alcohol or of genotype was observed in phosphorylated p38, phosphorylated JNK, phosphorylated ERK, or total p38 in cerebellum samples (alcohol treatment, $p = 0.17, 0.78, 0.64$, and 0.98, respectively; genotype, $p = 0.86, 0.34, 0.45$, and 0.88, respectively) (data not shown).

**Genetic deficiency of TLR4 or MyD88, or (+)-naloxone treatment do not influence peripheral or brain alcohol pharmacokinetics**

No significant differences in serum or brain alcohol concentrations (n = 4-5) were observed between (+)-naloxone-treated [(+)-naloxone/WT] vs. saline-treated WT mice (saline/WT) (serum, $p = 0.35$; brain, $p = 0.24$), or between WT mice and either $Tlr4^{-/-}$ (saline/$Tlr4^{-/-}$) (serum, $p = 0.053$; brain, $p = 0.099$) or $Myd88^{-/-}$ mice (saline/$Myd88^{-/-}$) (serum, $p = 0.075$; brain, $p = 0.15$) (Fig. 4a and e).
The serum alcohol AUC values of saline/WT, (+)-naloxone/WT, saline/Tlr4-/-, and saline/Myd88-/ groups were 9.8, 10.1, 9.2, and 9.3 mg·mL⁻¹·h, respectively, and the slopes of the concentration-time curves were -0.47 ± 0.06, -0.46 ± 0.08, -0.45 ± 0.10, and -0.67 ± 0.06 h⁻¹, respectively. The brain alcohol AUCs of saline/WT, (+)-naloxone/WT, saline/Tlr4-/-, and saline/Myd88-/ groups were 345, 398, 327, and 387 mg·100 mg⁻¹·h, respectively, and the slopes of the concentration-time curve were -0.18 ± 0.05, -0.25 ± 0.10, -0.14 ± 0.07, and -0.24 ± 0.08 h⁻¹, respectively.

Serum and brain samples (n = 4-5) were also collected when the mice awoke after each treatment. Significant differences in the alcohol concentrations were found in serum samples (p = 0.019; 95% CI, 392-469, 430-490, 368-434, and 399-574 mg·100 mL⁻¹ in saline/WT, (+)-naloxone/WT, saline/Tlr4-/-, and saline/Myd88/- groups, respectively; Fig. 4b), but not in brain samples (p = 0.88; 95% CI, 106-206, 80-187, 62-234, and 59-257 mg·100 mg⁻¹ in saline/WT, (+)-naloxone/WT, saline/Tlr4-/-, and saline/Myd88/- groups, respectively; Fig. 4d) tested with one-way ANOVA. However, no significant difference was observed between the null mutant groups or (+)-naloxone-treated WT group and saline-treated WT controls with Bonferroni’s multiple comparison test.

**Discussion and conclusions**

The current study shows that inhibition of acute alcohol-induced pro-inflammation through the use of mice with genetic deficiency of TLR4 or MyD88, or treatment with the TLR4 antagonist (+)-naloxone, was successful in attenuating acute alcohol dose-induced sedation and motor dysfunction in mice, as measured by duration of LORR and rotarod performance, respectively.
These behavioural actions were unlikely due to changes in the peripheral or central pharmacokinetics of alcohol. In addition, we have demonstrated at the cellular level, that IκBα protein levels were elevated in response to 30 min of alcohol exposure in mixed hippocampal cells from WT mice, but not from Tlr4-/ or Myd88-/ mice. However, acute alcohol exposure did not alter p38, JNK, and ERK phosphorylation in vitro or ex vivo. These results provide a mechanistic hypothesis underlying the behavioural observations. Together, these findings suggest that alcohol is able to induce rapid modification of pro-inflammatory mediator signalling within the brain through the TLR4-MyD88 pathway, and subsequently alter animal motor behaviours.

**Acute alcohol exposure activates the TLR4-MyD88-NFκB signalling pathway in the brain**

Although the activation of brain TLR4 signalling, including MAPK and NFκB pathways, by alcohol exposure has been demonstrated in vitro after acute alcohol exposure (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009), as well as in vivo and ex vivo with chronic models (Alfonso-Loeches et al., 2010; Liu et al., 2011; Pascual et al., 2011; Valles et al., 2004), it still remains unknown as to whether this activation mechanistically contributes to the acute behavioural effects induced by alcohol. In this study, we have taken one step further by demonstrating that such signalling can occur after even one dose of alcohol. Importantly, our data indicated that the TLR4 signalling in vivo occurred rapidly, as the robust difference between the WT and null mutant groups started from 20 min of alcohol administration in rotarod tests and about 30 min in LORR tests.

To further explore the link between our behavioural findings and TLR4-MyD88 signalling, we analyzed a number of cell signalling proteins that could be up-regulated by TLR4 signalling in
Chapter III TLR4-MyD88 signalling mediates acute behavioural effects of alcohol

the cerebellum and hippocampus. The cerebellum was chosen as it is generally considered to control motor activity (Valenzuela et al., 2010) in the brain regions influenced by alcohol (Vilpoux et al., 2009), and we assessed the modification of motor function by alcohol in this study. The hippocampus was investigated since hippocampal microglial activation was induced by adolescent binge alcohol exposure in rats (McClain et al., 2011). As attenuation of microglia, the prime component of the brain’s immune system (Streit et al., 2004), inhibited acute alcohol dose-induced sedation in mice (Wu et al., 2011), the activation of TLR4-MyD88-NFκB signalling may occur in microglia.

Thus, due to the rapid activation of TLR4 signalling by alcohol suggested from the behavioural data, we assessed the phosphorylation of p38, JNK, and ERK in MAPK pathway ex vivo in hippocampal or cerebellum tissue as well as in mixed hippocampal cells in vitro following alcohol exposure in an attempt to delineate the mechanism responsible. However, we did not observe any change in p38, JNK, or ERK phosphorylation by acute alcohol exposure, which differs from previous reports using chronic alcohol treatment ex vivo (Alfonso-Loeches et al., 2010; Valles et al., 2004) and foetal microglial or astrocyte cultures in vitro (Blanco et al., 2005; Fernandez-Lizarbe et al., 2008). This implies that non-MAPK signalling cascades, such as phosphoinositide 3 kinase (PI3K)/AKT pathways (Hua et al., 2007), may be involved in the acute alcohol-induced signalling downstream from TLR4. Recently it was found that acute alcohol challenge induced a robust AKT phosphorylation in mouse striatum (Bjork et al., 2010), further highlighting the involvement of the non-MAPK pathways. It is possible that the disparity between our studies and the previous study may be related to different phenotypes between adult and neonatal glia (Beauvillain et al., 2008). Nonetheless, it is important to note that the
concentration of alcohol (50 mM) used in all of the *in vitro* experiments is based on the maximum serum (85–100 mM) and brain (30–35 mM) alcohol concentrations observed in our pharmacokinetic study, which also show maximal activity in activating immune signalling in glial cells (Blanco *et al.*, 2005; Fernandez-Lizarbe *et al.*, 2008).

Furthermore, IκBα protein levels were determined *in vitro* in mixed hippocampal cells from WT, *Tlr4-*/- and *Myd88-*/- mice. Our previous study demonstrated that alcohol-induced cellular IκBα protein levels changed in a time-dependent manner with an increase at 15 and 30 min, and a decrease at 45 and 60 min following alcohol exposure in WT mouse mixed hippocampal cells (Wu *et al.*, 2011). The time point of 30 min was chosen to match the behavioural response we observed, and we hypothesized that the increased IκBα protein levels following 30 min of alcohol exposure might be as a result of NFκB activation leading to IκBα protein stabilization, free IκBα from nuclear NFκB, or increased transcription of IκBα mRNA (Ferreiro *et al.*, 2010; Scott *et al.*, 1993). In this study, we have shown that the elevated cellular IκBα protein levels by alcohol in WT cells were not observed in cells from *Tlr4-*/- or *Myd88-*/- mice. As IκBα is the main inhibitory protein of NFκB (Sun *et al.*, 1993), these results imply that acute alcohol exposure may induce a modification to the NFκB cascade following activation of TLR4-MyD88 signalling. In addition, chronic alcohol treatment elevated pro-inflammatory cytokine levels, such as TNF-α, IL-1β, and IL-6, in the brains of WT mice (Alfonso-Loeches *et al.*, 2010), which may due to alcohol-induced TLR4-NFκB activation.

