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Exploring the Pharmacology of (+)-Naltrexone on Alcohol Reward and Anxiety Behaviours

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Abstract

Reward and anti-reward are two key processes mediating occasional and long-term consumption of alcohol. Traditionally, neurons were thought to be the exclusive mediators of reward and anti-reward. However, emerging evidence has highlighted the importance of the neuroimmune system, specifically, an innate immune receptor (Toll-like receptor 4) in mediating these phenomena. Toll-like receptor 4 (TLR4) is a pattern recognition receptor that detects conserved molecular epitopes expressed on pathogens, danger molecules and drugs of abuse. In response to alcohol the downstream signalling pathways of TLR4 (MyD88 and TRIF pathways) are activated. This culminates in the expression of classical pro-inflammatory cytokines and type-one interferons respectively. These immune molecules act via multiple pathways to influence neuronal activity thereby altering alcohol-related behaviour. No study has currently examined the relative contribution of each signalling pathway to alcohol-induced reward and anti-reward behaviours. Therefore, the aim of this thesis was to investigate the role of the TLR4-TRIF pathway in mediating acute alcohol-induced reward; reward priming following acute alcohol exposure; and long-term alcohol-induced reward and anti-reward behaviours in mice. The studies presented herein demonstrate pharmacologically attenuating TLR4-TRIF signalling via (+)-Naltrexone; reduces behavioural markers of acute alcohol-induced reward such as conditioned place preference and two-bottle choice – an effect dependent on the time-of-day; prevents acute alcohol-induced sensitisation during adolescence and some but not all markers of reward-like behaviour later in life; and lastly, did not alter behavioural indices of reward and anti-reward behaviour following long-term alcohol consumption. Collectively, the results highlight the importance of the TLR4-TRIF pathway in mediating the acute, but not necessarily chronic effects of alcohol reward and anti-reward.

Declaration

“I 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 institution 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. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.”

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Statement of authorships

Mr Jonathan Henry Webster Jacobsen had major input in the experimental design, behavioural testing, molecular and statistical analysis, graphic presentation and prepared the manuscript for submission. Overall percentage: 85 %

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Professor Mark Hutchinson assisted in the experimental design, data interpretation and preparation of the manuscript.

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Dr. Femke Buisman-Pijlman assisted in data interpretation and preparation of the manuscript.

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Dr. Kenner Rice provided (+)-Naltrexone and assisted in the preparation of the manuscript.

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By signing the Statement of Authorship, each author certifies that the candidate's stated contribution to the publication is accurate; permission is granted for the candidate to include the publication in the thesis; and the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Additional publications

During my PhD, I published several reviews that are not presented in this thesis. These publications are listed below:

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"*This too shall pass*"

Edwards Fitzgerald

Polonius: A Collection of Wise Saws and Modern Instances

1852

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Abbreviations

[^{35}S]GTPγS	Guanosine-5'-O-[gamma-thio]triphosphate
ADH	Alcohol dehydrogenase
AIM2	Absent in melanoma 2
AMPA	α-amino-3-hydroxyl-5methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP-1	Activator protein-1
ARC	Activity-regulated cytoskeleton-associated protein
ATP	Adenosine triphosphate
BAC	Blood alcohol concentration
BDNF	Brain-derived neurotrophic factor
BK Channel	Big potassium channel; Ca^{2+} activated K^{+} channel
BMAL1	Brain and muscle ARNT-like 1; Aryl hydrocarbon receptor nuclear translocator-like protein-1
BNST	Bed nucleus of the stria terminalis
Ca^{2+}	Calcium
CBP	CREB binding protein
CCL2	Chemokine (C-C motif) ligand 2
CCP	Conditioned place preference
CCR	Chemokine receptor
CD	Cluster of differentiation
Cd5K	Cyclin-dependent kinase 5
cDNA	Complementary DNA
CeA	Central nucleus of the amygdala
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nervous system
COX	Cyclooxygenase

CREB	cAMP response element-binding protein
CRF/CRH	Corticotrophin releasing factor/hormone
CRHR1	Corticotrophin releasing hormone receptor 1
CRHR2	Corticotrophin releasing hormone receptor 2
CRY	Cryptochrome
CT	Cycle threshold
DAMP	Danger associated molecular pattern
DNA	Deoxyribonucleic acid
DNMT	DNA methyl-transferase
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
EAAT1	Excitatory amino acid transporter 1
ERK	Extracellular signal-regulated kinase
FOS	FBJ murine osteosarcoma viral oncogene homolog
GABA	Gamma-aminobutyric acid
GABRA1	Gamma aminobutyric acid A receptor, subunit alpha 1
GABRA2	Gamma aminobutyric acid A receptor, subunit alpha 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
GHRL	Ghrelin
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GRIA1	Glutamate receptor, ionotropic, AMPA1 (alpha 1)
GRIN1	Glutamate receptor, ionotropic, NMDA1 (zeta 1)
HDAC	Histone deacetylase
HEK cell	Human embroyonic kidney cell

HMGB1	High mobility group box 1
HPA axis	Hypothalamic-pituitary adrenal axis
i.g	Intragastric
i.p	Intraperitoneal
Iba-1	Ionised calcium-binding adapter molecule 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Interferon gamma-induced protein 10
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
Iκβ	Inhibitor of κβ
JNK	c-Jun N-terminal kinases
LEP-R	Leptin receptor
LPS	Lipopolysaccharide
LPS:RS	Lipopolysaccharide from *Rhodobacter Sphaeroides*
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemotactic protein (CCL2)
MD	Lymphocyte antigen 96
MyD88	Myeloid differentiation primary response gene 88
NAcc	Nucleus accumbens
NAD	Nicotinamide adenine dinucleotide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAAA	National Institutes of Alcohol and Alcoholism
NIH	National Institutes of Health
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
NPY	Neuropeptide Y

NPYR1	Neuropeptide Y receptor 1
NPYR2	Neuropeptide Y receptor 2
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2
OPRK1	Kappa (κ) opioid receptor
OPRM1	Mu (μ) opioid receptor
P	Postnatal day
PAMP	Pathogen associated molecular pattern
PER	Period
PFC	Prefrontal cortex
PKC	Protein kinase C
RAGE	Receptor for advanced glycation endproducts
Rel	Proto-oncogene c-Rel
RIG-1	Retinoic acid-inducible gene
RNA	Ribonucleic acid
Rxfp1	Relaxin/insulin-like family peptide receptor 1
SCN	Suprachiasmatic nuclei
SEAP	Secreted embryonic alkaline phosphatase
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TH	Tyrosine Hydroxylase
TIR	Toll-interleukin receptor
TIRAP	Toll-interleukin 1 receptor domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	Translocation associated membrane protein
TRIF	TIR-domain-containing-adapter-inducing interferon β

URN	Urocortin transcript variant
VTA	Ventral tegmental area
XAMP	Xenobiotic associated molecular pattern
ZT	Zeitgeber time

Chapter 1: Literature Review

1.1 Introduction

The consumption of alcoholic beverages has, and continues to be, an integral activity engaged in by members of both ancient and modern societies. Traditionally, alcoholic beverages were consumed primarily as a celebratory activity during large social gatherings (Boyle et al., 2013). However, the modernisation of fermentation and distillation processes, has enabled the widespread manufacturing of these beverages. Consequently, the way individuals, think, approach and consume alcohol has changed. While the tradition of consuming alcoholic beverages during celebrations continues, the recreational consumption of alcohol (primarily for its euphoric properties) has grown considerably. For example, 70 per cent of surveyed American adults report consuming alcohol within the past year, with a further 56 per cent reporting moderate to low monthly consumption (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2017). Higher and more frequent use is problematic and increasingly prevalent not only among adults, but also among adolescents. Approximately 27, 37 and 13 per cent of adults (18+ years old), college students (18 – 22 years old) and adolescents (12 – 20 years old) engage in monthly binge drinking respectively (SAMHSA, 2016) (in the United States of America binge drinking is defined as achieving a blood ethanol content of 0.08g/dL in 2 h – equivalent to 4 and 5 drinks for women and men, respectively) (NIAAA, 2004).

Every society that consumes alcohol demonstrates large health, social and economic costs attributable to its use (both occasional and excessive) (World Health Organisation, 2014). This problem is exacerbated as public policy largely ignores these problems owing to engrained cultural attitudes towards alcohol – particularly in western countries. This is concerning given the health costs associated with alcohol use are

extremely, and unnecessarily high. For example, the consumption of alcohol results in 3.3 million deaths annually, and consistently ranks within the top five risk factors for disease, disability and death worldwide (World Health Organisation, 2014). It is therefore unsurprising that the consumption of alcohol is causally associated with approximately 200 diseases, of which neuropsychiatric disorders account for the largest proportion of diseases. Within neuropsychiatric disorders, addiction and depression are the most frequently diagnosed disorder attributable to alcohol use (World Health Organisation, 2014). Given the large neuroanatomical overlap between these two disorders, it highlights that alcohol selectively modifies the brain's limbic system – a region governing mood, emotion, learning and memory (Oscar-Berman & Marinković, 2007).

However, discerning the detrimental effects of alcohol on the brain's limbic system in clinical and preclinical research is complex as the term "alcoholic beverages" encompasses a broad range of solutions including beer, wine and spirits; and uncommon forms of alcohol such as moonshine and homemade spirits. These beverages contain hundreds to thousands of unique chemical entities (Buglass, 2015) – some of which have both beneficial and detrimental effects on the body (see for example, IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2010; Brown et al., 2009). Interestingly, the detrimental and psychoactive properties of alcoholic beverages are often attributed to a single molecule: ethanol (ethyl alcohol, CH_3CH_2OH). Ethanol is a small, polar, hydrophilic molecule, the concentration of which fluctuates substantially among alcoholic beverages (Ferreira & Willoughby, 2008). For example, beer typically contains 3 to 7 per cent ethanol while spirits are often greater than 50 per cent (Buglass, 2015). The chemical properties of ethanol facilitate its fast absorption and preferential displacement to areas where water quantity is high (Ferreira & Willoughby, 2008) – for example the blood and brain. Given its high

concentration and preferential movement to areas most affected by the consumption of alcoholic beverages (the brain), ethanol has become the main focus of preclinical research assessing the effects of alcoholic beverages on the body.

Ethanol's action upon the brain, and more specifically the mesocorticolimbic system are complex and are unlike any other drug of abuse such as cocaine or opioids (Harrison, 2007; Nestler, 2005). Ethanol interacts with numerous neurotransmitter, neurotrophic and neuropeptide systems to generate complex behaviours including, anhedonia (reduced ability to feel pleasure), anxiety, dependence, motor impairment, pain and reward (Costardi et al., 2015; Tabakoff & Hoffman, 2013). Many of these behaviours are beyond the scope of this thesis. Please refer to Neupane, (2016) and Egli et al., (2012) for examples of reviews summarising ethanol-induced depression and pain respectively. Rather, this thesis will focus on the effects of ethanol on reward and anti-reward behaviours.

1.2 Liking, wanting and reward

The initial, occasional and long-term consumption of alcohol are driven in part by the rewarding properties of ethanol (herein referred to as alcohol). Alcohol-induced reward is comprised of two psychological phenomenon; "liking" and "wanting" (Berridge & Robinson, 2016; Robinson & Robinson, 2013). "Liking" is the pleasurable impact of alcohol consumption. Specifically, it is the hedonic sensation an individual feels upon consuming alcohol and other sweet compounds such as sucrose or saccharin. It is important to note that "liking" can be influenced by taste and thirst under specific circumstances (Berridge & Robinson, 2016). Historically "liking" behaviour was assessed in rodents by examining affective facial reactions following the consumption of alcohol or sucrose (Berridge & Grill, 1983). However, "liking" (hedonic) behaviour is more frequently inferred by using short two-bottle choice paradigms in which rodents

have access to two bottles – one contains water and the other a palatable/rewarding or aversive solution (Willner et al., 1987). By contrast, longer testing sessions are used to infer both “liking” and “wanting” behaviour (Tabakoff & Hoffman, 2000).

“Wanting” or incentive salience is the motivation or desire (expectation based on memory) to obtain rewarding stimuli (Berridge & Robinson, 2016). It is the attachment of significance or value to an object or cue that is associated with the rewarding compound. “Wanting” behaviour is typically assessed using conditioning paradigms such as conditioned place preference. In brevity, conditioned place preference pairs a distinct environmental cue with a potentially rewarding stimulus, and another environmental cue with a vehicle control. Over repeated sessions, the animal learns to associate the discrete environmental cue with the desired rewarding effect. On the last day of testing, mice have access to both the drug- and vehicle-paired environments with the amount of time spent in the drug-paired chamber (seeking behaviour) an inference of “wanting” behaviour (Tzschentke, 2007; 1998).

“Liking” and “wanting” are essential for generating reward. Under homeostatic conditions, brain regions governing “liking” and “wanting” are activated by natural rewards such as food, water and sex (Berridge & Robinson, 2016). Alcohol, like all other drugs of abuse, acts upon, and hijacks these brain regions. Importantly, drugs of abuse activate these pathways to a greater extent than natural rewards and are consequently preferred (Wise, 2004).

However, “liking” and “wanting” are neurobiologically distinct and initially occur independent from one another. Typically, the “liking” regions are activated first followed by the “wanting” regions. Consequently, the initial consumption of alcohol is governed predominately by “liking” component of reward, however the “wanting” pathways are

additionally activated overtime – alcohol sensitises the “wanting” pathway while inducing tolerance to the “liking” pathway. Therefore, the individual shifts away from consuming alcohol because they “like” it to a state where they “want” and need it (Berridge & Robinson, 2016). This transition underlies the shift from impulsive to compulsive use and is a key component of addiction and dependence (Berridge & Robinson, 2016; Koob & Le Moal, 2001).