Collectively, the current results demonstrate that both a binge drinking dose (3.5 and 4.5 g·kg⁻¹) and a lower moderate dose (2.0 g·kg⁻¹) of alcohol rapidly activates pro-inflammatory signalling
cascades within the brain, which appear to be critical to alcohol-induced sedation and motor impairment through activation of TLR4-MyD88-dependent signalling and NFκB. The possible mechanisms between this immune activation and behavioural effects of alcohol are discussed below. It has been hypothesized that the acute activation of NFκB leads to the release of pro-inflammatory cytokines, which in turn could modulate neuronal activity in the brain, although the mechanism by which this modulation occurs is only beginning to be understood (Ren et al., 2008). Interestingly, interleukin-1β (IL-1β) signalling, which was activated by acute alcohol administration in our previous study (Wu et al., 2011), drove excitotoxic motor neuron injury (Prow et al., 2008). Furthermore, chemokine (C-X-C motif) ligand 12 (CXCL12) may enhance GABA synaptic activity at serotonin neurons in rats (Heinisch et al., 2010). Therefore, cytokines and chemokines could alter neuronal receptor functions, and these actions raise the possibility that pro-inflammatory mediators could facilitate the activation of GABA_\text{A} receptors by acute alcohol exposure (Ikonomidou et al., 2000; Mukherjee et al., 2008). Thus, apart from directly acting on neurons, alcohol could also modify neuronal receptor signalling, via NFκB and cytokine signalling induced by alcohol exposure, and subsequently sedation and motor behaviours.

_Alcohol-induced behavioural changes are protected by (+)-naloxone treatment_

Signalling by TLR4 occurs in response to both clinically-employed opioid antagonists [(-)-isomers] and their non-opioid receptor (+)-isomers (Hutchinson et al., 2010b). In this study, we show firstly that in contrast to WT mice, there is no effect of (+)-naloxone treatment in the LORR test when mice are deficient in TLR4 or MyD88. This is consistent with the specificity of (+)-
naloxone for the TLR4-MyD88 signalling cascade. Secondly, (+)-naloxone induced an increase in \( \text{IкB}\alpha \) protein levels at 30 min following initial (+)-naloxone exposure, implicating the mechanism of (+)-naloxone action may be related to interference of \( \text{IкB}\alpha \) protein synthesis or degradation. Thirdly, this alteration of \( \text{IкB}\alpha \) protein levels by (+)-naloxone was TLR4-MyD88-dependent. To maintain physiological relevance, the (+)-naloxone concentration in our in vitro experiments was equivalent to blood (-)-naloxone concentrations in previous rodent pharmacokinetic study (Kleiman-Wexler et al., 1989), due to the lack of (+)-naloxone pharmacokinetic data available at the time of this study.

Behavioural changes are not a result of modified alcohol pharmacokinetic profiles in null mutant or (+)-naloxone treated animals

To confirm that the behavioural changes induced by (+)-naloxone and genetic deficiency of either TLR4 or MyD88 were not simply a result of modifying the peripheral or central pharmacokinetics of alcohol, we measured alcohol concentrations following the dosing regimens used in LORR tests (3.5 g∙kg⁻¹ of alcohol). Overall, neither (+)-naloxone treatment nor the absence of TLR4 or MyD88 altered alcohol concentrations in either serum or brain samples.

Because of the decreased alcohol pharmacodynamic responses and unchanged alcohol pharmacokinetics in TLR4 signalling attenuated groups compared to controls, we expected that mice which awoke earlier in the LORR test would have higher peripheral and brain alcohol concentrations following their awakening. However, there was no significant difference in serum or brain alcohol concentrations between groups at the time of waking from alcohol-induced sedation, which may be due to the shallow slopes of the alcohol concentration-time curves.
TLR4-MyD88 signalling plays pivotal roles in acute behavioural actions of alcohol

Amongst the acute behavioural effects of alcohol, sedation and motor incoordination are likely responsible for traffic accident-related deaths in humans, and accompany self-administration of alcohol in mice (Chuck et al., 2006). Thus, our results not only suggests that the initial effects of alcohol are related to TLR4 signalling, but also may have important clinical applications in binge drinking-related brain conditions and alcohol dependence, which may culminate in preventing traffic accidents and decreasing social burden of alcohol abuse.

In conclusion, the current study provides new evidence linking the contribution of TLR4-MyD88-dependent signalling to the behavioural response induced by acute alcohol administration. The consequences of blocking TLR4 signalling that support this theory include the inhibition of the influence of alcohol on IκBα protein levels, and the reduction in the sedative and motor effects of alcohol. Therefore, novel pharmacological strategies targeting TLR4 signalling, such as (+)-naloxone, may have important and highly relevant clinical application. Use of TLR4 antagonists would potentially also reduce alcohol-induced peripheral TLR4 signalling in the liver and gut (Szabo et al., 2010).
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Figure 1 Mice deficient in TLR4 or MyD88 had an enhanced ability to recover from the behavioural effects associated with alcohol treatment. *tlr4/- mice and *myd88/- mice both had shorter duration of alcohol-induced LORR (sedation, panel a, n = 6-13), and recovered from alcohol-induced deficits in rotarod performance quicker (motor dysfunction, panel b, n = 6-9). Data are presented as mean ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.
Figure 2 The treatment with (+)-naloxone significantly reduced duration of LORR (sedation, panel a, n = 6-13) and deficits in rotarod performance (motor dysfunction, panel b, n = 9) in WT mice after a dose of alcohol. Data are presented as mean ± SEM. *, p < 0.05, **, p < 0.01, ***, p < 0.001.
Figure 3 Cellular IκBα protein levels were differentially regulated by alcohol and (+)-naloxone in WT (a and b) but not in TLR4 deficient (c and d) or MyD88 (e and f) deficient mixed hippocampal cell samples *in vitro*. Band densities are presented graphically as a percentage of the density of the loading control β-actin. In WT groups (a and c), the relative IκBα levels were increased at 30 min post-alcohol, and in the presence of (+)-naloxone, IκBα levels were decreased by 30 min of alcohol exposure, while (+)-naloxone alone increased IκBα levels (n = 3). Data are presented as mean ± SEM. *, p < 0.05, **, p < 0.01.
Figure 4 Genetic deficiency of TLR4 or MyD88 or (+)-naloxone treatment did not influence peripheral or brain alcohol pharmacokinetics. As serum and brain alcohol concentrations assessed at 0.25, 1, 2 and 3 h post-alcohol exposure, no significant difference was observed in either serum samples (a) or brain samples (c) collected from Tlr4-/- mice, Myd88-/- mice, (+)-naloxone-treated WT mice, or saline-treated WT controls. Serum (b) or brain (d) alcohol concentrations were not significantly different across the groups (n = 4-5) at the time of righting following the dose of alcohol. Data are presented as mean ± SEM.
Conflicts of interest

None
Chapter IV TLR2 and MyD88 mediate alcohol’s action and alcohol-morphine interaction


As demonstrated in Chapter III, activation of the TLR4 signalling cascade could be induced by acute alcohol administration. Similarly, morphine triggered both TLR2 and TLR4 activation (Hutchinson et al., 2010b; Zhang et al., 2011). Since alcohol and morphine share central glial TLR4 signalling (section 1.4.2 and 1.5.3), alcohol may also have TLR2 activity. Furthermore, the similar immune mechanism of alcohol and morphine led to the hypothesis that the interaction between alcohol and morphine was TLR signalling-mediated. Due to the significant clinical implication of an alcohol-morphine interaction (Hickman et al., 2008), understanding its initial mechanism is key for preventing and treating alcohol-induced opioid-related deaths.

This study aimed to examine if genetic deficiency in TLR2 or both TLR2 and TLR4 modulated the duration of alcohol-induced sedation (LORR test, sleep time test) in mice, since such an effect in all the other genetic deficient mouse strains used in this study had been tested in Chapter III. In addition, it intended to investigate the alteration of alcohol-induced sedation by morphine administration. Furthermore, it aimed to determine if this acute interaction between alcohol and morphine is influenced by inhibiting TLR4, IL-1 receptor, or both MOR and TLR4.
pharmacologically by (+)-naloxone (Hutchinson et al., 2008b; Hutchinson et al., 2010b), IL-1ra, or (-)-naloxone, respectively. Finally, to further characterise the role of TLRs in the alcohol-morphine interaction, various null mutant mice, including TLR2-deficient, TLR4-deficient, TLR2 and TLR4 double-deficient, and MyD88-deficient mice, were utilised.