1.3 Addiction and dependence

It is important to highlight that drug addiction is clinically and neurobiologically distinct from an initial drug taking experience. While these two events share similar neurobiological substrates, addiction is a complex, relapsing disorder resulting from numerous neuroadaptations due to prolonged drug exposure (Koob & Le Moal, 1997). These adaptions are complex, persist beyond acute withdrawal and are thought to involve environmental, psychological (stress, learning and conditioning) and genetic (predisposition to drug taking) elements (Koob & Volkow, 2009). Furthermore, in humans most drug users do not become addicted with a similar scenario observed in animals. Stable drug intake can be observed within animals without indications of addiction even in paradigms designed to recapitulate addictive scenarios (limited access, operant self-administration). This reflects the complex aetiology of drug addiction (Koob & Le Moal, 1997).

The Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) recognizes drug dependence and substance abuse disorders as a chronic disorder which is characterized by an impaired control over taking, and an increased craving for, drugs of abuse; a withdrawal syndrome upon cessation; tolerance - with a larger dose required to achieve the desired psychological effect; a disproportionate time seeking

and consuming the drug and persistence of drug-use despite negative consequences (American Psychiatric Association, 2013; World Health Organization, 1993).

From a psychological standpoint, drug addiction is thought to reflect a shift from impulsive to compulsive behaviour (Koob & Le Moal, 1997). Impulsive consumption is thought to be driven primarily by the "liking" component of reward (Berridge & Robinson, 2016). After repeated intake, the individual becomes tolerant to the "liking" component of alcohol due to pharmacokinetic and pharmacodynamic adaptions to alcohol. For example, alcohol dehydrogenase and aldehyde dehydrogenase, enzymes responsible for metabolism of alcohol and acetaldehyde (a metabolite of alcohol) respectively, are upregulated, thus increasing the rate of alcohol's catabolism - decreasing blood alcohol concentration (Tabakoff et al., 1986; Misra et al., 1971). Furthermore, the activity of receptors interacting with alcohol for example, the μ opioid receptor, are reduced owing to internalisation processes and a reduction in the expression of signalling proteins. This is designed to limit the effects of alcohol on the brain (He & Whistler, 2011; Gianoulakis, 2001). Over time, the "wanting" component of reward is slowly engaged which assists in the transition to compulsive behaviour (Berridge & Robinson, 2016; Koob & Le Moal, 1997). The "wanting" component of reward typically arises in the absence of alcohol, and can under periods of addiction, manifest as anxiety, stress and anhedonia. To alleviate these adverse sensations, alcohol is sought after and consumed. Thus, the shift from impulsivity to compulsivity reflects a change in motivation state from positive to negative reinforcement (Koob & Le Moal, 2001; 1997).

1.3.1 Opponent-process theory in addiction

Several theories have been applied to explain the psychological and biological mechanisms underlying the change from impulsive to compulsive behaviour observed

during addiction. And, although many theories account for some aspects of drug addiction, they cannot in their entirety account for the disorder as a whole. Therefore, while I acknowledge the presence and importance of other theories, for example, associative-learning disorder (Di Chiara, 1999), I have chosen to predominately examine and apply one leading theory of addiction (opponent process theory). However, aspects of incentive-sensitisation will additionally be incorporated (Robinson & Berridge, 2001).

The opponent-process theory (Solomon & Corbit, 1974) is particularly relevant to drug addiction. Opponent-process theory states, hedonic, affective or emotional states are automatically opposed by CNS mechanisms, which reduce the intensity of these states, both pleasant and aversive to maintain homeostasis. In brief, the theory consists of two processes termed A and B. A-process consists of the initial positive or negative hedonic response to a stimulus. The magnitude of response correlates with the quality and duration of the stimuli and can, overtime exhibit tolerance. B-process occurs after the A-process has finished, is sluggish in onset, is slow to build up and decay, and is opposite in response to A. However, if the stimulus is consistently repeated, A-process becomes weaker, and the B-process becomes stronger and long lasting (allostasis) (Koob & Le Moal, 1997).

In the context of addiction, alcohol produces an initial “liking” experience (euphoria) (and “wanting” to some extent), which serves as a positive reinforcer (A-process). However, the user subsequently experiences negative hedonic affect (anhedonia and dysphoria, first encountered during a “hangover”), which acts a negative reinforcer (B-process) (Solomon & Corbit, 1974). Koob & Le Moal, (1997) furthered this concept by theorizing as occasional drug use transitions into a compulsive habit, this homeostatic process becomes dysregulated and breaks. The initial “liking”, euphoric sensation

begins to weaken (tolerance), and the negative hedonic experiences associated with anhedonia, anxiety and “wanting” occur more rapidly, are enduring, more intense and fail to return to base line, creating a new allostatic state. Consequently, the body must actively compensate for the dysregulation of the reward pathway by creating a new-set point that is now below the original reward value (allostatic point). Collectively, these processes alter the overall drug taking experience. What was once considered rewarding to take, is now taken in an attempt to return to a baseline state. Continual resetting contributes to the pathology of addiction (Koob & Le Moal, 2001; 1997).

Two key cellular processes are hypothesised to underlie allostasis in alcohol addiction; within and between-system adaptations (Koob & Le Moal, 1997). In the presence of constant alcohol exposure, a within-system adaption refers to the brain’s attempt to neutralise the drug’s effect by altering the primary responding circuitry that is producing desired effects (“liking” and “wanting”). In the context of alcohol addiction, a within-system adaption is hypothesised to include cellular changes within the mesolimbic pathway (primary responding circuit). A within-system adaption contributes to drug tolerance. The individual must therefore consume higher doses with increased drug taking frequency to achieve the same desired effect. However, the persistence of the within-system adaptions in the absence of a drug may contribute to anhedonia, anxiety and “wanting”. By contrast, a between-system adaption is a different cellular system, which has opposing effects to the primary response element. A between-system adaptation is aimed to limit the reward by inducing a stressful or dysphoric response (anti-reward). In the absence of alcohol, the system still persists, exacerbating the “wanting” of alcohol and stressful and dysphoric sensations (Koob & Le Moal, 1997).

1.4 Neurobiological basis of reward, anti-reward and dependence

Alcohol-induced reward and anti-reward are created and sustained by multiple brain regions. Specifically, the mesolimbic pathway mediates the “liking” and “wanting” components of alcohol-induced reward. Therefore, this pathway governs the initial, occasional and long-term (chronic) use of alcohol. As previously mentioned, acute alcohol activates this pathway generating reward. However, as alcohol use becomes more frequent, neuroplastic events within the mesolimbic pathway and brain regions governing stress and pain pathways (amygdala, bed nucleus of the stria terminalis and locus coeruleus) occur to limit the effects of alcohol. A by-product of these consequences, is the creation of anti-reward – a sensation characterised by anhedonia, stress and anxiety (Fein & Cardenas, 2015; Seo & Sinha, 2015; Koob & Le Moal, 2001). Importantly, the effects of alcohol on the reward and anti-reward pathways are influenced by many variables including the duration of exposure, route of administration, the time of day and the age of the individual (for example, Chatterjee et al., 2014; Alaux-Cantin et al., 2013; Veeraiah et al., 2012; Spanagel et al., 2004). The effects of duration of exposure on reward and anti-reward will be discussed in depth in the proceeding sections. The effects of time-of-day and age will be discussed as preludes to chapters 3 and 5 respectively.

1.4.1 Reward following acute alcohol exposure

Dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens are a key cellular mechanism underlying the “wanting” component of alcohol-induced reward (Di Chiara & Imperato, 1988; Imperato & Di Chiara, 1986). In response to natural rewards such as food, water and sex, the action potential frequency of these cells is increased causing a subsequent elevation in extracellular dopamine within the nucleus accumbens (Wise, 2013; 2006; Olsen, 2011; Kelley & Berridge, 2002; Melis & Argiolas, 1995). The rise in extracellular dopamine functions

as a learning mechanism, conferring motivational importance to specific stimuli (the "wanting" component of reward) (Wise, 2004). As such, an individual will "want" to seek out these rewarding stimuli. All drugs of abuse converge and act upon this pathway. While each drug differs in the biological mechanism, they all dose-dependently increase the concentration of extracellular dopamine in the nucleus accumbens (Imperato & Di Chiara, 1986). Importantly, the amount of dopamine release is typically greater following exposure to a drug of abuse than that of natural stimuli (Wise, 2004). Consequently, alcohol, and other drugs of abuse are imbued with a higher motivational value (Nestler, 2005; Wise, 2004).

In regards to alcohol's effect on this pathway, research has demonstrated microinjection of alcohol into the VTA but not the nucleus accumbens dose-dependently increases accumbal dopamine (Di Chiara & Imperato, 1988; Imperato & Di Chiara, 1986). This idea is further supported as research has shown the enhanced dopamine release is not due to the inhibition of dopamine's re-uptake in the nucleus accumbens (Yim & Gonzalez, 2000). This suggests alcohol specifically targets the soma (cell body) that reside in the VTA and not the terminals of dopaminergic neurons in the nucleus accumbens. Further, pharmacological or genetic inhibition of dopamine D1, D2 or D3 receptors and siRNA knockdown of D2 in the nucleus accumbens alters alcohol-induced conditioned place preference and 24 h intake of alcohol - key indices of "wanting" behaviour (Sciascia et al., 2013; Bahi & Dreyer, 2012; García-Tornadú et al., 2010; Hamlin et al., 2007; Boyce-Rustay, 2003; Liu & Weiss, 2002; Cunningham et al., 2000; Risinger et al., 2000; Dyr et al., 1993). Additionally, inhibition of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, significantly reduces alcohol intake (Myers & Veale, 1968). Collectively, these results suggest alcohol acts upon the cell body of VTA dopaminergic neurons to increase the extracellular release of dopamine within the nucleus accumbens. Dopamine's effects are subsequently

transduced by D1 and D2 receptors primarily by medium spiny neurons (Jeanes et al., 2014) to generate alcohol-“wanting” behaviour.

There are multiple mechanisms underlying alcohol-induced firing of dopaminergic neurons. Of particular importance are voltage-gated potassium channels (BK channels) and $GABA_A$ receptors (Martin, 2010; Harris et al., 2008; Brodie et al., 2007a; Chester & Cunningham, 2002). Alcohol modulates the lipid microenvironment surrounding BK channels enhancing its activity in the soma but not dendrites of dopaminergic cells (Yuan et al., 2008; Crowley et al., 2005). Increasing activation of this channel reduces the inward potassium current preventing the after-hyperpolarisation following action potentials (Brodie et al., 2007b; Martin, 2004). This increases action potential frequency and dopamine release (Brodie et al., 1999). Alcohol additionally acts as a functional agonist on $GABA_A$ receptors expressed by medium spiny interneurons (GABAergic interneurons) in the VTA (Harris, 1999). As such the sensitivity towards GABA is increased, leading to an influx of chlorine ions; and decreasing the excitability of these cells (Wallner et al., 2014; Sundstrom-Poromaa et al., 2002;). The loss of inhibitory function by medium spiny interneurons decreases GABA release on to dopaminergic neurons (Patton et al., 2016; Davies, 2003). Therefore, dopaminergic cells exhibit a spike in activity; increasing dopamine release in the nucleus accumbens (Kalivas et al., 1990). $GABA_A$ receptors are expressed on dopaminergic neurons as well. However, the subunit composition of these receptors differs to those expressed by medium spiny interneurons resulting in different molecular and electrophysiological properties. Consequently, dopaminergic neurons are less sensitive to the agonistic interaction induced by alcohol (Davies, 2003).

While emphasis has been placed upon dopamine as the key mediator of alcohol-induced “wanting” behaviour, there are additional neurotransmitter systems which

influence this response. For example, serotonergic and cholinergic pathways in the VTA and nucleus accumbens regulate the activity of medium spiny neurons and can cause dopamine-dependent and independent effects which influence alcohol “wanting” behaviour in rodents (Ding et al.,2015; Liu et al., 2013; Rodd et al., 2010). However, a complete discussion of these effects are beyond the scope of this thesis, please refer to Bell et al., (2013) for a comprehensive summary.

Dopamine mediates the “wanting” but not “liking” component of alcohol reward (Berridge & Robinson, 2016). “Liking” occurs in discrete regions within the nucleus accumbens, ventral palladium and the prefrontal cortex. Interestingly, these nuclei act in concerted manner, with stimulation of one area activating another, amplifying the “liking” signal (Berridge & Kringelbach, 2015). On a molecular level, current evidence suggests “liking” is mediated by the endogenous opioid system (Berridge & Robinson, 2016). Alcohol increases the extracellular concentration of endorphins and enkephalins and potentiates endorphin receptor binding in the nucleus accumbens (Jarjour et al., 2009; Méndez et al., 2001; Olive et al., 2001; Anwer & Soliman, 1995). Antagonising opioid receptors or delivering μ opioid receptor antisense oligonucleotides in the nucleus accumbens inhibits voluntary alcohol consumption (Pastor & Aragón, 2008; Lasek et al., 2007; Becker et al., 2002). Further, administration of an opioid receptor antagonist, (-)-Naltrexone, but not selective removal of dopaminergic terminals in the nucleus accumbens blocked alcohol intake in the same rodents – suggesting that “liking” is a dopamine-independent (Koistinen et al., 2001). However, opioid receptor antagonists attenuate alcohol-induced dopamine release suggesting a complex interaction between the “liking” and “wanting” systems (Benjamin et al., 1993; Devine et al., 1993). Endocannabinoids and orexins are additionally implicated in “liking” behaviour (Ho & Berridge, 2013; Mahler et al., 2007). However, their precise function remains to be fully elucidated.

1.4.2 Reward sensitisation following acute alcohol exposure

Acute (repeated) exposure followed by a period of significant deprivation can result in sensitisation of the reward pathway, an effect particularly apparent during periods of neurodevelopment such as adolescence. Following alcohol exposure, there are alterations in the gene expression of endocannabinoid, opioid and dopamine D1 and D2 receptors within the brain (for example, McClintick et al., 2016). These are key mediators underlying the molecular basis of “liking’ and “wanting”. Thus, re-exposure to alcohol or other drugs of abuse (cross-sensitisation) induces a higher “liking” and “wanting” response. Interestingly, alcohol exposure during adolescence results in a persistent rise in the expression of these receptors – an effect which can last through adulthood (Alaux-Cantin et al., 2013; Hargreaves et al., 2009; Pascual et al., 2009). By contrast, alcohol consumption in adults often results in transient increases in the activity of these systems; reinforcing the concept of ontological-dependent sensitivities towards drugs of abuse (see Spear & Swartzwelder, 2014 for review).

1.4.3 Reward following chronic alcohol exposure

As alcohol use becomes more frequent, the brain attempts to counteract the rewarding effects of alcohol. As such the same brain regions and molecular processes mediating reward undergo within-system adaptions to limit the effect of alcohol. Long-term exposure to alcohol increases brain reward threshold. This is attributable to decreased activity of dopaminergic neurons in the VTA (Weiss et al., 1996; Diana et al., 1993; Shen & Chiodo, 1993) and a reduction in the amount of extracellular dopamine released in the nucleus accumbens (Weiss et al., 1996). Consequently, larger and more frequent doses of alcohol are required to achieve the same rewarding effect. In the absence of alcohol, a state of anhedonia occurs. Therefore, alcohol is again

consumed to alleviate these adverse sensations and assisting the “wanting” mechanism.

There are multiple mechanisms underscoring alcohol-induced dopamine deficits. For example, hyperactive L-type calcium channels hyperpolarise dopaminergic neurons; reducing the frequency of action potentials and in turn dopamine release (Rossetti et al., 1999). Further, the expression of tyrosine hydroxylase is decreased and the expression of dopamine receptors and transporters are increased (Chatterjee et al., 2014; Sari et al., 2006; Djouma, 2002; Lograno et al., 1993). This suggests a reduction in dopamine synthesis and an increase in dopamine reuptake from the synapse (Weiss & Porrino, 2002; Rothblat et al., 2001). Importantly, the mechanisms underlying dopamine deficits can persist for prolonged periods, increasingly the susceptibility of relapse (Berridge & Robinson, 2016).

Prolonged alcohol use additionally lessens the “liking” component of alcohol (pharmacodynamic tolerance). For example, chronic alcohol exposure reduces the expression of μ and δ opioid receptors and increases the uncoupling of opioid receptors from G_i class of G proteins, their signal transducers, within the nucleus accumbens, cortex and hippocampus – mitigating the effects of alcohol reward (He & Whistler, 2011; Saland et al., 2004; Chen & Lawrence, 2000). Further, the down-regulation of endogenous cannabinoids and their receptors increases GABA signalling in the nucleus accumbens and VTA (Talani & Lovinger, 2015; Melis & Pistis, 2012; González et al., 2002; Basavarajappa & Hungund, 1999; Basavarajappa et al., 1998). It is hypothesised that increased GABA signalling may further inhibit endorphin or dopaminergic responding neurons; limiting the “liking” and “wanting” component of reward. Given the effects of pharmacodynamic tolerance (the down-regulation of opioid and cannabinoid receptors) higher concentrations of alcohol are required to

induce “liking”. This effect is exacerbated owing to pharmacokinetic tolerance (increased rate of alcohol metabolism) further reducing the circulating concentration of alcohol – increasing the amount required to achieve the desired pharmacodynamic effects (Tabakoff et al., 1986; Misra et al., 1971).

Collectively, within-system adaptions to the nucleus accumbens and VTA create a state of heightened “wanting” at the expense of “liking”; altering the rewarding properties of alcohol. These effects are primarily attributable to a reduction in dopaminergic and opioidergic signalling within this pathway (Diana et al., 1996). Therefore, an alcohol-dependent individual is in a state where they are constantly chasing the original hedonic sensation felt by alcohol however, they are unable to achieve it (Berridge & Robinson, 2016).

1.4.4 Anti-reward following chronic alcohol exposure

Prolonged alcohol use also results in the recruitment of anti-reward pathways. Anti-reward is based on a concept that brain systems are in place to limit reward triggered by excessive activity within the reward pathway (B-process) (Koob & Le Moal, 2001). Several anatomical sites mediate the anti-rewarding properties of alcohol such as the hypothalamic-pituitary-adrenal (HPA)-axis, locus coeruleus, the extended amygdala (BNST, central nucleus of the amygdala (CeA) and the nucleus accumbens (Koob & Volkow, 2009). It is important to highlight that these brain regions are also involved in arousal, stress, fear, anxiety and the emotional component of pain (Koob & Le Moal, 2001).

Activation of the brain’s anti-reward pathway has been hypothesized to overcome the chronic presence of alcohol and restore homeostasis. However, in the absence of alcohol, these systems remain active and are hypothesised to produce aversive,

anhedonic, dysphoric and stress-like behaviour (Koob & Le Moal, 2001). For example, corticotrophin-releasing factor (CRF) and neuropeptide Y (NPY) are key stress and anti-stress neuropeptides (respectively) of the brain stress system and the HPA axis (Shekhar et al., 2005). In the absence of alcohol, the expression and release of NPY and CRF in the amygdala are decreased and increased respectively. The subsequent binding of these molecules to their cognate receptors (CRFR1 – 2 and NYPR1 – 5) and activation of intracellular signalling pathways are thought to contribute to the anxiety and anhedonia experienced by alcohol-dependent subjects (Gilpin, 2012; Sommer et al., 2008; Funk et al., 2006; Primeaux et al., 2006; Roy & Pandey, 2002; Zorrilla et al., 2001 Pich et al., 1995). In support of this, mice deficient in CRF-1 receptors or antagonism of CRF-1 or -2 receptors decreases alcohol withdrawal behaviour (Pastor et al., 2011; Huang et al., 2010; Lowery et al., 2010; Wills et al., 2010; Funk & Koob, 2007; Overstreet et al., 2004) and increasing NPY or genetic knock out of NPY receptor 1 and 2 receptors decrease and increase withdrawal-induced anxiety in mice, respectively (Gilpin, 2012; Cippitelli et al., 2011; Bhisikar et al., 2009).

Brain regions involved in anti-reward may also govern aspects of reward following chronic exposure to alcohol. Particular emphasis has been placed upon dopaminergic neurons extend from the nucleus accumbens and BNST to the CeA. Similar to alcohol exposure in the nucleus accumbens, alcohol increases dopamine release in the CeA which assists in mediating long-term motivational behaviours towards alcohol (Koob & Volkow, 2009). However, this behaviour is additionally mediated by opioid, neuropeptide Y and GABAergic systems as antagonising either of these systems in the nucleus accumbens, BNST or CeA can reduce alcohol self-administration (Pleil et al., 2015; Marinelli et al., 2010; Hyytiä & Koob, 1995).

In summary, the initial consumption of alcohol is driven by a complex interaction of "liking" and "wanting" generating alcohol-induced reward. Repeated cycles of alcohol consumption followed by periods of abstinence, results in neuroplastic events recruiting the anti-reward pathway and altering the reward pathway. Collectively, these processes reduce the ability to feel the "liking" component of reward, promote the "wanting" of alcohol and induce feelings of anxiety and anhedonia. This triggers the loss of control over intake associated with dependence (Koob & Le Moal, 2001).

Importantly, many of the mechanistic- and system-based schemas driving the initial consumption of alcohol and dependence were developed in a model devoid of the neuroimmune system. Nevertheless, it is apparent that these theories have served, and continue to serve, as foundational intellectual stepping-stones upon which modern reward and dependence research has been based. Therefore, to progress in our understanding of the mechanisms underpinning initial alcohol consumption and dependence, we need to integrate the emerging importance of the neuroimmune system with the current established models of initial consumption and dependence.

1.5 The Neuroimmune system

The neuroimmune system is an incredibly intricate and diverse system comprised of endogenous and exogenous immune functioning cells and their associated signalling molecules and receptors within the central nervous system (Pacheco et al., 2012). These cells and their immune components are crucial for maintaining homeostasis in the brain and spinal cord (Ousman & Kubes, 2012). Following detection of injury or infection, these cells facilitate the removal and elimination of pathogens and initiate and propagate repair processes (Ransohoff & Brown, 2012; Bailey et al., 2006). As will be alluded to, alcohol manipulates the neuroimmune system creating a state of persistent immune activation.

1.5.1 Cells of the neuroimmune system

Almost all cells within the brain and spinal cord are part of the neuroimmune system. However, the degree to which these cells participate varies substantially (Waisman et al., 2015; Tian et al., 2012). Therefore, this thesis will focus upon three key cell types: neurons, microglia and astrocytes. Together these cells form the tetrapartite synapse an important communication bridge connecting immune cells, neurons and behaviour (Dityatev & Rusakov, 2011; De Leo et al., 2006).

1.5.1.1 Neurons

Neurons were traditionally considered the basic functional unit of the central nervous system. These cells transmit and integrate sensory information in the form of electrical impulses modified by neurotransmitters, neuropeptides and neurotrophic factors to generate cognition and behaviour. From an immunological perspective, neurons were traditionally viewed as bystanders during and proceeding an immune response (Galea et al., 2007). However, it is now recognized that neurons express a broad array of immune molecules and receptors (see for example Leow-Dyke et al., 2012; Gosselin et al., 2005; Viviani et al., 2003) and can perpetuate an inflammatory response via neurogeneic neuroinflammation (peripheral nerve fibres release neuropeptides to trigger a localised inflammatory response) (Xanthos & Sandkühler, 2013) or by modulating neighbouring immune cells via paracrine signalling involving neurotransmitters and cytokines (Becher et al., 2016; Lee, 2013).

1.5.1.2 Microglia

Microglia are macrophage-like cells constituting approximately 10 per cent of the cellular population in the central nervous system (Ransohoff & Brown, 2012). These cells colonise the brain during early embryonic development where they migrate to

their final destination and begin to self-renew (Kierdorf et al., 2013; Ginhoux et al., 2010). During development, the activity and activation state of microglia varies substantially, reflecting the individual timing of each brain regions development (Hanisch, 2013; Schwarz & Bilbo, 2012). As brain development continues from early embryogenesis to adulthood, microglia phagocytise cellular debris and apoptotic cells, assist in cellular differentiation and synaptic migration, elimination and guidance (Choi et al., 2017; Neher et al., 2011; Stellwagen & Malenka, 2006; Bishop et al., 2004). In addition to their role in development, microglia constantly survey their microenvironment by extending and retracting their philophodia searching for perturbations in homeostasis (Nimmerjahn et al., 2005). Further, microglia are in intimate contact with synapses and can clear metabolic waste, modulate synaptic pruning, provide trophic support and regulate basal synaptic transmission (Ribeiro Xavier et al., 2015; Pascual et al., 2012; Paolicelli et al., 2011; Ragozzino et al., 2006; Takahashi et al., 2005).

1.5.1.3 Astrocytes

Astrocytes are the most abundant cell type within the central nervous system (Oberheim et al., 2006). These cells are derived from a specific population of neural progenitor cells and typically emerge towards the end of embryonic development (Tien et al., 2012; Bushong et al., 2004). Astrocytes do not reach functional maturity until the end of adolescence mimicking the slow development of neurons (Bushong et al., 2004). Astrocytes were traditionally classified as supportive, trophic cells owing to their physical proximity to neurons (one astrocyte can contact up to 100,000 and 1,000,000 synapses in the rodent and human brain respectively (Halassa et al., 2007; Bushong et al., 2002; Ogata & Kosaka, 2002)). However, these cells also express a broad array of neurotransmitters, trophic factors and their cognate receptors. Consequently, astrocytes actively participate in neuronal function and development. For example,

they regulate synaptic plasticity, synaptogenesis, synaptic scaling and the regulation of ion and glutamate concentrations within the synapse (Hu et al., 2007; Christopherson et al., 2005; Duan et al., 1999; Berbel & Innocenti, 1988). Astrocytes are also in contact with endothelial cells lining the blood brain barrier and can regulate it's opening and closing (Takano et al., 2005; Kacem et al., 1998).

Microglia and astrocytes (glial cells) are considered the primary immunocompetent cells within the central nervous system (Rivest, 2009; Carpentier et al., 2004). These cells survey their surrounds searching for tissue injury or infection (Ousman & Kubes, 2012; Nimmerjahn et al., 2005). Key to immune surveillance are intercellular and membrane-bound pattern recognition receptors including Nucleotide oligomerisation domain-like (NOD), Retinoic acid-inducible gene 1-like (RIG) and Toll-like receptors (TLR) (Kigerl et al., 2014; Carpentier et al., 2008). These receptors recognise conserved molecular epitopes expressed by dead and stressed host cells (danger-associated molecular patterns (DAMPs)) and exogenous bacteria, viruses and yeasts (pathogen-associated molecular patterns (PAMPs)) (Takeuchi & Akira, 2010). Binding of PAMPs and DAMPs to pattern recognition receptors initiates an inflammatory response from microglia and astrocytes (for example, Suh et al., 2009; Sterka et al., 2005; Olson & Miller, 2004). Immunological activation of these cells changes their morphology, rate of proliferation and gene expression. Morphologically, glial cells change from a ramified (resting) to an amoeboid (activated) state. This is characterised by a retraction of their processes and a rounding of cell body (Ransohoff & Brown, 2012; Sofroniew & Vinters, 2009). In addition, these cells increase the transcription, translation and release of immune mediators including cytokines and proteases (for example, Gorina et al., 2010; Olson & Miller, 2004). Immune mediators aim to eliminate the pathogen and enhance repair. However, prolonged immune activation is detrimental to the surrounding microenvironment as high levels of inflammatory

mediators can; distract glia and neurons from performing their housekeeping functions; degrade the myelin sheath; and induce cell death (Chien et al., 2016; Chatterjee et al., 2013; Sheng et al., 2003).

As the immune response resolves, glia change their phenotypes to one more conducive to repair with increased expression of anti-inflammatory mediators such as interleukin-10 (IL-10) and transforming growth factor β (TGFβ) (Norden et al., 2015; Cherry et al., 2014; Hashioka et al., 2013; Vincent et al., 1997). Once the immune event is resolved, glia can return to basal behaviour or remain in a sensitised, primed state (Bilbo, 2009). The precise mechanism underlying the switch to either a basal or primed state remains disputed. Primed glia appear morphologically active in the absence of an immune stimuli but these cells do not overproduce immune mediators basally (Bilbo, 2009). However, epigenetic processes may have occurred facilitating an increase in immune-related gene transcription but not translation. This ensures a faster, stronger response upon re-exposure to an immune stimulus.