I showed that mice deficient in TLR2 or both TLR2 and TLR4 had shorter duration of LORR following single administration of alcohol. This result is similar to that which had been shown in TLR4- or MyD88-deficient mice in Chapter III. Notably, this indicated a novel TLR2-dependent mechanism of alcohol’s effects. Furthermore, morphine administration potentiated the duration of alcohol dose-induced LORR. This interaction was abolished by genetic deficiency of TLR2, combined TLR2 and TLR4, or MyD88. In contrast, mice deficient in only TLR4 did not have a reduced duration of LORR induced by alcohol and morphine co-administration. These findings suggested that, in contrast to the effect of alcohol alone, TLR2, instead of TLR4, was critical in the interaction between alcohol and morphine. Pharmacological blockade by the administration of (-)-naloxone, but not (+)-naloxone or IL-1ra, was capable of inhibiting the interaction between alcohol and morphine in wild-type mice completely. This result highlighted the importance of MOR, instead of TLR4 and IL-1 receptor, in the alcohol-morphine interaction.

As such, this study demonstrated for the first time the role of the innate immune TLR2 cascade in the mechanisms of alcohol’s effect and the interaction between alcohol and morphine. These data reveal a novel neuroimmunopharmacologic mechanism of alcohol and opioids especially in combination.
TLR2 and MyD88 mediate the sedative effect of alcohol and interaction between alcohol and morphine in mice

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Chapter IV TLR2 and MyD88 mediate alcohol’s action and alcohol-morphine interaction

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Summary

BACKGROUND AND PURPOSE
There is increasing evidence that toll-like receptors 2 (TLR2) and 4 (TLR4) signalling is induced by morphine, and TLR4 signalling by alcohol. The current study aimed to assess the effect of TLR2-deficiency on alcohol-induced sedation, the potentiation of alcohol-induced sedation by morphine, and the inhibition of TLR2, TLR4, MyD88, IL-1 receptor, and μ opioid receptor (MOR) activities on such potentiated sedation in mice.

EXPERIMENTAL APPROACH
The loss of righting reflex (LORR) test was used to assess the acute sedative effect of alcohol and the sedative interaction between alcohol and morphine. Mice deficient in TLR2, TLR4, both TLR2 and TLR4, or MyD88 were utilised. (-)-Naloxone was used as the MOR antagonist and as a TLR4 signalling inhibitor, (+)-naloxone was only a TLR4 signalling inhibitor, and IL-1ra was a IL-1 receptor antagonist.

KEY RESULTS
TLR2-deficient mice had reduced duration of alcohol-induced (4.5 g·kg⁻¹) LORR by 13% compared to wild-type mice (p < 0.001). Morphine (5 mg·kg⁻¹) increased the alcohol-induced (2.5 g·kg⁻¹) duration of LORR in wild-type mice by 400% (p = 0.0057). This interaction was not found in the MyD88-, TLR2-, or TLR2/TLR4-deficient and (-)-naloxone-treated wild-type mice, but was observed in TLR4-deficient mice (p < 0.001).

CONCLUSIONS AND IMPLICATIONS
These findings demonstrate the key role of TLR2 and MyD88 in the acute behavioural effects of alcohol and alcohol plus morphine in mice. The data reveal a novel
neuroimmunopharmacological mechanism of alcohol and opioids’ actions especially when present in combination.

**Keywords**

Alcohol-Morphine Interaction, TLR2, TLR4, MyD88, IL-1, Naloxone, Sedation, Loss of Righting Reflex, Mice Study

**Abbreviations**

CNS, central nervous system; GABA, gamma-aminobutyric acid; IL-1ra, interleukin-1 receptor antagonist; MOR, µ opioid receptor; MyD88, myeloid differentiation primary response gene 88; NMDA, N-methyl-D-aspartate; LORR, loss of righting reflex; TLR, toll-like receptor.
Introduction

Among a variety of alcohol-drug interactions, alcohol increases the risk of heroin-related deaths (Hickman et al., 2008; Levine et al., 1995), which is not, as anticipated, due to any pharmacokinetic interactions (Darke et al., 1997; Fugelstad et al., 2003). Knowledge of the mechanisms of such a pharmacodynamic interaction between alcohol and opioids in the central nervous system (CNS) is surprisingly limited (White et al., 1999), but includes the mechanism of a potentiated alcohol-induced sedation by morphine in mice. Neuronal gamma-aminobutyric acid (GABA) receptors, N-methyl-D-aspartate (NMDA) receptor, and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signalling have been demonstrated to modulate the sedative effect of alcohol (Boyce-Rustay et al., 2005; Sharko et al., 2008; Wand et al., 2001), while morphine is capable of rapidly modifying the binding affinity of GABA for its receptors differently in various brain regions (Sivam et al., 1982; Ticku et al., 1980). Moreover, morphine alone can cause sedation (Craft et al., 2006; Kissin et al., 1991), the mechanism of which is invariably due to activation of the μ opioid receptor (MOR). Collectively, the modulated GABA receptor activities and the activated MOR by acute morphine exposure may serve as part of the neuronal mechanisms of the sedative effect induced by acute dosing of alcohol and morphine.

Apart from these neuronal mechanisms, recent evidence has indicated that both opioids and alcohol are able to activate the central immunocompetent cells, glia, through toll-like receptors (TLRs) and myeloid differentiation primary response gene 88 (MyD88)-dependent pathways. Hutchinson et al. (2010b) showed that TLR4-deficient mice had significantly increased morphine-induced analgesia compared to wild-type animals, which was likely to be a result of
attenuated proinflammatory cytokine signalling (Hutchinson et al., 2008a). In addition, evidence suggested that opioids might also have TLR2 activities. Li et al. (2010; 2009) demonstrated that morphine increased TLR2 expression and that TLR2-deficiency prevented morphine-induced apoptosis in vitro in neurons. Zhang et al. (2011) showed that mice deficient in TLR2 had 3- to 5-fold lower morphine-induced interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) expression in the nucleus accumbens, less microglial activation (quantified by CD11b levels), and morphine withdrawal compared to their wild-type counterparts. On the other hand, we have shown that acute alcohol-induced sedation and motor dysfunction were decreased by pharmacological blockade of or genetic deficiency in microglia, TLR4, MyD88, or IL-1 signalling (Wu et al., 2011a; Wu et al., 2011b). Altogether, the activation of TLR signalling cascades by alcohol and opioids may shed light on a new mechanism behind the interactions between alcohol and opioids.

The possible link between the neuronal and glial effects of alcohol and opioids may reside in cytokine signalling, as proinflammatory cytokines produced by activated glia result in a modification of neuronal receptor signalling. We recently showed that, in mice, inhibition of IL-1 receptor signalling by recombinant human IL-1 receptor antagonist (IL-1ra) significantly decreased acute alcohol-induced sedation and motor dysfunction; this suggested that alcohol was capable of rapidly inducing proinflammatory cytokine signalling (such as IL-1) in vivo (Wu et al., 2011b). Similarly, morphine, a MOR agonist, was able to activate glia and in turn lead to the release of proinflammatory cytokines from glia (Hutchinson et al., 2008a; Watkins et al., 2005). Taken together, both alcohol and opioids induce proinflammatory cytokine signalling in the CNS,
and this immune activation may subsequently alter the function of GABA receptors and MOR, which are responsible for the neuronal effects of either alcohol or opioids.

Hence, considering the growing evidence of alcohol-induced TLR4 signalling activation and morphine-induced TLR2 and TLR4 signalling activation, we hypothesised that blockade of TLR2 activity would decrease the sedative effect [the loss of righting reflex (LORR) test] of alcohol, and inhibition of TLR2, TLR4, MyD88, IL-1 receptor, and MOR signalling would attenuate such an effect induced by alcohol plus morphine. The aim of this study was, firstly, to examine if mice genetically deficient in TLR2 or combined TLR2 and TLR4 had decreased sedation to alcohol alone, since such an effect was reduced in all the other genetic deficient mouse strains used in this study in our previous experiments (Wu et al., 2011a). Secondly, we aimed to assess the effects of morphine on alcohol-induced LORR. Thirdly, we aimed to determine the effect of (+)-naloxone [a TLR4 signalling inhibitor, the MOR-inactive isomer (Hutchinson et al., 2008c; Hutchinson et al., 2010b)], (-)-naloxone (a TLR4 signalling inhibitor, the MOR antagonist), and IL-1ra on combined alcohol and morphine-induced sedation. Finally, we aimed to characterise the effect of genetic deficiency of TLR2, TLR4, combined TLR2 and TLR4, or MyD88 on the interaction between alcohol and morphine.

**Methods**

**Animals**

Pathogen-free male Balb/c wild-type mice, and mice with null mutations in *Tlr2* (*Tlr2-/-* mice), *Tlr4* (*Tlr4-/-* mice), *Tlr2* and *Tlr4* (*Tlr2-/-/Tlr4-/-* mice), and *Myd88* (*Myd88-/-* mice) (all 8–14 weeks old; n = 4–8) genes were used in the experiments. The genetically deficient mice, back
crossed onto Balb/c for more than ten generations, were sourced from Prof. Akira (Osaka University, Osaka, Japan), and the Tlr4, Tlr2/Tlr4, and Myd88 null mutant mice were supplied by Dr. Simon Phipps (University of Queensland, QLD, Australia) and Prof. Paul Foster (University of Newcastle, NSW, Australia). Mice were housed in temperature (23 ± 3 °C) and light/dark cycle (12/12 h) controlled rooms with standard rodent food and water available ad libitum. All animal studies were approved by the University of Adelaide Animal Ethics Committee.

Drugs

Human IL-1ra (Kineret®, Amgen Inc, Thousand Oaks, CA, USA) was purchased from the Queen Elizabeth Hospital Pharmacy (Woodville, SA, Australia). (+)-Naloxone was supplied by Dr. Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism National Institutes of Health, Rockville, MD, USA). (-)-Naloxone was purchased from Sigma (St. Louis, MO, USA). Morphine was obtained from Fauldings Australia (Adelaide, SA, Australia). Alcohol (ethanol) was obtained from Chem-Supply (99.5%, Gillman, SA, Australia). Sodium chloride was obtained from Merck (99.5%, Darmstadt, Germany). The receptor and channel nomenclature used in the paper follows Alexander et al. (2009).

Morphine (5 mg·kg⁻¹), (+)-naloxone (60 mg·kg⁻¹), (-)-naloxone (60 mg·kg⁻¹), and IL-1ra (100 mg·kg⁻¹) were injected intraperitoneally (i.p.) at 0.01 mL·g⁻¹. Alcohol was injected at 4.5 g·kg⁻¹ and 2.5 g·kg⁻¹ to assess the sedative effect induced by alcohol alone and the co-administration of alcohol and morphine, respectively. The volume for injection of alcohol (20%, v/v, i.p.) which was based on weight of animals varied. The weight of mice was 25 g on average, and ranged
from 22 to 30 g, and thus the volume for injection of alcohol was 0.40 mL (range: 0.35-0.48 mL) at 2.5 g·kg\(^{-1}\) and 0.71 mL (range: 0.63-0.86 mL) at 4.5 g·kg\(^{-1}\) of alcohol. Saline (0.9% sodium chloride) was used as the vehicle control.

**Experimental procedures**

Alcohol alone-induced sedation

To determine sedation induced by alcohol alone in wild-type, Tlr2-/-, and Tlr2-/-/Tlr4-/- mice, an alcohol dose (4.5 g·kg\(^{-1}\)) was administered.

Alcohol and morphine-induced sedation

To examine the effects of a single dose of morphine on alcohol-induced sedation in wild-type mice, mice were injected with morphine (5 mg·kg\(^{-1}\), -35 min) (morphine/saline/alcohol or morphine/alcohol/wild-type) or saline (saline/saline/alcohol or saline/alcohol/wild-type), prior to a dose of saline (-30 min) and a lower dose of alcohol (2.5 g·kg\(^{-1}\), 0 min).

To assess the influence of IL-1ra, (+)-naloxone, and (-)-naloxone on sedation induced by alcohol and morphine co-administration, wild-type mice were injected with morphine (-35 min), followed by IL-1ra (morphine/IL-1ra/alcohol), (+)-naloxone [morphine/(+)-naloxone/alcohol], or (-)-naloxone [morphine/(-)-naloxone/alcohol] administration (-30 min). Alcohol (2.5 g·kg\(^{-1}\)) was administered at 0 min.

To examine the effects of morphine on alcohol-induced sedation across different genetically deficient mouse strains, Tlr2-/-, Tlr4-/-, Tlr2-/-/Tlr4-/-, and Myd88-/- mice were treated with...
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TLR2 and MyD88 mediate alcohol’s action and alcohol-morphine interaction.

Morphine (5 mg·kg⁻¹, -35 min) (morphine/alcohol/Tlr2-/-, morphine/alcohol/Tlr4-/-, morphine/alcohol/Tlr2-/-/Tlr4-/-, and morphine/alcohol/Myd88-/-) or saline (saline/alcohol/Tlr2-/-, saline/alcohol/Tlr4-/-, saline/alcohol/Tlr2-/-/Tlr4-/-, and saline/alcohol/Myd88-/-), prior to a dose of saline (-30 min) and a dose of alcohol (2.5 g·kg⁻¹, 0 min). These data were compared to their wild-type counterpart data from morphine and low dose alcohol.

Measurement of the sedative effect of drugs (the LORR test)

After being injected with alcohol, mice were placed into separate cages with bedding. The duration of LORR was measured as the time from LORR to the time of righting themselves 3 times in 30 s (waking up) (Wu et al., 2011b).

Statistical analysis

Graphpad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Unpaired t-test, one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test, or two-way ANOVA followed by Bonferroni’s post hoc test were performed. Data are presented as mean with 95% confidence interval (95% CI) in brackets. p values of 0.05 or less were considered significant.

Results

TLR2-deficiency results in decreased duration of LORR induced by alcohol

The alcohol-induced duration of LORR was significantly different between wild-type, Tlr2-/-, and Tlr2-/-/Tlr4-/- mice (p < 0.0001, one-way ANOVA, Figure 1). A shorter duration of alcohol-
induced LORR (min) was found in Tlr2-/− and Tlr2-/−Tlr4-/− mice compared to their wild-type counterparts, when analysed by Bonferroni’s multiple comparison test (all n = 6): wild-type, 123 (114 to 132); Tlr2-/−, 16 (6 to 26), p < 0.001; Tlr2-/−Tlr4-/−, 74 (63 to 86), p < 0.001.

Potentiation of the duration of alcohol-induced LORR by morphine is inhibited by (-)-naloxone in wild-type mice

A low dose of morphine in wild-type mice significantly increased the duration of alcohol-induced LORR (min) p = 0.0057, n = 6, unpaired t-test: saline/saline/alcohol, 7 (-3 to 16); morphine/saline/alcohol, 28 (16 to 40). Furthermore, (-)-naloxone-treated mice showed a shorter duration of LORR (min) induced by alcohol and morphine co-administration when compared to morphine/saline/alcohol-treated animals, p = 0.002, n = 6, one-way ANOVA; p < 0.01, Bonferroni’s multiple comparison test: morphine/saline/alcohol, 28 (16 to 40); morphine/(-)-naloxone/alcohol, 0 (0 to 0). While (+)-naloxone or IL-1ra did not alter the potentiated duration of alcohol-induced LORR (min) by morphine, p > 0.05, n = 6, Bonferroni’s multiple comparison test: morphine/(+)naloxone/alcohol, 26 (5 to 46); morphine/IL-1ra/alcohol, 24 (16 to 32); Figure 2a.

Lack of enhanced duration of LORR by the combination of alcohol plus morphine in Tlr2-/−, Tlr2-/−Tlr4-/−, and Myd88-/− mice

Mice deficient in TLR2, combined TLR2 and TLR4, or MyD88 displayed no enhanced duration of alcohol-induced LORR (min) by morphine, while morphine-treated Tlr4-/− mice still showed significantly enhanced LORR duration, p < 0.0001, treatment, p < 0.0001, genotype, p < 0.0001, treatment and genotype interaction by two-way ANOVA: saline/alcohol/wild-type, 7 (-3 to 16),
n = 6 vs. morphine/alcohol/wild-type, 28 (16 to 40), n = 6, \( p < 0.001 \); saline/alcohol/\( Tlr4^{-/-} \), 0 (0 to 0), n = 4 vs. morphine/alcohol/\( Tlr4^{-/-} \), 42 (34 to 50), n = 6, \( p < 0.001 \); saline/alcohol/\( Tlr2^{-/-} \), 0 (0 to 0), n = 4 vs. morphine/alcohol/\( Tlr2^{-/-} \), 0 (0 to 0), n = 4, \( p > 0.05 \); saline/alcohol/\( Tlr2^{-/-}/Tlr4^{-/-} \), 0 (0 to 0), n = 4 vs. morphine/alcohol/\( Tlr2^{-/-}/Tlr4^{-/-} \), 0 (0 to 0), n = 5, \( p > 0.05 \); saline/alcohol/\( Myd88^{-/-} \), 0 (0 to 0), n = 4 vs. morphine/alcohol/\( Myd88^{-/-} \), 2 (-1 to 5), n = 5, \( p > 0.05 \), Bonferroni post hoc test, Figure 2b.