With respect to the inflammatory response, important differences exist between glial cells. For example, microglia are more sensitive and react faster to alterations in the central nervous system as they have a higher expression level of pattern recognition receptors and produce more inflammatory mediators basally (Tian et al., 2012). Astrocytes are thought to be slower to activate and exhibit more control over their activation (Jack et al., 2005; Lee et al., 1993). Furthermore, the expression of pattern recognition receptors and signal transduction pathways differ between these cells (Jack et al., 2005; Hua & Lee, 2000). For example, astrocytes, like neurons do not possess all the different intracellular signalling pathways for Toll-like receptors (Okun et al., 2011). Therefore, activation of this class of receptors on these cells may result in a different signalling outcome than is classically appreciated.

It is important to note glial cells are not the only cells within the central nervous system capable of mounting an immune response. Neurons, endothelial cells, oligodendrocytes, and infiltrating monocytes and T cells are all capable of mounting an immune response to some degree. However, the discussion of these cells is beyond the scope of this thesis. Please refer to Grace et al., (2014) for a recent review discussing the immunological capabilities of these cells.

1.5.2 Immune mediators of the neuroimmune system

The ability of the neuroimmune system to detect and respond appropriately to PAMPs and DAMPs is dependent on a repertoire of immunological receptors, signalling molecules and transcription factors (Kigerl et al., 2014). These factors act in a concerted manner to restore the system back to homeostasis as quickly and efficiently as possible. Discussion of all types of immune mediators and the peripheral immunology is beyond the scope of this thesis and only those pertinent to alcohol, reward and anti-reward will be discussed. An overview of the immune mediators will be provided with specific focus on the central nervous system.

1.5.2.1 Immune signalling molecules and their function within the central nervous system

Cytokines

Cytokines are a diverse class of signalling proteins involved in multiple cellular processes such as immune surveillance, induction of inflammation, cell adhesion, phagocytosis and cell death (Becher et al., 2016). Cytokines belong to one of five families based on structural homology. They include: chemokines, colony-stimulating factors, interleukins (IL), interferons (IFN), transforming growth factors (TGF) and tumour-necrosis factor (TNF) superfamily. Within each family, cytokines are typically

classified as either pro- or anti-inflammatory (Dinarello, 2007). However, this is a drastic oversimplification as the function of cytokines is dependent upon the inducing signal and the responding cell type. Thus, what is considered pro-inflammatory for one cell type may be anti-inflammatory for another (Shachar & Karin, 2013). Cytokines have high affinity for their corresponding receptors. Therefore, only small concentrations of cytokines are required to exert a large biological effect. Consequently, there are several mechanisms in place to limit the effect of these molecules including decoy receptors and regional differences in receptors and intracellular signalling protein expression (Kim, 2010).

All cells of the neuroimmune system express cytokines and their receptors. The response to cytokines is cell specific and often results in non-immunological effects. For example, binding of tumour necrosis factor α (TNFα) to its receptor on microglia or neurons results in the production of reactive oxygen species and endocytosis of $GABA_A$ and AMPA receptors respectively (Olmos & Lladó, 2014; Stellwagen, 2005; Dopp et al., 2002). Further, IL-1β activates MAPKs and CREB in hippocampal neurons to alter synaptic function, but activates NFκB in astrocytes to alter synaptic function and induce the release of inflammatory mediators (Srinivasan, 2004).

Chemokines

Chemokines are a class of cytokines characterised by the presence of three to four conserved cysteine residues. They can be further subdivided based on the N-terminal cysteine's position creating C-X-C, C-C, CX3C and xCL1 families (Groves & Jiang, 2016). Microglia are the main source of chemokines in the central nervous system with the highest expression of chemokine receptors detected on astrocytes and neurons (Banisadr et al., 2005b). Chemokines signal through G protein-coupled receptors to activate signalling pathways causing a broad array of cellular effects (Groves & Jiang,

2016). The key feature of chemokines is their role in chemotaxis. However, these molecules also play important roles in the initiation of haematopoiesis, adaptive immune responses and immune surveillance (Turner et al., 2014). Outside of their immunological role, chemokines influence neurotransmission and neuroplasticity. For example, application of CCL2 (MCP-1), increases the firing of dopaminergic neurons via closure of background potassium channels which in turn increases circling behaviour in rodents (Guyon et al., 2009).

Danger associated molecular patterns (DAMPS)

Endogenous danger signals are a diverse array of molecules and proteins predominately released from damaged or dying cells (Bianchi, 2006). However, these molecules are also secreted under normal, basal conditions (Reina et al., 2012). They range from purine metabolites such as adenosine triphosphate (ATP); DNA and RNA; protein chaperones (heat shock proteins) and chromatin binding proteins (high mobility box group 1) (Gadani et al., 2015). In addition to their traditional roles, for example, increasing the stability of proteins or DNA, these molecules act as signalling factors indicating to the central and peripheral immune system that homeostasis has been altered (Yang et al., 2015; Osmanov et al., 2013). This in turn initiates an inflammatory response.

1.5.2.2 Immune transcription factors

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)

The NFκB family is comprised of five related transcription factors (p50, p52, RelA (p65), c-Rel and RelB) which share a N-terminal DNA binding/dimerisation domain termed the Rel homology domain (S. Ghosh et al., 1998). The Rel homology domain enables the homo- or heterodimerisation of NFκB transcription factors and facilitates DNA binding to promoter, enhancer or repressor regions within the genome (Bonizzi

& Karin, 2004). The homodimers of RelA, c-Rel and RelB are considered activators facilitating transcription via their C-terminal transcriptional activation domain. By contrast, p50 and p52 lack this domain and thus function as repressors unless bound to RelA, c-Rel or RelB (Chen & Ghosh, 1999; Chen et al., 1998; Ghosh et al., 1995; Mercurio et al., 1993; Toledano et al., 1993).

Under basal conditions, NFκB is sequestered in the cytoplasm by inhibitor of kappa beta (IκB) proteins (Bonizzi & Karin, 2004). Upon activation by immunological and non-immunological signals (such as a burst of action potentials), NFκB is translocated to the nucleus inducing or repressing the expression of inflammatory-related genes including cytokines, proteases and adhesion molecules (Lawrence, 2009). Interestingly the activation of NFκB in neuronal cells can regulate synaptic plasticity, neurite outgrowth and the expression of neurotransmitters, neuropeptide or neurotrophic factors and their receptors (Yirmiya & Goshen, 2011). Persistent activation of NFκB resulting from ongoing inflammation can induce epigenetic processes that modify the folding of DNA and the remodelling of histones - facilitating either activation or repression of NFκB–targeted genes (Vento-Tormo et al., 2014; Gazzar et al., 2007).

Activator protein-1 (AP-1)

AP-1 represents a functional heteromer consisting of proteins belonging to the Fos, Jun, Maf and ATF families in the central nervous system (Karin et al., 1997). AP-1 contains two functional domains, a leucine zipper and a basic region that govern the protein dimerization and the DNA binding respectively (Chinenov & Kerppola, 2001). AP-1 is induced by a variety of immunological and non-immunological stimuli resulting in its translocation from the cytoplasm to the nucleus where it binds to specific regions of DNA regulating the expression of genes pertaining to a range of cellular process (for

example, Casals-Casas et al., 2009; Cavigelli et al., 1995; Abraham et al., 1991). All cells of the neuroimmune system express AP-1, with c-Jun/Fos the most commonly expressed heteromer (Shaulian & Karin, 2002). However, the composition of these heteromers varies according to cell type (Herdegen & Waetzig, 2001). Consequently, AP-1 has diverse roles including the regulation of immune-related processes, synaptic plasticity and cellular differentiation (Herdegen & Waetzig, 2001).

1.5.2.3 Immune receptors

Pattern recognition receptors

Within the central nervous system, one mechanism by which the neuroimmune system detects PAMPs and DAMPs is through pattern recognition receptors (Kigerl et al., 2014). Pattern recognition receptors include: C-type lectin receptors, RNA helicases, NOD-like receptors, RIG-I-like receptors and TLRs (Carpentier et al., 2008; Takeuchi & Akira, 2010). Each pattern recognition receptor recognises unique motifs expressed by bacteria, viruses, fungi and host cells, initiating intracellular signalling cascades culminating in the induction of inflammatory-related genes (Takeuchi & Akira, 2010). These receptors are considered the first line of defence against foreign entities and can subsequently act to recruit more specialised immunological cells (Takeuchi & Akira, 2010). In addition to the detection of pathogens and danger signals, it is becoming increasingly apparent that a specific class of pattern recognition receptors (TLRs) can detect and initiate inflammatory responses towards drugs of abuse such as opioids, cocaine, and amphetamines and alcohol (Bachtell et al., 2015).

Toll-like receptors

Toll-like receptors are a family of pattern recognition receptors that are expressed throughout cells of the neuroimmune system. Toll-like receptors belong to the Toll/interleukin-1 receptor superfamily, and are a type 1 integral membrane

glycoprotein (Akira & Takeda, 2004). These receptors consist of three key domains: N-terminal (extracellular or extra-endosomal), transmembrane and C-terminal (intracellular or intra-endosomal) domains (O'Neill & Bowie, 2007). The N-terminal domain consists of 16 – 28 leucine-rich repeats that form a horseshoe-like structure (Bell et al., 2003). It is hypothesised that the concave surface created by the leucine-rich repeats are directly involved in the recognition of pathogens. The C-terminal domain (the Toll/IL-1R (TIR) domain) binds adapter molecules regulating signal transduction (Botos et al., 2011). For signal transduction to occur, monomeric TLRs must dimerise. Most TLRs exist as homodimers. However, TLR2 can exist as heterodimers with TLR1, 4, 6 and 10 (Oosting et al., 2014; Fernandez-Lizarbe et al., 2013; Jin et al., 2007; Kang et al., 2009).

Following dimerization, adapter proteins are recruited to the TIR domain and are phosphorylated to induce their activation. TIR domain-containing adaptor protein (TIRAP) and TLR adaptor molecule (TRAM) are the main adapter proteins that bind to the TIR domain. These proteins subsequently recruit the myeloid differentiation primary response protein 88 (MyD88) and the TIR-domain-containing adapter-inducing interferon-β (TRIF) proteins respectively (Akira & Takeda, 2004; Beutler & Rietschel, 2003). Activation of TIRAP-MyD88 or TRAM-TRIF results in divergent signalling outcomes. For example, activation of the MyD88 pathway results in the phosphorylation of mitogen-associated protein kinases (MAPKs) p38, c-Jun, STAT and JNK, the formation and activation of AP-1, and early phase of NFκB (Sakai et al., 2017; Horng et al., 2002; Fitzgerald et al., 2001; Burns et al., 1998). This results in the transcription of genes relating to classical pro-inflammatory cytokines such as IL-1β, TNFα, and CCL2 and reactive oxygen-inducing enzymes such as COX-2 and iNOS (Sakai et al., 2017; Fitzgerald et al., 2001). In contrast, signalling via the TRAM-TRIF pathway results in the downstream activation of interferon response factor (IRF) 3 and

7, which in turn regulate the expression of CCL5 and type-1 interferons such as interferon-β (Sakai et al., 2017; Fitzgerald et al., 2003; Kawai et al., 2001). The TRAM-TRIF pathway additionally results in the late phase activation of NFκB that transcribes anti-inflammatory genes such as IL-10 (Fitzgerald et al., 2003). Collectively, these inflammatory mediators facilitate the removal of the inflammatory stimuli and initiate wound healing. TLR4 is the only TLR that engages both signalling pathways; TLRs 1, 2, 5, 6, 7, 8, 9 and 10 signal via MyD88 where as TLR3 signals via TRIF (Akira & Takeda, 2004).

1.5.2.4 Investigating TLR4 signalling in the CNS

TLR4 is unique among TLRs as it signals through both MyD88 and TRIF pathways, can reside on the cell surface or within endosomes and is widely expressed throughout the central nervous system (Buchanan et al., 2010; Kielian, 2006). Under basal conditions, TLR4 is predominately expressed on microglia, with low levels of expression detected on neurons and astrocytes (Bsibsi et al., 2002). It is unclear however, if astrocytes and neurons express MyD88, TRIF and other downstream signalling molecules necessary for signal transduction as studies conflict in regards to reporting the expression of these proteins (Okun et al., 2010; Rolls et al., 2007). Further, the expression of TLR4's co-receptors differ between neuroimmune cells. For example, neurons and glia express a combination of MD1, MD2 and CD14. Unlike neurons, MD-1 in neuroimmune cells is bound to exclusively to its co-receptor RP105 and not TLR4 (Okun et al., 2011; Divanovic et al., 2005). Therefore, the precise function of TLR4 may differ to those expressed on microglia. Lastly, while neurons and astrocytes are capable of inducing prototypical cytokines used as markers of MyD88 and TRIF activation, it is presently thought that these cells may not express proteins pertinent to the TLR4 pathway. Therefore it is unclear whether these cytokines are

produced due to TLR4 activation or whether another immune pathway caused their expression (Okun et al., 2011; Préhaud et al., 2005).

Within the central nervous system, TLR4 plays an important part in host immunity but also has crucial non-immunological roles such as the regulation of cellular development, differentiation and migration of neuronal precursor cells (Okun, 2014; Buchanan et al., 2010). However, discerning the molecular and behavioural consequences of TLR4 is difficult. For example, genetic knockout of TLR4 can result in compensatory mechanisms such as an upregulation of TLR2: confounding any conclusions (Okun et al., 2010; Rolls et al., 2007). Further, traditional pharmacological agonists and antagonists such as lipopolysaccharide (LPS), a cell wall component of gram negative bacteria, and LPS:RS, LPS from *Rhodobacter sphaeroides*, respectively, cannot cross the blood brain barrier thereby limiting the study of TLR4 respectively (Banks & Robinson, 2010). In spite of this, a large number of studies still infer central TLR4 activation via peripheral administration of LPS. At present there are three pharmacological antagonists which readily cross the blood brain barrier to inhibit TLR4: TAK242, T5432126 and (+)-Naltrexone.