**Discussion and conclusions**

The present study demonstrates that \( Tlr2^{-/-} \) and \( Tlr2^{-/-}/Tlr4^{-/-} \) mice display a reduced sedative effect induced by a single dose of alcohol, as measured by the duration of LORR. Furthermore, a single dose of morphine is sufficient to increase the sedative effect induced by a lower dose of alcohol in wild-type mice. This interaction between alcohol and morphine was abolished by genetic deficiency in TLR2, MyD88, or both TLR2 and TLR4. In contrast, mice genetically deficient in only TLR4 did not display a reduction of this interaction, which suggests a key role of TLR2 and MyD88, instead of TLR4. Moreover, blockade of MOR and TLR4 signalling with (-)-naloxone in wild-type mice also completely inhibited the sedative interaction between alcohol and morphine. In contrast, inhibiting TLR4 signalling only with (+)-naloxone or IL-1 receptor signalling using IL-1ra did not alter the interaction. Taken together, in contrast to the effects of alcohol or morphine alone involving both TLR2 and TLR4 (Hutchinson *et al.*, 2009; Hutchinson *et al.*, 2008b; Li *et al.*, 2010; Wu *et al.*, 2011a; Wu *et al.*, 2011b), the current results suggest that the co-administration of alcohol and morphine elicits the sedative effect only via the TLR2-MyD88 signalling cascade.
Alcohol-induced sedation is attenuated by a genetic deficiency in TLR2

We used Tlr2-/− and Tlr2-/−/Tlr4-/− mice in the current study as morphine can activate TLR2 signalling (Li et al., 2010). Similar to what has previously been shown in Tlr4-/− and Myd88-/− mice (Wu et al., 2011a), Tlr2-/− and Tlr2-/−/Tlr4-/− mice had a substantially reduced sedative effect of alcohol. This is the first evidence indicating the activation of TLR2 by alcohol.

The antagonist of MOR, (-)-naloxone, inhibits the sedative interaction between alcohol and morphine

Morphine is a MOR agonist, and has also the ability to activate central neuronal TLR2 (Li et al., 2010; Li et al., 2009) and glial TLR4 (Hutchinson et al., 2010a; Hutchinson et al., 2010b; Watkins et al., 2009). Our results showed that morphine significantly potentiated the sedative effect of alcohol, which could have been due to the activation of TLR2, TLR4, and MOR by morphine. Thus, we used (-)-naloxone, a MOR antagonist, to block both MOR and TLR4 signalling (Hutchinson et al., 2008c), and as a comparison, (+)-naloxone to block only TLR4 signalling (Hutchinson et al., 2008c). The results showed that (-)-naloxone, and not (+)-naloxone, completely blocked the sedative interaction between alcohol and morphine, which suggests a critical role of MOR, but not TLR4. In contrast, we have previously shown that (+)-naloxone reduced sedation caused by a high dose of alcohol (Wu et al., 2011a) and potentiated acute morphine analgesia (Hutchinson et al., 2010b). As the doses of both alcohol and morphine in this interaction study were much lower than those used in the previous studies that tested the acute effects of alcohol or morphine, the different results may imply that TLR4 signalling is differentially altered and dependent on the dose of alcohol and morphine. Although a higher dose
of morphine (ED$_{50}$, 51.5 ± 4.8 mg·kg$^{-1}$) causes LORR in male rats (Craft et al., 2006; Kissin et al., 1991), we have demonstrated that the dose of morphine used in the current study was not sufficient to induce LORR in wild-type mice (Figure 2a) or in any of the genetically deficient mice (data not shown). Further, the variability of duration of LORR in (+)-naloxone-treated group (range: 0 to 49 min) appeared to be increased compared to other treatment groups [range: saline/morphine/alcohol group, 16 to 44 min; IL-1ra/morphine/alcohol group, 15 to 34 min; (-)-naloxone/morphine/alcohol group, 0 to 0 min]. Further investigation is required to explain this observation.

**Differential effects of TLR2-MyD88 and TLR4-IL-1 signalling on the alcohol-morphine interaction**

Our findings together with others (Hutchinson et al., 2010b; Li et al., 2010; Shavit et al., 2005; Wu et al., 2011a; Wu et al., 2011b) indicate that the effects of alcohol or morphine alone and the interactions between alcohol and morphine may be through different mechanisms, that is different innate immune receptors, cell types, and cytokine signalling may be involved.

There are a few similarities but also some differences between TLR2 and TLR4 signalling pathways (Figure 3). TLR2 heterodimerises with TLR1 or TLR6 (Jin et al., 2007; Kang et al., 2009), whereas TLR4 is a homodimer (Kim et al., 2007; Park et al., 2009). All of the TLRs contain flexible leucine-rich repeats (LRRs) to allow ligand binding, such as lipopeptides which bind the lipid-binding pocket of TLR2 and lipopolysaccharide (LPS) which binds TLR4 with myeloid differentiation factor 2 (MD-2) (O’Neill et al., 2007). Thus, the finding that blockade of TLR4 did not attenuate the sedative interaction between alcohol and morphine is likely due to the
different ligand binding affinities and conditions of TLR2 and TLR4, as MD-2 facilitates TLR4 binding. The TLR4 downstream signalling is classified into a MyD88-dependent and a TIR domain-containing adaptor-inducing interferon-β (TRIF)-dependent signalling, while TLR2 downstream signalling only involves the MyD88-dependent signalling (Sheedy et al., 2007; Rakoff-Nahoum et al., 2009). As such, another possible reason for our different findings in Tlr2-/- and Tlr4-/- mice is that the TLR4-TRIF pathway may be differentially activated at various doses of alcohol and morphine.

With regard to the cell types where the alcohol and morphine interactions occur, both neurons and glia are likely to be involved. Li et al. (2010) showed in neurons that chronic morphine exposure increased TLR2 levels, while other studies showed TLR4 signalling activation by alcohol or morphine in glia (Alfonso-Loeches et al., 2010; Hutchinson et al., 2007). In addition, TLR2 and TLR4 may cross-talk, as the stimulation of TLR2 and TLR4 synergistically enhanced the production of TNF-α and IL-1β (Jung et al., 2009). Since a single high dose of either alcohol or morphine could trigger both TLR2 and TLR4 signalling, the amplified signalling by potential TLR2 and TLR4 cross-talk may serve as part of the mechanism behind the alcohol-morphine interaction.

Indeed, our observation showing Tlr2-/-, Tlr2-/-/Tlr4-/-, and Myd88-/- mice had an abolished sedative interaction between alcohol and morphine is possibly due to the increased potency of LORR in the null mutant mice. The reason for the lack of LORR in these genetically deficient mice may be that the alcohol dose (2.5 g∙kg⁻¹) used in the interaction study did not reach the dose sufficient to induce LORR. However, Tlr4-/- mice did not lose their righting reflex at 2.5 g∙kg⁻¹.
of alcohol alone either, yet they had ~ 42 min of LORR after the co-administration of alcohol plus morphine. Therefore, this result in Tlr4-/- mice suggests that the lack of LORR in Tlr2-/-, Tlr2-/-/Tlr4-/-, and Myd88-/- mice is not solely dependent on any altered alcohol and morphine potency in these mice and rather a specific pharmacodynamic action.

With regard to cytokine signalling, IL-1 has been related to acute behavioural effects of alcohol or morphine (Shavit et al., 2005; Wu et al., 2011). However, in this study, IL-1ra was not able to reduce the sedative interaction between alcohol and morphine. Since cytokine signalling has been considered to be a possible mechanism of glia-neuronal cross-talk in response to alcohol or morphine exposure (Blanco et al., 2005; Fernandez-Lizarbe et al., 2009; Hutchinson et al., 2008b), other cytokines and chemokines, such as TNF-α, chemokine (C-C) motif ligand 2 (CCL2), and CCL5, which can also be enhanced by alcohol or morphine (Avdoshina et al., 2010; El-Hage et al., 2005; El-Hage et al., 2006; Niwa et al., 2007; Sawaya et al., 2009; Turchan-Cholewo et al., 2009), are worthy of future investigations into the alcohol-morphine interaction.

In conclusion, the current study establishes the critical role of TLR2 and MyD88 in the acute sedative effects of alcohol and the interaction between alcohol and morphine in mice. Understanding how alcohol influences the CNS and how alcohol-morphine interaction occurs in the short term is critical to the characterisation of the initial effects of alcohol and morphine, which suggests the important and highly relevant clinical application of novel pharmacological strategies targeting TLR2-MyD88 signalling. As the current study has only assessed the enhancement of alcohol-induced sedation by morphine, the inverse action of enhancement of morphine’s effects by alcohol will require additional investigation in future studies.
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Figure 1. Decreased duration of LORR effect of alcohol in Tlr2-/- and Tlr2-/-/Tlr4-/- mice compared to wild-type mice (all n = 6). Data are presented as mean ± 95% CI. *, $p < 0.05$, ***, $p < 0.001$. 

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Figure 2. The effects of treatments with IL-1ra, (+)-naloxone, or (-)-naloxone and the genetic deficiency of innate immune genes in TLR signalling cascades on the increased duration of LORR interaction between alcohol and morphine. (a) In wild-type mice, (-)-naloxone completely blocked the potentiated duration of LORR effect of alcohol by morphine. (b) In Tlr2/-, Tlr2/-/Tlr4/-, and Myd88/- mice, the enhancement of alcohol-induced sedation by morphine was decreased (n = 4-6). Data are presented as mean ± 95% CI. **, p < 0.01, ***, p < 0.001.
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Figure 3. Schematic representation of the possible mechanisms of the acute effects of alcohol or morphine alone and the acute interaction between alcohol and morphine. Acute morphine administration induces MOR, TLR2, TLR4, and the MyD88-dependent signalling. On the other hand, acute alcohol administration potentiates GABA receptor, TLR2, TLR4, and the MyD88-dependent signalling, and reduces NMDA receptor activity. However, a reduced effect of alcohol and morphine occurs when the TLR2-MyD88 cascade or MOR is blocked. The role of TRIF-dependent signalling in the effect of alcohol and morphine is not clear. The alteration of GABA receptors, NMDA receptors, MOR, and TLRs may occur in either the same cell type or different cell types (neurons and glia). The TLRs activation elicits NFκB and its downstream
signalling, which may subsequently modulate GABA receptor, NMDA receptor, and MOR signalling. NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK, mitogen-activated protein kinase; solid line arrows, the involvement of such signalling is supported by evidence; dash line arrows, the involvement of such signalling is hypothesised.
Conflicts of interest

None
Chapter V Investigation of the association between TLR4 genetic polymorphisms and opioid or alcohol dependence

5.1. Introduction

Up to 60% of opioid and alcohol dependence is heritable (Dick et al., 2006; Lachman, 2006). However, investigation of genetic variants in metabolism pathways, transporters, and receptors has failed to explain a large percentage of this heritability. Hence other gene candidates are yet to be identified. Given the expanding role of glial signalling in mediating the effects of opioid and alcohol, which has been discussed in the introduction, genes within this glial activation and signalling cascade are likely candidates. Indeed, genetic variability of the IL1B gene has been associated with both opioid and alcohol dependence (Liu et al., 2009; Pastor et al., 2005). In addition, IL1RN (Pastor et al., 2000; Pastor et al., 2005; Saiz et al., 2009), IL10 (Marcos et al., 2008), and NFKB1 (Edenberg et al., 2008) polymorphisms have been demonstrated to be associated with alcohol dependence. These provide indirect evidence of a proinflammatory contribution to alcohol and opioid dependence.

TLR4 is the key receptor mediating opioid- and alcohol-induced glial activation (Fernandez-Lizarbe et al., 2009; Hutchinson et al., 2007). Although TLR4 genetic polymorphisms have been associated with numerous diseases, including cancers (Santini et al., 2008) and neuron regressive diseases (Balistreri et al., 2007), the relationship between TLR4 genetic polymorphisms and opioid and alcohol dependence is yet to be examined. The human TLR4 gene is highly polymorphic (225 SNPs in gene region, NCBI SNP database), however, most disease association studies to date have focussed on the Asp299Gly (rs4986790, A896G) SNP (see section 1.7.3).
Hence, if TLR4 activation is changed, it is possible that the glial activation signal following exposure to opioids and alcohol would also be altered to change reward and dependence propensities. On the other hand, if such a relationship between TLR4 genetic polymorphisms and alcohol and opioid dependence is detected, it will provide indirect evidence that TLR4 is involved in the mechanism of alcohol and opioid dependence, and TLR4 polymorphisms may be a predictor of alcohol and opioid dependence. Therefore, the aim of this study was to investigate the association between the Asp299Gly SNP and opioid or alcohol dependence in humans.

5.2. Methods

5.2.1. Subjects

This was a retrospective case-control association study in 99 opioid dependent subjects, 100 alcohol dependent subjects, and 56 non-dependent healthy control subjects who had previously taken part in clinical studies conducted by the Discipline of Pharmacology at the University of Adelaide. Subject demographics are shown in Table 5-1. More than 90% of the subjects were Caucasian, and there was no co-morbidity between alcohol and opioid dependencies in the subjects. The opioid dependent subjects were receiving methadone maintenance treatment at a stable dose for at least one month, and the alcohol dependent subjects were receiving (-)-naltrexone treatment. Studies were approved by the Royal Adelaide Hospital Research Ethics Committee, and all subjects provided written informed consent.
Table 5-1 Demographic data of opioid dependent (OD), alcohol dependent (AD) and non-dependent healthy control (HC) populations.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Female : Male</th>
<th>Age (years) (mean ± SD) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td>25 : 74</td>
<td>34.4 ± 8.3 (19–56)</td>
</tr>
<tr>
<td>AD</td>
<td>43 : 57</td>
<td>42.5 ± 8.8 (24–62)</td>
</tr>
<tr>
<td>HC</td>
<td>23 : 33</td>
<td>33.3 ± 16.7 (17–77)</td>
</tr>
</tbody>
</table>

SD, standard deviation.

5.2.2. TLR4 genotyping

Genomic DNA was isolated from whole blood with a QIAamp DNA Mini Kit (Qiagen Pty Ltd., Doncaster, VIC, Australia). TLR4 Asp299Gly SNP genotypes were determined by a polymerase chain reaction (PCR)-restriction fragment length polymerase (RFLP) assay previously described (Lorenz et al., 2002) with the following optimised cycling conditions: 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C; and a final step of 10 min at 72°C. All PCR reactions were run with a negative control (no genomic DNA template) to ensure that there was no contamination of reagents, and positive control samples (of known genotype previously determined by ABI Prism BigDye v3.0 sequencing analysis).

5.2.3. Statistical analysis

Genotype distribution was examined for significant departure from Hardy-Weinberg equilibrium by Fisher’s exact test. Allele and genotype frequencies between the groups were compared using the Fisher’s exact test with OR [95% confidence interval (CI)]. p values of < 0.05 were
considered to be statistically significant (GraphPad Prism 5.02, GraphPad Software Inc., San Diego, CA, USA). The statistical power of the study was estimated by GraphPad StatMate 2.00 (GraphPad Software Inc., San Diego, CA, USA).

5.3. Results

No significant deviation was observed from Hardy-Weinberg equilibrium ($p = 1$). The allele and genotype frequencies did not differ ($p > 0.05$) between the opioid [allele: $p = 0.39$, OR = 0.52 (0.14-1.92); genotype: $p = 0.38$, OR = 0.50 (0.13-1.91)] and alcohol dependent [allele: $p = 0.27$, OR = 0.43 (0.12-1.56); genotype: $p = 0.26$, OR = 0.42 (0.11-1.54)] and non-dependent healthy control populations (Table 5-2).

Table 5-2 Comparison of $TLR4$ Asp299Gly allele (A) and genotype (B) frequencies in opioid dependent (OD), alcohol dependent (AD) and non-dependent healthy control (HC) populations.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Allele [n, (%)]</th>
<th>OR (95% CI) versus HC</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>188 (94.9)</td>
<td>10 (5.1)</td>
<td>0.52 (0.14-1.92)</td>
</tr>
<tr>
<td>AD</td>
<td>188 (94.0)</td>
<td>12 (6.0)</td>
<td>0.43 (0.12-1.56)</td>
</tr>
<tr>
<td>HC</td>
<td>109 (97.3)</td>
<td>3 (2.7)</td>
<td>-</td>
</tr>
</tbody>
</table>
Chapter V Investigation of the association between TLR4 genetic polymorphisms and opioid or alcohol dependence

(B)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Genotype [n, (%)]</th>
<th>OR (95% CI) versus HC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/A</td>
<td>A/G</td>
<td>G/G</td>
</tr>
<tr>
<td>OD</td>
<td>89 (89.9)</td>
<td>10 (10.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AD</td>
<td>88 (88.0)</td>
<td>12 (12.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HC</td>
<td>53 (94.6)</td>
<td>3 (5.4)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

The statistical power of this study was 10-20%, comparing the allele frequencies between the opioid dependent and non-dependent healthy control populations, and 20-30%, comparing the allele frequencies between the alcohol dependent and non-dependent healthy control populations (using a two-tailed significance level of 0.05).