TAK 242 blocks the interaction between TLR4 and its adapter proteins TIRAP and TRAM stopping the activation of the MyD88 and TRIF pathways (Matsunaga et al., 2011). By preventing this interaction, TAK242 inhibits the activation NFκB and IRF3 and subsequent release of inflammatory cytokines (Li et al., 2006). However, while this drug has provided insight into TLR4's function (Wang et al., 2013), clinical trials using this drug for the treatment of sepsis have failed (Rice et al., 2010). T5342126 inhibits the interaction between TLR4 and MD2 preventing the subsequent rise in LPS-induced IL-6, IL-8, TNFα and Akt-1. Unfortunately, the relatively fast metabolism limits the efficacy of this drug (Chavez et al., 2011). (+)-Naltrexone is an enantiomer of the μ

opioid receptor antagonist (-)-Naltrexone. Both isomers are TLR4 antagonists, however, the spatial rotation of a hydroxyl and amine group prevents the (+)-isomer from binding to μ opioid receptors (Selfridge et al., 2014; 2015). Interestingly, (+)-Naltrexone is currently thought to be a biased TLR4-antagonist preferentially attenuating the TRIF pathway. In BV2 cells, a microglia-like cell line, (+)-Naltrexone reduced LPS-induced NO and IFNβ production an effect attributable to a decrease in IRF3. This drug did not attenuate IL-1β production, NFκB, p38 or JNK (Wang et al., 2016). *In vivo*, this drug prevented LPS induced changes to microglial morphology and can reverse chronic constriction injury-induced pain and cocaine and opioid self-administration (Northcutt et al., 2015; Hutchinson et al., 2012; 2008;). The precise mechanism of (+)-Naltrexone's biased antagonism remains to be fully elucidated. There are no specific MyD88 pharmacological inhibitors that can readily cross the blood brain barrier.

Studies using (+)-Naltrexone have revealed an interesting interaction between TLR4 and modern pharmaceuticals. Opioid receptor agonists and antagonists potentiate and inhibit TLR4 signalling (Hutchinson et al., 2010a; 2010b). This highlights that TLR4 serendipitously interacts with a third class of molecular patterns, xenobiotic-associated molecular patterns (XAMPs) (Hutchinson et al., 2011; Buchanan et al., 2010). XAMPs represent a broad range of pharmaceuticals such as opioids, cocaine, methamphetamines and alcohol. These pharmaceuticals share one common characteristic: they are all drugs of abuse.

Interestingly, TLR4 is not activated by other n-chain alcohols including methanol, propanol or butanol nor ethanol's short-lived metabolites acetate or acetaldehyde (Blanco et al., 2005; Jacobsen unpublished). However, n-chain alcohols have immune altering capabilities with the degree of immunomodulation relating to the length of the

hydrocarbon backbone (Carigan et al., 2013). All n-chain alcohols (aside from methanol) dysregulate the peripheral immune system with attenuated cytokine production and immune-related transcription factor translocation (Carignan et al. 2013; Hoyt et al. 2013). By contrast methanol and ethyl-glucuronide, a minor, long-lasting metabolite of ethanol, augment the release of inflammatory cytokines and increase the expression and activity of immune related transcription factors (Lewis et al., 2013; Desy et al. 2010). Whether n-chain alcohols or ethanol's minor metabolites alter the neuroimmune system remain to be determined.

1.6 Parallels between an immune response to a lipopolysaccharide and alcohol within the central nervous system

Within the central nervous system, an alcohol-induced TLR4 signal closely resembles that of an LPS-induced TLR4 signal. The extent to which TLR4 is engaged following LPS or alcohol exposure however is dependent upon: age, anatomical location (central vs. peripheral), concentration, cell type, extracellular protein co-factors and duration of exposure (Doremus-Fitzwater et al., 2015; Pascual-Lucas et al., 2014; Alfonso-Loeches et al., 2010; Mandrekar et al., 2009). The consequences of alcohol- and LPS-induced TLR4 signalling on the neuroimmune system may be broken down into morphological and functional changes, the effects on receptors and signal transduction pathways and immune-related molecules.

1.6.1 Morphological and functional changes

1.6.1.1 Acute exposure to alcohol or LPS

Microglia

Exposure to alcohol or LPS activates microglia in a time- and dose-dependent manner *in vitro* and *in vivo*. For example, a single dose of LPS alters the morphology of microglia from ramified to amoeboid, and increases the expression of microglial

markers Iba-1 and CD11b *in vivo*. Functional markers of microglia activation such as ED-1, a phagocytic marker, are additionally increased (Hoogland et al., 2015). The response to LPS is rapid, occurring three hours post exposure and persists for up to a week (Hoogland et al., 2015). On the contrary, four doses of alcohol are required to change microglia from a ramified to amoeboid state and increase cellular expression of Iba-1 and CD11b *in vivo* (Marshall et al., 2013; McClain et al., 2011). However, functional markers are unaffected (Marshall et al., 2013). Despite the lack of change in functional markers when using histological approaches, *in vitro* experiments suggest acute alcohol (and LPS) increase the phagocytic abilities of microglia (Fernandez-Lizarbe et al., 2009). Collectively, this suggests alcohol exposure results in a similar immune response to a classical immunogen.

Astrocytes

Akin to microglia, astrocytes are activated by acute exposure to LPS or alcohol. Both compounds increase the expression of GFAP, an astrocyte marker, and alter their morphology to a more amoeboid-like shape. Unlike microglia, the response towards LPS or alcohol is not rapid, typically beginning approximately 12 h post exposure (Pascual-Lucas et al., 2014; Blanco et al., 2005). LPS and alcohol transiently interfere with the ability of astrocytes to perform housekeeping functions such as maintaining glutamate homeostasis (Ayers-Ringler et al., 2016; Tilleux & Hermans, 2007). Collectively, alcohol, like LPS, alters the phenotype of astrocytes to one that is immunologically more reactive at the expense of basal, homeostatic functions.

Neurons

Research has primarily focused on the susceptibility of neurons towards the neurotoxic effects of LPS and alcohol, with few studies considering whether these cells alter their phenotype to one more in line with to an immune response.

In general, acute exposure to LPS results in cell death. Interestingly, cell death occurs in a regional specific manner with the dopaminergic cells innervating the substantia nigra, particularly sensitive to the immune consequences of LPS (Zhou et al., 2012; Jeong et al., 2010). By contrast, neurons in the hippocampus or cortex are more robust and are unaffected by a single exposure to systemic LPS (Kim et al., 2000). This effect has been attributed to the number of glial cells within these brain regions; the substantia nigra and cortex exhibit the highest and lowest number respectively (Lehnardt et al., 2003). Notably, dopaminergic cells that survive LPS exposure exhibit transient alterations to their functionality. For example, tyrosine hydroxylase expression and dopamine re-uptake are reduced (Kim et al., 2000). In contrast, an acute dose of alcohol does not result in neurodegeneration *in vivo*. *In vitro*, co-culture of neurons with glial cells results in neuronal cell death following 24 h of alcohol exposure (Fernandez-Lizarbe et al., 2009). However, caution must be used when interpreting *in vitro* results, as the concentration of alcohol employed typically exceeds that found *in vivo* and LPS does not enter the brain. Therefore, unlike exposure to LPS, acute alcohol does not result in high levels of cell death and long-term alterations to cellular functionality. This suggest the immune altering effects of acute alcohol to neurons is low.

1.6.1.2 Chronic exposure to alcohol or LPS

Microglia

Chronic exposure to LPS or alcohol results in exaggerated and prolonged activation of microglia. Chronic administration of these compounds intensifies Iba-1, CD11b and CD68 staining, causes a retention of an amoeboid morphology in the absence of the compound and produces irregular cell shapes and sizes (Hoogland et al., 2015; Pascual et al., 2011). Activation markers such as ED-1 are persistently elevated

following chronic LPS exposure (Hoogland et al., 2015). However, it is unclear whether this marker is altered following chronic alcohol exposure. Chronic LPS exposure results in widespread changes to glial morphology throughout the brain. By contrast the response to alcohol is area specific. For example, chronic alcohol administration alters microglial staining in the hippocampus, ventral tegmental area, cingulate cortex and midbrain but not the amygdala (He & Crews, 2008). Just like acute exposure, chronic exposure to alcohol shifts microglia to an inflammatory phenotype. This phenotype is long-lasting and persists in the absence of the drug (McClain et al., 2011).

Astrocytes

Chronic administration of LPS and alcohol results in long-term alterations to astrocyte morphology such as increased GFAP expression and a retraction of their processes creating an amoeboid-like shape (Adermark, 2015; Bull et al., 2015; Hauss-Wegrzyniak et al., 1998; Ortiz et al., 1995). These effects persist for prolonged periods (days – weeks) before returning to baseline (Adermark, 2015; Zamanian et al., 2012). Chronic stimulation with either LPS or alcohol inhibits the ability of these cells to perform housekeeping functions such as maintaining glutamate homeostasis and synaptogenesis; and can result in astrocyte cell death (Ayers-Ringler et al., 2016; Shen et al., 2016; Bull et al., 2015; Sharma et al., 2015). Therefore, chronic exposure induces immunological activation of astrocytes an effect that persists even after the initial stimulus is removed.

Neurons

Chronic administration of LPS or alcohol results in high levels of neurodegeneration (Alfonso-Loeches et al., 2013; 2010). The prefrontal cortex is particularly sensitive to the effects of chronic LPS and alcohol, exhibiting the greatest loss in cell number (Vetreno et al. 2015; de Pablos et al., 2006; Fan et al., 2005). Furthermore, chronic

immune stimulation of neurons, whether by LPS or alcohol alters the functional properties of these cells. For example, chronic administration of LPS or alcohol alters membrane resistance, action potential threshold, slows the frequency of action potentials (Bajo et al., 2014; Hellstrom et al., 2005), increases the aggregation of poly-ubquitinated proteins, down-regulates the autophagy pathway (Pla et al., 2016; 2014) and reduces the myelination of neurons (Alfonso-Loeches et al., 2010; Hellstrom et al., 2005).

1.6.1.3 Priming following exposure to alcohol or LPS

Following administration of LPS or alcohol, microglia often do not return to their basal state. Rather, these cells can remain in a 'primed' state of partial activation (Bilbo, 2009). For example, administration of alcohol during adolescence leads to long-lasting increases in microglial Iba-1 or CD11b expression and alters microglia morphologically to a more amoeboid shape in adulthood (Cruz et al., 2017; Norden & Godbout, 2013; McClain et al., 2011). It is hypothesised that these cells do not exhibit basal increases in immune mediators and receptors. Subsequent immunological activation whether by the original or new stimuli (cross-sensitisation) results in an exaggerated inflammatory response. There is some evidence to suggest astrocytes may also exist in a primed state (Hennessy et al., 2015; Norden & Godbout, 2013). However, it is unclear whether this process is due to ageing or can occur at any ontological period. It is presently unclear whether neurons exhibit a priming effect following exposure to LPS or alcohol.

1.6.2 Receptor signalling and alterations to signal transduction pathways

1.6.2.1 Acute exposure to alcohol or LPS

Receptors

Acute LPS and alcohol facilitate the translocation of TLR4 and TLR2 in to lipid rafts within the plasma membrane (Fernandez-Lizarbe et al., 2013). In brief, lipid rafts are

sub-domains of the plasma membrane and serve as signalling platforms enabling the co-localisation of specific proteins expediting their interaction to increase signalling transduction (Katagiri et al., 2001). Following stimulation with LPS or alcohol, TLR4 and TLR2 are rapidly translocated to caveloae-enriched lipid rafts, promoting the recruitment of downstream adaptor molecules onto the cytoplasmic side of these microdomains. The raft is subsequently endocytosed and trafficked to the endoplasmic reticulum, golgi and the nucleus in astrocytes and microglia where downstream signalling molecules can exert their biological function (Pascual-Lucas et al., 2014; Fernandez-Lizarbe et al., 2013). Thus, acute stimulation of LPS or alcohol results in a rapid decrease in the surface expression of TLR4 owing to its internalisation. Interestingly, higher concentrations of alcohol but not LPS are known to disrupt the lipid membrane resulting in a loss of lipid rafts (Fernandez-Lizarbe et al., 2013). LPS and alcohol's effect on TLR4 signalling have been characterised using astrocytes and microglial cells both *in vitro* and *in vivo* (Pascual-Lucas et al., 2014; Fernandez-Lizarbe et al., 2013). However, the effect of alcohol or LPS on neuronal TLR4 is presently unknown.

At the gene-level, acute administration of alcohol increases the mRNA expression of multiple TLRs including TLR2, 3, 4 and 7 (Crews et al., 2017). These experiments have been conducted in brain homogenates. Therefore, the cell type underlying the gene elevations is unknown. By contrast, studies have shown LPS causes an increase and decrease in the expression of TLRs mRNA in astrocytes and microglia respectively (Marinelli et al., 2015).

Signalling pathway

LPS and alcohol trigger the translocation, recruitment and phosphorylation of early TLR4 signalling molecules MyD88, TRAF-6 and IRAK from the plasma membrane to

TLR4-rich lipid rafts within microglia and astrocytes (Fernandez-Lizarbe et al., 2013; Blanco et al., 2008). Recruitment of these signalling molecules occurs rapidly (within 10 mins) and is short-lived – resolving within an hour. Following internalisation and activation of adaptor molecules, alcohol and LPS induce the phosphorylation of Fos and MAPKs: ERK, p38 and JNK. The phosphorylation of these proteins peak at 30 mins and decline 3 to 24 h post exposure (Fernandez-Lizarbe et al., 2013; Blanco et al., 2008). In contrast, mixed hippocampal cells isolated from mice demonstrated no increase in JNK, ERK or p38 when stimulated with 50mM alcohol for 30 min (Wu et al., 2012) suggesting this response may be specific to some but not all cells of the neuroimmune system.