5.4. Discussion

This is the first study to investigate the possible association between the TLR4 Asp299Gly SNP and opioid and alcohol dependence. A variant allele frequency of 2.7% was observed in the non-dependent healthy control population, which is similar to that previously reported in Caucasians in Hapmap (3.5%, http://hapmap.ncbi.nlm.nih.gov/). The Asp299Gly mutant showed decreased response to LPS and two structurally unrelated TLR4 agonists in vitro, however, no differences in TLR4 protein expression were found between the wild-type and TLR4 mutant cells (Rallabhandi et al., 2006). This finding suggests that this reduced response is less likely due to a change in ligand, such as LPS, specific binding sites of TLR4 or the decreased TLR4 expression by these mutations. The functional consequence of Asp299Gly mutant has been hypothesised to be a disrupted interaction between such mutant and a serum component (e.g., CD14, LBP, or
MD-2), as reduction of the serum concentration in the culture medium had a much more profound effect on LPS responsiveness (Rallabhandi et al., 2006) (section 1.7.3). In contrast to previous studies that have associated the TLR4 Asp299Gly SNP with various diseases, this study did not observe an association with opioid and alcohol dependence. This is surprising considering the pivotal role of the TLR4 receptor in glial activation following opioid and alcohol exposure, and the prior role of immunogenetics observed with IL1B gene (Liu et al., 2009; Saiz et al., 2009). However, the molecular mechanism of xenobiotic (e.g. opioids and alcohol) TLR4 activation may differ from that of endogenous danger signals associated with other pathologies, rendering the Asp299Gly SNP in isolation to minor importance with regard to direct opioid- and alcohol-induced TLR4 signalling. For example, alcohol- and opioid-induced TLR4 activation and activation by LPS may not require the same serum components. One of the limitations of the current study however, is the sample size, with power analysis revealing that if there was a statistically significant association between the TLR4 Asp299Gly SNP and opioid or alcohol dependence the current study would not have been adequately powered to detect this (500 and 1000 subjects to shown a 2-fold difference with 80% power). As such, the lack of association between TLR4 Asp299Gly SNP and opioid or alcohol dependence, which was found in this study, does not rule out the pivotal role of TLR4 in mediating the effects of alcohol or opioids. Future studies incorporating other SNPs of TLR4 (see 1.7.3) are required together with a substantially larger population cohort to confirm this preliminary finding.
Chapter VI Conclusions

Prior to recent research, the general assumption has been that purely neuronal mechanisms account for all of the behavioural effects of alcohol and the interaction between alcohol and opioids. It has been demonstrated that alcohol triggers glial TLR4 activation and morphine induces both TLR2 and TLR4 signalling. However, the behavioural consequences of such activation remained to be determined. These behavioural effects following alcohol and morphine-induced neuroimmune activation in the CNS are important missing pieces in the current understanding of the mechanisms of alcohol and opioids’ effects.

In this thesis, I focused on investigating the role of TLR2- and TLR4-MyD88-IL-1 receptor signalling in the mechanisms of alcohol dose-induced sedation and motor dysfunction as well as the interaction between alcohol and morphine by acute administration. In the studies assessing the effects of alcohol (Figure 6-1), blockade of microglial activation, TLR2, TLR4, combined TLR2 and TLR4, MyD88, or IL-1 receptor by pharmacological or genetic means decreased acute alcohol dose-induced sedation or motor impairment in mice. Furthermore, IκBα protein levels were time-dependently altered by alcohol exposure in vitro. The increase of such protein levels was blocked by inhibition of microglial activation, TLR4, MyD88, or the IL-1 receptor signalling. On the other hand, in the study investigating the interaction between alcohol and morphine (Figure 6-2), lack of the alcohol-morphine interaction as assessed by sedation was found in mice with attenuated TLR2, combined TLR2 and TLR4, and MyD88, but not TLR4 or IL-1 receptor, signalling cascade. These different results suggest different mechanisms of effects between alcohol’s actions and the alcohol-morphine interaction. The human genetic data indicated lack of
association between TLR4 Asp299Gly SNP and alcohol or opioid dependence. However, inadequate statistical power was also found, suggesting that larger population cohort is required in future studies to confirm this result.

6.1. New mechanisms for the acute effects of alcohol

6.1.1. The role of Microglia in the acute effects of alcohol

To assess the role of microglial activation in the behavioural effects following acute alcohol administration, minocycline was used as a microglial attenuator. Differential effects of minocycline administration on acute alcohol dose-induced sedation and motor dysfunction as well as the reduction of alcohol sedation by alcohol re-administration were investigated, the results of which are as follows:

1) Three daily doses of minocycline, but not a single dose, reduced acute alcohol dose-induced sedation.

2) Such repeated minocycline administration had no effect on the reduction of alcohol’s sedative effect by alcohol re-administration.

3) Three daily high doses of minocycline increased alcohol-induced motor dysfunction, while a single dose or repeated low doses did not alter this effect.

These findings implied that minocycline had both positive and negative effects on the acute behavioural effects of alcohol, which was dependent on the type of behavioural tests used. Specifically, the role of microglia in sedation and motor dysfunction induced by alcohol
administration was demonstrated by the current data. Notably, as the mechanisms of minocycline’s effects are not fully understood, the differential effects of minocycline in different behavioural models may be due to some unspecified effects of minocycline, such as neuronal effects.

6.1.2. TLR2- and TLR4-MyD88 mediate the acute effects of alcohol

A range of genetic deficient mouse strains were utilised to determine the role of innate immune TLR2 and TLR4 signalling cascades in acute behavioural effects of alcohol. Pharmacological blockade of TLR4 by (+)-naloxone was also used. The results, that TLR2, TLR4, combined TLR2 and TLR4, or MyD88-deficient mice and (+)-naloxone-treated wild-type mice had decreased alcohol-induced sedation or motor impairment, showed that alcohol was capable of activating both TLR2 and TLR4 via the MyD88-dependent pathway. This activation contributed to acute behavioural effects of alcohol.

Recent findings showed that mice lacking TLR4 were protected against inflammatory damage and behavioural associated effects as induced by chronic alcohol consumption (Pascual et al., 2011). Moreover, selective blockade of TLR4 in central nucleus of the amygdala inhibited binge drinking in alcohol-preferring rats (Liu et al., 2011). These data, as well as the current findings, highlighted the role of TLR4 in the behavioural effects of alcohol. Furthermore, the data from this thesis revealed, for the first time, the importance of TLR2 in alcohol’s effects of sedation and motor dysfunction.

Various sources of evidence have indicated the possible link between neuronal and non-neuronal mechanisms of alcohol’s effects. Binge drinking was associated with GABA_Aα2-regulated TLR4
expression (Liu et al., 2011). Interestingly, different from what has been widely accepted that GABA receptors were solely neuronal receptors (Theile et al., 2011), Lee et al. (2011) have demonstrated that GABA receptors are also expressed on microglia and astrocytes. These new findings revealed the relationship between the glial TLR activation by alcohol and the previous neuronal hypotheses of alcohol’s effects. Future studies should focus on whether the neuronal and glial GABA receptors are both involved in the effects of alcohol, and how TLR signalling converges with GABA receptor cascades.

6.1.3. IL-1 receptor signalling mediates the acute effects of alcohol

Pharmacological blockade of IL-1 receptor signalling with a single dose of IL-1ra administration decreased sedation and motor impairment in wild-type mice following a single dose of alcohol injection. This result suggested that alcohol activated IL-1 receptor-mediated signalling within a rapid time frame. As such, the signalling of proinflammatory cytokines and their receptors, such as IL-1 receptor, may account for the cross-talk between neuronal NMDA and GABA receptors and glial TLRs in the effects of alcohol.

6.1.4. The involvement of IκBα in the acute effects of alcohol

As the main inhibitory protein of NFκB, IκBα was studied to assess the acute modification of NFκB signalling by alcohol exposure in vitro. The results highlighted the rapid and time-dependent response of NFκB signalling to alcohol stimulation. In the behavioural studies, acute alcohol-induced behavioural differences between mice inhibited in microglial, TLR4, MyD88, or IL-1 receptor signalling and controls was observed at 30 min post-alcohol administration. As such, 30 min was chosen as the time point to assess the effect of alcohol on IκBα protein levels in
In mixed hippocampal cells, an increase in \(\text{IκB}_\alpha\) protein levels was found at 30 min of alcohol exposure. Such an increase in \(\text{IκB}_\alpha\) protein levels was blocked in cells from wild-type mice in the presence of minocycline or IL-1ra, and from mice deficient in TLR4 or MyD88. This finding indicated that the rapid alteration of \(\text{IκB}_\alpha\) protein levels by alcohol exposure was microglial and TLR4-MyD88-IL-1 receptor-dependent.

Interestingly, \(\text{IκB}_\alpha\) protein levels were acutely modulated by (+)-naloxone, a TLR4 signalling inhibitor (Hutchinson et al., 2008b). This preliminary finding indicated that the mechanism of (+)-naloxone’s effect was associated with alteration in \(\text{IκB}_\alpha\) protein levels. However, the questions of how (+)-naloxone changes \(\text{IκB}_\alpha\) protein levels and what role this alteration of \(\text{IκB}_\alpha\) plays in the effects of alcohol remain unanswered.

The effect of alcohol exposure on brain MAPK pathways was determined by measuring the phosphorylation of p38, JNK, and ERK \textit{ex vivo} and \textit{in vitro}. Surprisingly, no effect of alcohol on MAPK pathways was found, which suggested alcohol could activate NFκB signalling via non-MAPK pathways. It is therefore suggested that evidence using other techniques and a time course study are needed to confirm this negative finding.

6.1.5 Future directions for studies of the acute effects of alcohol

To sum up, data generated in this thesis have shown that the central innate immune signalling activation through TLR2- and TLR4-MyD88-IL-1 receptor-\(\text{IκB}_\alpha\) pathways plays a key role in the acute behavioural effects of alcohol. Microglial activation has also been demonstrated to be involved in these novel immune mechanisms (Figure 6-1). As such, pharmacological strategies,
such as minocycline, (+)-naloxone, and IL-1ra, that target these signalling cascades within the CNS may prove beneficial in various alcohol abuse-related conditions.

Indeed, these findings raise further questions, which need to be answered by future studies.

1) Due to the role of glial TLR signalling in alcohol-induced sedation, motor impairment (Wu et al., 2011a; Wu et al., 2011b), chronic withdrawal (Pascual et al., 2011), and minocycline-induced decreased alcohol consumption (two-bottle choice test) (Agrawal et al., 2011), other acute and chronic behavioural effects of alcohol, such as locomotor activity (open-field test), anxiety-like behaviour (elevated plus maze test), and positive and negative rewards (conditioned place preference), are worth investigating.

2) Other TLR signalling inhibitors and glial attenuators, as described in section 1.2.4, could be evaluated.

3) The specific brain regions and cell types where this alcohol-induced neuroinflammation occurs, as well as the relationship between the behavioural effects of alcohol and their corresponding action sites, need to be investigated.

4) Determination of the influence of alcohol on cell signalling proteins in PI3K/AKT pathways is required to fully understand whether the non-MAPK pathways are involved in the acute activation of TLR-dependent NFκB pathway by alcohol.
Figure 6-1. Modified diagram of the hypotheses of alcohol’s effects on neurons and microglia (Figure 1-4) based on the current results. The hypotheses that alcohol activates microglia via the TLR4-MyD88-dependent signalling, and that such activation induces IL-1 receptor signalling activation have been proven by the animal behavioural data in this thesis. The role of NFκB has been demonstrated using the changes of IκBα protein levels by alcohol exposure in vitro.
5) Apart from IL-1 receptor signalling, the signalling of other cytokines, chemokines, and their receptors, such as TNF-α and CCL2, have been demonstrated to be altered by alcohol exposure (section 1.4.2). Thus, the behavioural consequences of inhibiting such cytokine signalling on the effect of alcohol could be investigated.

6) The modification of neuronal or non-neuronal GABA and NMDA receptors by alcohol-induced glial TLR activation and the modification of TLR signalling by GABA and NMDA receptor activation remain unclear. Understanding the mechanism of this glial-neuronal crosstalk will fulfil the knowledge of alcohol’s effect.

7) The behavioural effects following acute alcohol administration have been attenuated by pharmacological treatment in mice. These drugs, including minocycline and IL-1ra, have good clinical safety records (section 1.4.4). Although (+)-naloxone has not been used in humans, its (-)-isomer has been proven to be well-tolerated (section 1.4.4). Therefore, future human clinical studies, assessing the influence of minocycline, IL-1ra, and (+)-naloxone treatment on the effects of alcohol, should be conducted. The CNS activities of these drugs in humans need to be assessed, such as the BBB permeability of (+)-naloxone.

6.2. New mechanisms for the interaction between alcohol and morphine

Given the findings of alcohol-induced acute neuroinflammation as summarised above and the growing evidence of the TLR2 (Zhang et al., 2011) and TLR4 (Hutchinson et al., 2010b) activities of morphine, these raised the possibility of a new mechanism for alcohol-morphine interactions.
6.2.1. TLR2-MyD88 cascade mediates the alcohol-morphine interaction

The sedative interaction between alcohol and morphine was tested across different genotypes in mice, including wild-type and TLR2-, TLR4-, combined TLR2 and TLR4-, or MyD88-deficient mice. Pharmacological attenuation of TLR4, MOR, or IL-1 receptor activity was obtained by administration of (+)-naloxone, (-)-naloxone, or IL-1ra, respectively. The results showed that the MOR, TLR2, and MyD88, but not TLR4, were critical in the alcohol-morphine interaction (Figure 6-2). Notably, this is different from what has been found in the behavioural studies of alcohol that both TLR2 and TLR4 signalling cascades were involved. These differences indicated that the effect of alcohol or morphine alone and the interaction between alcohol and morphine may be through different mechanisms.

Indeed, there is the possibility that the observation of lower sedative effects of alcohol and morphine in TLR2-, combined TLR2 and TLR4-, or MyD88-deficient mice is due to the increased potency to alcohol dose-induced sedation, since the dose of alcohol used in the interaction study did not induce any sleep time in these genetic deficient mice. Further experiments that test a range of alcohol and opioid doses with the alcohol-morphine interaction model are required to confirm the current results.
Figure 6-2. Modified diagram of hypotheses of the alcohol-morphine interaction (Figure 1-5) based on current results. The hypotheses, that alcohol and morphine activate the TLR2-MyD88 signalling and MOR, and that such activation triggers an increased duration of LORR, have been proved by the animal behavioural data in this thesis. However, the role of TLR4 or IL-1 receptor in alcohol-morphine interactions has not been evident in this model.
6.2.2 Future directions for studies of alcohol-morphine interactions

As the neuroimmune mechanisms of alcohol-morphine interaction had not been investigated before this thesis, many more studies are urgently required in this new research area. Similar to what has been suggested in the alcohol studies as per section 6.1.5, other behavioural effects, TLR signalling inhibitors and glial attenuators, cell signalling proteins, and cytokines need to be evaluated in future studies of alcohol-opioid interactions. Moreover, the brain regions and cell types, where alcohol and morphine-induced neuroinflammation occurs, are yet to be determined. The modification of neuronal opioid, GABA, and NMDA receptors by glial TLR activation as induced by alcohol and morphine is also yet to be understood. Furthermore, as this thesis focused on investigating the effect of morphine administration on the effect of alcohol, experiments designed for assessing the modification of morphine’s effects by alcohol administration need to be conducted. The knowledge gained from such studies will help in understanding of the mechanisms of alcohol-induced opioid-related deaths.

6.3. Alcohol and opioid pharmacogenetics

Due to the inadequate statistical power of this study, the data from this thesis did not show an association between TLR4 Asp299Gly SNP and opioid or alcohol dependence. As such, future studies using multivariate analysis reflecting TLR4 SNPs together with the genes as listed below are required to be investigated with a substantially larger population cohort.

1) Alcohol and opioid metabolic enzyme and transporter genes, such as CYP2E1, ABCB1, ADH, and ALDH.
2) Genes coding for neuronal receptors of alcohol and opioids, such as *OPRM1* and *GABRA6*.

3) Immune genes which have been demonstrated to play a role in alcohol and opioid dependence by pharmacogenetic evidence (section 1.7.2), such as *NFKB1*, *IL10*, and *IL1RN*.

4) *TLR2* gene coding for another TLR which can be activated by opioids (section 1.5.3).

Thus, a full picture of the genetic influence on alcohol and opioid dependence could be drawn from these data.

### 6.4. Summary

Overall, results of the current thesis revealed the cell type, receptors, intracellular signalling cascades, and cytokines involved in the acute sedative and motor effects of alcohol. A slightly different neuroinflammatory mechanism has been demonstrated in the sedative interaction between alcohol and morphine. Together, the behavioural preclinical evidence highlights the importance of alcohol and opioid neuroimmunopharmacology. These results offer novel insights and approaches that form the foundation of a new era of research in the pursuit of more effective pharmacotherapies for alcohol and opioid abuse.
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