The hallmark of TLR4-MyD88 signalling is the downstream activation of NFκB and AP-1. Alcohol and LPS induce the phosphorylation and translocation of the p65 subunit of NFκB to the nucleus in microglia and astrocytes (Fernandez-Lizarbe et al., 2009; Blanco et al., 2005). Further, the DNA binding activity of NFκB is increased following alcohol and LPS exposure indicating activated gene transcription (Crews et al., 2006; Heese et al., 1998). Microglia or astrocytes isolated from mice deficient in TLR4 or inhibiting TLR4 activation via antibodies or siRNA inhibits the recruitment of adapter proteins, activation of intracellular signalling pathways and the translocation of transcription factors to the nucleus (Fernandez-Lizarbe et al., 2013; Alfonso-Loeches et al., 2010; Blanco et al., 2005).

In addition to the MyD88 dependent pathway, alcohol and LPS activate the TRIF pathway in microglia (Regen et al., 2011). This process is not well characterised *in vivo* however. Alcohol and LPS significantly increase the formation and phosphorylation of IRF-3 dimers approximately 3 h post exposure. This coincides with increases in STAT-1 protein and *Ifnb* and *Ip10* mRNA levels (Lawrimore et al., 2017; Fernandez-Lizarbe

et al., 2013; Tarassishin et al., 2011; Kozela et al., 2010; Qin et al., 2005). It is presently unclear why the onset of TRIF signalling is slower than that of MyD88; whether late phase NFκB is activated; and the cell or signalling location of TRIF following alcohol and LPS exposure in microglia.

It is important to note that the temporal dynamics of the response towards alcohol and LPS is unique to each cell type; with microglia and astrocytes exhibiting differences in terms of the expression and activation kinetics of MAPKs (Norden et al., 2015; Fernandez-Lizarbe et al., 2009; Blanco et al., 2005) with microglia exhibiting faster and more robust induction of MAPKs compared to astrocytes. Furthermore, the recruitment of TLR4's signalling molecules is significantly lower for alcohol than LPS (Fernandez-Lizarbe et al., 2009; Blanco et al., 2008) and therefore, the overall systems response towards the two stimuli will differ. This furthers the idea that activating a receptor with different ligands is not going to give an identical response. The molecular response is dependent upon the binding affinity and binding site of the ligand. These variables influence the conformation of the receptor, impacting which signalling proteins are recruited and the subsequent biological outcome.

1.6.2.2 Chronic exposure to alcohol or LPS

Unlike the acute effects of alcohol or LPS, long-term exposure to these compounds results in pharmacodynamic alterations influencing receptor-mediated signalling events in neuroimmune cells. This in turn impacts molecular and behavioural outcomes attributed to each compound.

Receptors

Chronic administration of LPS (greater than two doses or 18 h of exposure) reduces or does not alter surface expression of TLR4 (Cuschieri et al., 2006; Nomura et al.,

2000). There are however, notable reductions of TLR4 dimers in lipid rafts suggesting the ability to induce transduction is impaired. Chronic administration of alcohol increases the expression of *TLR4* mRNA and protein on astrocytes, microglia and neurons (Vetreno & Crews, 2012). Interestingly, unlike previous studies demonstrating the highest level of TLR4 expression on microglia (Bsibsi et al., 2004), in this study the highest expression of TLR4 was found in neurons rather than glia. It is presently unclear whether the signal transduction properties of TLR4 is altered following chronic alcohol exposure.

Signalling pathway

LPS tolerance is a phenomenon in which chronic administration of LPS results in a diminished immune response. Interestingly, the tolerance induced by LPS results in heterologous desensitisation toward related inflammatory stimuli such as TNFα but not IFNβ. This indicates that LPS tolerance predominately modifies the MyD88 but not TRIF pathways. While LPS tolerance has been demonstrated in microglia following chronic LPS administration (Chu et al., 2016), the underlying mechanisms are unknown. In the periphery however, the effects of LPS tolerance are well characterised. Given that microglia are hypothesised to be of myeloid-origin (Ginhoux et al., 2010), it is possible that some of the mechanisms underlying peripheral LPS-tolerance are conserved in this cell type. In the periphery, there are multiple mechanisms underlying LPS tolerance including; reduced TLR4-MyD88 interaction (as inferred by immunoprecipitation), decreased IRAK, p38, ERK1/2 and JNK expression and upregulation of negative regulatory proteins such as IRAK-M (see Seeley & Ghosh, 2017 for review). Tolerance is also associated with changes in the subunit composition of NFκB: the expression of the p65-p50 heteromer is decreased and the p50 homodimer, a transcriptional repressor, expression is increased. Further, histone modification, nucleosome remodelling, miRNA binding to promoter regions and DNA

methylation are also modified repressing the transcription of inflammatory-related genes (Chen et al., 2009; Gazzar et al., 2007).

The effects of chronic alcohol administration on TLR4s downstream signalling pathway are not well characterised. Chronic administration of alcohol increases the expression of MyD88, MAPKs and NFκB and alters epigenetic processes *in vivo* (Montesinos et al., 2016; Pascual et al., 2011). This suggests a lack of TLR4 tolerance towards alcohol (Alfonso-Loeches et al., 2010). No study has conclusively demonstrated that the TRIF pathway is activated following chronic alcohol exposure *in vivo*. However, preliminary *in vitro* evidence indicates 24 h of alcohol exposure increases IRF3 expression in microglia- and neuronal- like cells (Lawrimore et al., 2017). Future studies are required to fully characterise the MyD88 or TRIF response to chronic alcohol. This is of particular importance as TLR4 acting compounds can potentially exhibit preferential augmentation or attenuation MyD88 or TRIF pathways (biased ligands) (Wang et al., 2016).

1.6.2.3 Priming following alcohol or LPS

Receptors

The effects of alcohol-induced priming on TLR4 expression have predominately been characterised in studies assessing the effects of adolescent binge drinking and later life behaviours. Adolescent alcohol exposure results in a persistent upregulation of *TLR4*, *TLR3*, *TLR2* and *RAGE* mRNA through adulthood (Vetreno & Crews, 2012). Similarly, morphine, another XAMP and TLR4 agonist, induces the persistent upregulation of microglial *TLR4* mRNA in the nucleus accumbens (Schwarz & Bilbo, 2013). In contrast, a persistent upregulation of *TLR4* mRNA or protein is not observed following LPS exposure (Cardoso et al., 2015). Interestingly, in studies designed to examine the long-term effects of sepsis, LPS results in prolonged increases in TLR4's,

endogenous agonist HMGB1, within the central nervous system (Chavan et al., 2012). This indicates while LPS may not directly increase TLR4 expression, there is an ongoing sensitisation event which may alter immune activation.

Signalling pathway

At present, no study has examined whether acute exposure to alcohol or LPS during adolescence or adulthood results in a persistent upregulation of TLR4 signalling pathway proteins (MyD88 or TRIF) later in life.

1.6.3 Expression of immune proteins

1.6.3.1 Acute exposure to alcohol or LPS

The cytokine response following LPS and alcohol exposure is dependent upon cell-type, duration of exposure and the age of the animal. *In vitro* acute LPS increases IL-6, IL-1β, TNFα, CCL2 and IL-10 but not TGFβ protein expression from microglial cells. The increase occurs within 2 h, and persists for, 2 and 12 h (Norden et al., 2015). By contrast, astrocytes exhibit a decreased and slower inflammatory response towards LPS (12 – 24 h post exposure), with elevations in TNFα, CCL2 and TGFβ but not IL-6, IL-1β or IL-10 (Norden et al., 2015). Further *in vitro* experiments demonstrate that acute alcohol increases *Il1b*, *Il6* and *Ip10* mRNA from astrocytes and microglia (Pascual-Lucas et al., 2014; Boyadjieva & Sarkar, 2010). It is unclear whether the expression of other cytokines differ following alcohol exposure in astrocytes or microglia. Again, caution must be used when interpreting *in vitro* results, as alcohol concentrations typically exceed those in an *in vivo* setting, and as a result potentially exacerbate an alcohol mediated TLR4 signal.

In vivo, a single dose of LPS increases the protein and mRNA levels of pro-inflammatory cytokines and type one interferons (Püntener et al., 2012; Qin et al.,

2008). A single dose of alcohol increases the mRNA, but not protein of classical pro-inflammatory, interferon and anti-inflammatory products (Qin et al., 2008). In addition, the expression profile differs from LPS. For example, a single dose of LPS increased the mRNA expression of *Il1b*, *Il10*, *Ccl2*, *Tnfa*, *Cox-2* and *gp91^{phox}* and the protein expression of IL-1β, CCL2 and TNFα in the brain. By contrast, a single dose of alcohol increased the mRNA of *Tnfa*, *Ccl2* and *Cox-2* but not *Il1b*, *Il10* or *gp91^{phox}*. Further, the protein concentration of IL-1β, TNFα or CCL2 was unaltered (Qin et al., 2008). Four doses of alcohol is sufficient to induce protein change indicating the immunological stimulatory effects of alcohol are not as pronounced compared to LPS (Qin et al., 2008). Furthermore, acute administration of LPS increases the expression of IFNβ from neurons (Lynch et al., 2004; Gahring et al., 1996). However, conjecture remains whether these cells produce IFNβ from a traditional manner given these cells are thought to lack certain TLR4 signalling pathway proteins (Okun et al., 2011). It is further unclear whether acute alcohol alters cytokine production from neurons.

Interestingly, the age of the mouse also influences the neuroimmune response towards alcohol and LPS. For example, acute LPS and alcohol induces a reduced IL-6, CCL2 and TNFα response in adolescent mice compared to adults (Doremus-Fitzwater et al., 2015; Kane et al., 2013) suggesting that adolescent mice are some-what protected against the effects of LPS and alcohol compared to adult mice.

1.6.3.2 Chronic exposure to alcohol or LPS

Owing to the effects of endotoxin tolerance, chronic exposure to LPS reduces the expression of classical pro-inflammatory molecules but not type 1 interferons (Gazzar et al., 2007). By contrast, chronic administration of alcohol increases the mRNA and protein of pro-inflammatory and type-1 interferon's, with a reduction in anti-

inflammatory proteins (Pascual et al., 2015). However, it is unclear whether the inflammatory response differs between cell types.

1.6.3.3 Priming following alcohol or LPS exposure

The priming effect of LPS and alcohol differs. Acute but not chronic LPS induces a priming response. For example, postnatal administration of LPS leads to persistent upregulation of cytokines in the hippocampus of mice - an effect that persists until adulthood (Musaelyan et al., 2014). Furthermore, a single dose of LPS caused a persistent elevation in TNFα for ten months (Qin et al., 2007). However, acute alcohol does not induce a priming response. For example, four days of alcohol exposure did not result in a persistent upregulation of IL-6 or TNFα protein in the hippocampus or entohiroinal cortex of adult mice (Marshall et al., 2013). Similarly, an adolescent alcohol binge did not result in long-lasting cytokine increases at the protein level (Zhao et al., 2013; McClain et al., 2011). Despite an absence of inflammatory cytokines, HMGB1 remains persistently elevated in adulthood following chronic alcohol exposure during adolescence suggesting a degree of immune-sensitisation (Montesinos et al., 2016; 2015). The differences between LPS and alcohol may be due to the nature of the inflammatory response. Acute alcohol reflects a low transient rise in immune mediators. By contrast, chronic alcohol increases the expression and release of immune mediators similar to an acute LPS-induced immune response. The severity of the immune response cannot be readily overcome with anti-inflammatory feedback mechanisms, resulting in long lasting (priming) changes to inflammatory gene transcription. However, chronic LPS alerts an immunological safety threshold. Regulatory mechanisms are activated in an attempt to limit inflammation and promoting the survival of the individual, thus preventing TLR4-based priming (Chu et al., 2016).

Overall, acute and chronic alcohol and acute but not chronic LPS induce TLR4 signalling resulting in an increase in inflammatory-related transcription factors and molecules. While both stimuli initiate and perpetuate an inflammatory response as indicated in the aforementioned section (Table 1), there are crucial differences between the two that lead to fundamentally different behavioural effects. For example:

1. The initial immune response towards LPS is significantly greater compared to alcohol. A single dose of LPS results in a profound upregulation and release of inflammatory mediators within the central nervous system. By contrast a single dose of alcohol results in a moderate increase in mRNA of some but not all immune genes. Importantly, protein level is not increased following a single dose of alcohol. This suggests alcohol is not as strong an immunogen as LPS; rather alcohol creates a sub-inflammatory response (Qin et al., 2008).
2. Acute administration of LPS results in cellular and molecular changes throughout most brain regions (Qin et al., 2007). Acute alcohol, results in cellular and molecular changes within discreet brain regions associated with reward and anti-reward (He & Crews, 2008).
3. Chronic administration of LPS results in tolerance (Gazzar et al., 2007). Owing to the comparatively lower level of immune system engagement, it is unlikely alcohol has crossed the immunological threshold for inducing tolerance.
4. Unlike LPS, additional TLRs may be involved in the response towards alcohol such as TLR2 and TLR7 (Coleman et al., 2017; Pascual et al., 2015).
5. While both LPS and alcohol promote the clustering of TLR4 on lipid rafts (Fernandez-lizarbe et al., 2009) the mechanism underlying alcohol-induced TLR4 signalling is vastly different. LPS directly interacts with LPS-binding protein, CD14 and MD2 to transduce a signal via TLR4 (Kim & Kim, 2017; Beutler & Rietschel, 2003). Like LPS, alcohol-induced TLR4 activation requires co-receptors CD14 and MD2. However the precise mechanism remains unclear

(Alfonso-Loeches et al., 2010). We have hypothesised that alcohol induces reactive oxygen species (owing to local metabolism) or alters the osmotic stress place on neuroimmune cells (Yu et al., 2015; Tsung et al., 2007). This in turn causes the release of DAMPs such as HMGB1, a TLR4 agonist thereby initiating an immune response. Alcohol additionally, induces a “leaky gut” resulting in the translocation of bacterial, viral and fungal proteins and lipids from the gut to blood and the liver. Activation of resident and circulating peripheral immune cells by these proteins are hypothesised to activate the neuroimmune system (see Crews & Vetreno, 2015 for review).

6. Unlike alcohol, LPS does not readily cross the blood brain barrier. LPS-TLR4 binding occurs in the periphery which subsequently activates the neuroimmune system by circulating cytokines crossing the blood brain barrier (circumventricular organs); active transport of cytokines across the brain endothelium; activation of peripheral nerve fibres such as the vagal nerve, which transmit cytokine signals to specific brain regions; activation of endothelial cells and peripheral macrophages with the cerebral vasculature to produce inflammatory mediators within the brain; or migration of peripheral immune cells into the brain by extravasation (Quan & Banks, 2007) or by the brain’s lymphatic system (Louveau et al., 2015).

Exposure to LPS results in much higher levels of inflammatory mediators and generates a state of neuroinflammation. Neuroinflammation has profound effects on both human and animal behaviours. For example, the high levels of inflammatory mediators observed during pathological neurodegenerative disease states such as Alzheimer’s and Parkinson’s disease cause neuronal cell death, which in turn directly affects an individual’s memory, language, and mood (McGeer & McGeer, 2010; Rogers et al., 2007). However, an alcohol-induced immune response is relatively

smaller and occurs in discrete neuroanatomical areas compared to that of a LPS-induced immune response. As such, it has been hypothesized that the immune response is acting in a manner similar to neurotransmission (central immune signalling) than neuroinflammation.

Table 1 Summary neuroimmune responses towards alcohol and LPS

	Acute		*Chronic*		*Priming*	
	Alcohol	*LPS*	*Alcohol*	*LPS*	*Alcohol*	*LPS*
Morphological and functional changes						
Astrocytes	- Cell death ↑ Amoeboid morphology ↓ Glutamate homeostasis	— Cell death ↑ Amoeboid morphology ↓ Glutamate homeostasis	↑ Cell death ↑ Amoeboid morphology ↓ Glutamate homeostasis	↑ Cell death ↑ Amoeboid morphology ↓ Glutamate homeostasis	No data	No data
Microglia	↑ Amoeboid morphology ↑ Phagocytosis	↑ Amoeboid morphology ↑ Phagocytosis	↑ Amoeboid morphology No data	↑ Amoeboid morphology ↑ Phagocytosis	↑ Amoeboid morphology	↑ Amoeboid morphology
Neurons	— Cell death — Basal function	↑ Cell death ↓ Basal Function	↑ Cell death ↓ Basal Function	↑ Cell death ↓ Basal Function	No data	No data
Receptor signalling and alterations to signal transduction pathways						
TLR4 expression	↑ dimerisation ↑ internalisation ↑ mRNA	↑ dimerisation ↑ internalisation ↑↓ mRNA	? dimerisation ? internalisation ↑ mRNA	↓ dimerisation ↓ internalisation — mRNA	↑ mRNA	? mRNA

MyD88 pathway	↑ signalling pathway ↑ early NFκB	↑ signalling pathway ↑ early NFκB	↑ signalling pathway ↑ early NFκB	↓ signalling pathway ↓ early NFκB	No data	No data
TRIF pathway	↑ signalling pathway ? NFκB ↑ IRF-3	↑ signalling pathway ↑ late NFκB ↑ IRF-3	? signalling pathway ↑ IRF-3	↑ signalling pathway ↑ IRF-3	No data	No data
Expression of immune proteins						
MyD88 pathway	— protein ↑ mRNA	↑ protein ↑ mRNA	↑ protein ↑ mRNA	↓ protein ↓ mRNA	? protein ↑ mRNA	↑ protein ↑ mRNA
TRIF pathway	? protein ↑ mRNA	↑ protein ↑ mRNA	? protein ↑ mRNA	— protein ↑ mRNA	? protein ↑ mRNA	↑ protein ↑ mRNA

↑, increase; ↓, decrease; —, no effect; ?, unknown effect

1.7 What do these immune mediators do?

The parallels listed above focus primarily upon the traditional immunological role of TLR4, its transcription factors and signalling molecules. In addition to this role, it is becoming increasingly apparent that immune molecules act as neuromodulators capable of modifying the electrophysiological properties of neurons, the release of neurotransmitters and neuropeptides which under specific circumstances can alter behaviour (Miller et al., 2013; Parsadaniantz & Rostène, 2008; Rostène et al., 2007). Prolonged exposure to immune mediators results in transcriptional and epigenetic processes altering the expression of genes related to neurotransmitters and receptors. This reinforces or diminishes synaptic activity, thus modulating plasticity and further modifies neuronal behaviour.

The proceeding sections focus primarily on how immune mediators can alter the behaviour of neurons. It is important to note, neurons can additionally modulate the activity of glial cells and their immune responses. However, this area is beyond the scope of this thesis, please refer to Neumann, (2001) for review.

1.7.1 Acute exposure to immune molecules

Acute exposure to alcohol results in an increase in immune-related molecules such as cytokines, and reactive oxygen creating enzymes (Fernandez-Lizarbe et al., 2009; Qin et al., 2008; Blanco et al., 2005). Emerging data suggests these immune molecules; specifically cytokines, exhibit characteristics similar to neurotransmitters (Miller et al., 2013; Rostène et al., 2007). For example, they are located in nerve-terminal vesicles, are often co-localised to phenotypically defined neurons such as dopaminergic or cholinergic neurons, are released following membrane depolarisation and can bind to post-synaptic receptors activating intracellular signalling pathways and membrane depolarisation (Knapp et al., 2011; Guyon et al., 2009; Banisadr et al., 2005b; de Jong, 2005).

1.7.1.1 Cytokines and neuromodulation

The neuromodulatory properties of cytokines are becoming increasingly appreciated. Particular emphasis has been placed on CCL2, a chemokine, with well characterised neuronal function. For example, CCL2 and its receptor CCR2 are expressed by glia and neurons respectively (Banisadr et al., 2005b; Oh et al., 1999; Hayashi et al., 1995). In particular, CCR2 co-localises to dopaminergic and cholinergic neurons within the substantia nigra and VTA (Banisadr et al., 2005b; 2005a). Application of CCL2 into the substantia nigra closes K^+ channels in dopaminergic neurons increasing their membrane resistance. This leads to increased frequency of pacemaker and burst action potentials potentiating dopamine release and in turn increases circling

behaviour in rodents (Guyon et al., 2009). CCL2 additionally acts via T-type Ca^{2+} and Na^{+} channels in neurons in the cortex, hippocampus, hypothalamus and mesencephalon (Belkouch et al., 2011; You et al., 2010; Gosselin et al., 2005; van Gassen et al., 2005). However, the behavioural and functional consequences of these interactions remain to be fully elucidated.

Interestingly, CCL2 expression is increased following alcohol exposure – an effect dependent on TLR4 (Pascual et al., 2015; Qin et al., 2008). Further, mice deficient in CCR2 exhibit reduced alcohol intake (Blednov et al., 2005). Given the importance of dopamine in alcohol reward, it is likely that a lack of CCL2 signal in $CCR2^{-/-}$ mice reduces extracellular dopamine, alcohol-induced reward and consequently the intake of alcohol is attenuated.

The neuromodulatory properties of the TNF superfamily are crucial for the generation of anti-reward behaviour. For example, administration of TNFα into central amygdala (CeA) increased the amplitude but not frequency of miniature excitatory postsynaptic currents (mEPSCs) and decreased the threshold for triggering action potentials without altering membrane properties of CeA neurons – an effect mediated by glutamate and PI3K (Ming et al., 2013). This resulted in the release of GABA and CRF (an anti-reward peptide) from presynaptic neurons. Attenuating microglial activation using minocycline attenuated the effects of TNFα on CeA neurons suggesting microglia are involved in mediating this process (Ming et al., 2013; Knapp et al., 2011).

TNFα is increased in the brain following chronic alcohol exposure and remains elevated once the drug has been cleared (Qin et al. 2008). Given CRFs pivotal role in mediating anti-reward behaviours such as anxiety, alcohol-induced TNFα signalling could potentiate its release in the CeA further exacerbating the symptoms of anti-

reward (Chen et al., 2013; Knapp et al., 2011). Further, the increased TNFα-induced GABAergic tone may contribute to anhedonia as this process could potentially reduce CeA dopaminergic neurotransmission – a key component of chronic alcohol intake.

Aside from cytokines, glial derived neurotrophic factor (GDNF) is gaining increasing importance in coupling the effects of glia and neurons following alcohol exposure (Ron & Barak, 2016; Lin et al., 1993). GDNF and its receptor are highly expressed in the VTA and nucleus accumbens respectively (Glazner et al., 1998). This protein regulates the survival and maintenance of dopaminergic neurons and is involved in learning, memory, synaptic plasticity, all key variables influencing the "wanting" component of reward (Ghitza et al., 2010). Infusion of GDNF into the VTA increases the spontaneous activity of dopaminergic neurons increasing the concentration of extracellular dopamine in the nucleus accumbens via an ERK1/2 dependent process (Wang et al., 2010). Administration of GDNF during alcohol withdrawal, a period characterised by dopamine deficiency, normalises the level of extracellular dopamine potentially attenuating relapses to alcohol drinking (Barak et al., 2014; 2011) Crucially, ibudilast, a microglial specific anti-inflammatory agent increases GDNF expression (Mizuno et al., 2004) which may assist in this drugs ability to reduce alcohol drinking (Bell et al., 2015) and the self-administration of, and relapse to cocaine and methamphetamines (Poland et al., 2016; Snider et al., 2012; Beardsley et al., 2010).

1.7.2 Chronic exposure to immune molecules

Chronic exposure to alcohol results in higher levels of inflammatory mediators that persist even in the absence of the alcohol (Pascual et al., 2016; 2015). Prolonged exposure to immune-related molecules results in long-term adaptions on a cellular, epigenomic and genomic level (Hennessy & McKernan, 2016; Carson et al., 2014; Hellstrom et al., 2005).

1.7.2.1 Cellular changes

Neuroimaging studies in rodents and humans consistently demonstrate a reduction in white matter content; myelinated neurons; and the astrocyte expression marker GFAP following prolonged alcohol exposure (Vetreno et al., 2015; Alfonso-Loeches et al., 2013; 2012; 2010; Blanco et al., 2005; Korbo, 1999). The loss of myelin, neurons and astrocytes is attributable to the persistent expression of inflammatory molecules and reactive oxygen species which activate caspases (cell death proteins) and reduced the expression of myelin synthesising enzymes (Alfonso-Loeches et al., 2013; Fernandez-Lizarbe et al., 2009). These losses, which are particularly evident in the frontal cortex, striatum and hippocampus, have functional consequences (Nixon, 2006; Crews et al., 2004). For example, mice exposed to long-term alcohol exhibit impairments in tasks assessing memory and cognitive function as well as increases in anxiety-like and reward-seeking behaviours. This effect is mitigated by genetic knock out of immune receptors (Alfonso-Loeches et al., 2012; 2010; Pascual et al., 2011). This suggests that the loss of neurons, myelin and support from astrocytes reduces neuroplasticity; creating a brain more hardwired towards obtaining reward and generating anti-reward.

Glial cells are crucial to the homeostatic functioning of neurons (Kettenmann et al., 1996). Chronic alcohol exposure alters the phenotype of these cells, causing them to neglect many of their normal housekeeping functions such as maintaining glutamate homeostasis and clearance of metabolic waste (Ayers-Ringler et al., 2016; Pla et al., 2014; Spanagel & Rosenwasser, 2005). Under basal conditions astrocytes regulate the clearance of extracellular glutamate through GLAST (EAAT1) and GLT-1 (EAAT2) astrocyte-specific glutamate transporters (Rothstein et al., 1996). These transporters co-transport Na^+, which in turn activates neuronal Na^+/K^+ - ATPase pump, reducing neuronal excitability via clearance of K^+ from the extracellular space (Kanai & Hediger, 1992; Storck et al., 1992). Following acute alcohol exposure, the expression of GLAST

and GLT-1 is increased in organotypic cortical cell cultures to remove excessive glutamate induced by alcohol (Adermark & Bowers, 2016). Chronic consumption, however, decreases the expression of these transporters, causing the concentration of extracellular glutamate to rise. Increased extracellular glutamate causes excitotoxicity and neurodegeneration (Sattler & Tymianski, 2001). Restoring GLAST and GLT-1 function in the nucleus accumbens decreases glutamate concentration and prevents withdrawal-induced drinking and seizures (Hakami et al., 2017).

1.7.2.2 Transcription factor changes

NFκB

NFκB has primarily been characterised in terms of its ability to transcribe genes pertaining to an immune response. It is therefore unsurprising that this transcription factor contributes to alcohol-induced neurodegeneration (Pascual et al., 2011; Fernandez-Lizarbe et al., 2009; Blanco et al., 2008). However, NFκB has many other roles beyond the immune system such as its expression within neurons and the regulation of synaptic function, plasticity and expression of neurotransmitters and their receptors (Nennig & Schank, 2017; Yirmiya & Goshen, 2011). NFκB binding sites have been found in the promoter region of β-endorphin, opioid, neuropeptide Y and glutamate receptor genes (Chiechio et al., 2006; Karalis, 2004; Kraus et al., 2003; Richter et al., 2002; Musso et al., 1997). Given that chronic alcohol increases the expression and activity of NFκB, this may increase transcription (or repression) of opioid- and neuropeptide Y-related genes (Nennig & Schank, 2017), which in turn, alters the “liking” component of alcohol reward and anxiety behaviour respectively.

NFκB transcription factor activity in neurons is additionally required for memory formation and consolidation – important processes generating the “wanting” component of reward. For example, the activity of Iκβ (a regulator of NFκB activity) in

neurons and microglia modifies hippocampal long-term potentiation during associative learning (Kyrargyri et al., 2014). Further, NFκB is required for spine formation and synaptogenesis –important processes underlying plasticity and memory formation. While data on alcohol is lacking, studies examining cocaine have shown attenuating NFκB reduced cocaine-induced reward; an effect which coincided with the decreased cocaine-induced formation of dendritic spines in the nucleus accumbens (Russo et al., 2009). This indicates the ability of the mouse to form associative memories was reduced.

cAMP response element binding protein (CREB)

CREB is a key transcription factor expressed in brain regions associated with reward and addiction (Olson, 2005; Pandey et al., 2003; 2001; Misra et al., 2001). CREB regulates genes involved with vesicle transport, synaptic transmission, cell growth/differentiation, cell adhesion/motility, cellular stress and the immune response. Given the breadth of processes regulated by CREB, it is unsurprising, that this transcription factor is involved in alcohol-induced plasticity, memory and learning – necessary processes to create addiction (McClung & Nestler, 2007). Importantly, this transcription factor is activated by TLR4 (Avni et al., 2010; Park et al., 2005) and alcohol (Wand et al., 2001; Pandey et al., 1999). Both promote its activation and translocation to the nucleus (Park et al., 2005; Hsu et al., 2004; Wand et al., 2001). Activation of CREB limits the rewarding, and reinforces the anti-rewarding, effects of alcohol (McPherson & Lawrence, 2007). For example, CREB decreases the expression of reward-related proteins such as dopamine D1 and D2 receptors on medium spiny neurons; and increases the expression of anti-reward related proteins such as CRF (McPherson & Lawrence, 2007; Nestler, 2005).

1.7.2.3 Epigenetic modifications

Epigenetics is an emerging field in addiction research. It is principally concerned with modifications to the structure of chromatin that can repress or enhance transcription factor binding. There are numerous types of epigenetic modifications that occur in mammalian cells such as DNA methylation and histone modifications (Bernstein et al., 2007). Importantly, chronic exposure to immune molecules and alcohol can induce long-term changes to the function and behaviour of neurons by altering their epigenome specifically around genes mediating reward and anti-reward (Pandey et al., 2008a).

Methylation modifications to DNA can regulate the transcription of genes. Typically, DNA methylation occurs via DNA methyltransferases (DNMT), an enzyme which adds a methyl group to cytosine flanked by a guanine molecule (CpG dinucleotides). These CpG nucleotides are largely found in promoter regions and can interfere with transcription by blocking transcription factor binding. Methylation additionally recruits histone deacetylase complexes (HDACs) to assist in the remodelling of chromatin that further regulates transcription factor binding (see Bernstein et al., 2007 for review).

Hypermethylation of genes associated with synaptic plasticity and neurotransmission such as opioid, dopamine and GABA receptors have been identified following alcohol exposure (Xu et al., 2017; Zhang & Gelernter, 2016; Barbier et al., 2015). This effect may be attributable to immune-molecules as they are known to regulate the methylation process. For example, LPS stimulation alters CpG methylation and decreases the expression of DNMT and HDACs potentially restricting synaptic plasticity (Shen et al., 2016).

Another form of epigenetic modifications involves chromatin modification. Chromatin is a form of packaged and condensed DNA. It consists of DNA wound around histone proteins. Post-translational modification to the histone proteins via acetylation or methylation can cause chromatin to be remodelled forming a more or less condensed state respectively. This in turn facilitates or restricts access of transcription factors, enhancer or repressor proteins to promoter regions. Acetylation and methylation of histones are controlled by histone acetyltransferase (HAT) and HDACs respectively. Acetylation generates an open, transcriptionally active state, while removal of the acetyl group closes chromatin, repressing transcription (see Li, 2002 for review).

Importantly, immune molecules and alcohol exposure modifies the state of chromatin particularly around genes relating to neuronal differentiation and synaptic plasticity. For example, alcohol exposure inhibits HDAC activity, induces the acetylation of histone H3 and H4, and increases the expression of CREB-binding protein, a histone acetyltransferase within the amygdala (Moonat et al., 2013; Pandey et al., 2008b). These alterations cause chromatin remodelling to a less condensed state facilitating the binding of CREB and CBP. CREB-CBP increases the expression of neuropeptide Y, prodynorphin, BDNF and Arc in the amygdala (Sakharkar et al., 2014; Legastelois et al., 2013; You et al., 2013). This leads to an increase in dendritic spine density (a marker of plasticity) and a reduction in anxiety-like and drinking behaviours (Moonat et al., 2013). In contrast, chronic alcohol exposure induces tolerance to alcohol and normalises molecular signatures of the epigenome. Upon withdrawal, HDAC activity is increased, histones are deaceylated and condense, CREB-CBP activity is reduced as is the expression of NPY and BDNF (Moonat et al. 2013; Roy & Pandey 2002). This in turn decreases dendritic spine density (reduces plasticity) increasing anxiety-like and drinking behaviours (Berkel & Pandey, 2017). Additionally, inhibiting HDAC activity

prevents GABA hyposensitivity of dopaminergic neuronal firing in the ventral tegmental area that typically accompanies chronic alcohol exposure (Berkel & Pandey, 2017).

CREB and CBP are downstream products of the TLR4 signalling pathway. Activation of TLR4 results in the phosphorylation of CREB and CBP (Wen et al., 2010). CREB then competes for CBP, a cofactor required for NFκB signalling. Therefore, chronic alcohol exposure may decrease CREB, and cause CBP to bind preferentially to NFκB. This may create a state of heightened inflammation as transcription of inflammatory molecules is potentially increased (Wen et al., 2010). The increased inflammatory response would increase HDAC activity reducing plasticity and decreasing genes important to reward such as tyrosine hydroxylase, and enkaphalins. However, HDACs reciprocally influence glial inflammatory response. For example, LPS-activated microglia exhibit decreased histone acetylation activity (Correa et al., 2011) and HDAC inhibitors impair NFκB dependent synthesis of IκBα and reduce the DNA binding of p65, c-FOS, c-Jun and FRA2. This in turn limits inflammation as indicated by a downregulation in IL-1β and COX-2 (Faraco et al., 2009).

1.8 Linking TLR4 to the neurobiological basis of reward and anti-reward

Alcohol exposure induces an inflammatory response from astrocytes, microglia and neurons. The preceding sections have highlighted how inflammatory mediators alter behaviour by switching the phenotype of astrocytes, inducing epigenetic remodelling and transcription factor activity surrounding genes relating to reward and anti-reward processes, and can under some circumstances induce neurodegeneration. Collectively, these processes assist in creating and maintaining reward and anti-reward behaviours. However, there is limited evidence directly implicating alcohol-induced TLR4 signalling in creating and altering the neurobiological processes

underlying reward and anti-reward. Thus, the following sections are designed to highlight potential links between alcohol, TLR4, reward and anti-reward.

1.8.1 TLR4-Dopamine

Preliminary evidence suggests there is an interaction between dopamine, the key molecule mediating the “wanting” component of reward and alcohol-induced TLR4 signalling. For example, alcohol-induced TLR4 signalling in VTA dopaminergic neurons induces the expression of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis via protein kinase C (PKC) and CREB (Aurelian et al., 2016). However, microdialysis studies are needed to confirm whether this translates to increased dopamine release within the nucleus accumbens. Associative evidence demonstrates that attenuating TLR4 via (+)-Naloxone reduces morphine and cocaine-induced dopamine release (Northcutt et al., 2015; Hutchinson et al., 2012). Importantly, these drugs of abuse activate TLR4, thereby furthering the link between TLR4 activation and dopamine signalling.

Studies examining the direct effects of TLR4 on the dopaminergic system following chronic alcohol are lacking. A study using LPS to mimic the high levels of inflammatory mediators observed during chronic alcohol administration demonstrated a reduction in alcohol-induced VTA dopaminergic neuron firing seven to ten days post exposure (Blednov et al., 2011a). This paradoxically coincided with elevated alcohol intake and reduced alcohol conditioned taste aversion (an inverse marker of “likability”). It was hypothesised that the high level of inflammatory mediators reduced basal dopaminergic neurotransmission thus contributing to the anhedonia (anti-reward). Therefore, upon re-exposure to alcohol mice exhibited potentiated intake to reduce the anhedonic sensations and restore dopamine levels (Blednov et al., 2011a). Thus alcohol-induced TLR4 signalling may mediate both acute and chronic effects of alcohol

on the dopaminergic system. Again however, given the discrepancies between an immune response to LPS and alcohol this finding must be interpreted with caution.

1.8.2 TLR4-GABA$_A$

GABA is a major inhibitory neurotransmitter system within the central nervous system and contributes to both reward and anti-reward behaviours associated with alcohol use (Harris et al., 2008; Davies, 2003). Of particular importance is GABA$_A$α2, a subunit of GABA receptors that is strongly expressed in reward and anti-reward regions such as the nucleus accumbens, amygdala, BNST and hypothalamus (Davies, 2003). Genetic association studies repeatedly demonstrate an association between GABA$_A$α2 and the behavioural consequences of alcohol use such as dependence (Soyka et al., 2008; Edenberg et al., 2004). Furthermore, mice with impaired GABA$_A$α2 function demonstrate reduced alcohol intake and preference (Blednov et al., 2013). Interestingly, siRNA knock down of GABA$_A$α2 in the CeA but not ventral pallidum decreases the expression of TLR4 and reduces binge-like alcohol drinking (June et al., 2015; Liu et al., 2011). Electrophysiological evidence further supports a link between alcohol, GABA$_A$α2 and the TLR4 signalling pathway. However, the results are far from uniform. For example, alcohol-induced TLR4 signalling alters inhibitory post-synaptic currents in GABAergic cells but does not influence baseline GABA release or excitatory post-synaptic potentials (Yan, 2015; Bajo et al., 2014). (+)-Naloxone, a TLR4-TRIF antagonist, blocks early low-dose alcohol-induced potentiation of GABAergic transmission (Bajo et al., 2014). However, other studies using CeA slices from TLR4$^{-/-}$ mice have cast doubts upon these findings with no alterations in GABA activity compared to wildtype mice (Harris et al., 2017). Therefore, authors have postulated that CD14 or IL-1β may be responsible for alterations in GABA signalling and not TLR4 itself (Harris et al., 2017). Crucially, CD14 mediates acute alcohol- or LPS-induced excitatory postsynaptic potentials from GABAergic cells and deletion of IL-1β's

negative regulator IL-1Rn alters basal phasic and tonic GABAergic transmission and alcohol-induced facilitation of phasic GABAergic transmission. Administration of IL-1Ra reverses and restores alterations in GABA signalling (Bajo et al., 2015a; 2015b; 2014; Wang et al., 2000). Collectively the results suggest TLR4, its co-receptors and downstream signalling pathways modulate GABAergic signalling within the CeA. This modulation may cause increased disinhibition of dopaminergic or CRF-expressing neurons further increasing the release of reward and anti-reward-related neurotransmitters and neuropeptides respectively (June et al., 2015; Liu et al., 2011). This in turn would modulate the behavioural effects of reward, anxiety and anhedonia in mice.

1.8.3 TLR4-NMDA

Alcohol has differential effects on glutamate reflecting acute or chronic exposure. For example, acute alcohol exposure reduces glutamatergic signalling in the nucleus accumbens and other limbic regions (Möykkynen & Korpi, 2012). However, withdrawal from alcohol results in a hyperglutamatergic state owing to the constant suppression during alcohol exposure (Piña-Crespo et al., 2014). This in turn is implicated in alcohol-induced excitotoxicity and seizures in the absence of alcohol (Piña-Crespo et al., 2014) (however, recent findings cast doubts on this hypothesis (Collins and Neafsey, 2016)). Importantly, TLR4 and glutamate are linked. For example, $TLR4^{-/-}$ mice do not exhibit increased expression of GluR1, NR1 or GluR1/NR1 heteromers following adolescent alcohol consumption – an effect which coincided with alterations in reward and anxiety-behaviour in adulthood (Montesinos et al., 2016). While studies linking alcohol-TLR4-NMDA and behaviour are lacking, experiments examining kainate-induced seizures can be used to infer interactions between TLR4 and NMDA. Similar to chronic alcohol, inflammatory molecules are elevated during a seizure. Under these conditions, IL-1β and HMGB1 (TLR4 signalling molecule and agonist respectively) reduce NMDA-

induced outward current via p38, a MAPK, and enhance NMDA Ca^{2+} influx – collectively increasing neuronal excitability that initiates and propagates seizures (Carmignoto, 2014; Maroso et al., 2011; Viviani et al., 2003). Given that withdrawal from alcohol results in high levels of IL-1β and HMGB1 (Pascual et al., 2015; Whitman et al., 2013; Qin et al., 2008), these TLR4 signalling molecules could potentiate alcohol-induced NMDA currents following withdrawal from chronic alcohol consumption. This may, in turn, contribute to alcohol-induced excitotoxicity and seizures (Maroso et al., 2011).

1.8.4 TLR4-epigenetic modifications

The adverse effects following chronic alcohol consumption result from long-term epigenetic changes to genes governing plasticity and inflammation in brain regions involving reward, stress and learning (Moonat et al. 2013; Roy & Pandey, 2002). The epigenetic modifications typically involve chromatin remodelling owing to histone modifications. TLR4 regulates many alcohol-induced epigenetic processes including the expression of histone acetyl-transferase and the acetylation of histone H3 and H4 in the medial prefrontal cortex - an effect that coincided with increased anxiety- and reward-like behaviour in mice (Pascual et al., 2011). Interestingly, adolescent alcohol exposure increased acetylation of histone H3 and H4 and decreased methylation of H3 (Kyzar et al., 2016; Montesinos et al. 2016). This promoted long-term alterations in the expression of BDNF, its receptor TrκB and transcription factors FosB, ΔFosB and Cd5k – all of which are implicated in addiction-related plasticity (McClung & Nestler, 2007). This coincided with alterations in glutamatergic and dopaminergic signalling in the nucleus accumbens, prefrontal cortex and striatum, which may underlie the predisposition of these mice to seek rewarding stimuli and exhibit increased anxiety behaviour later in life (Montesinos et al., 2016). Importantly, mice deficient in TLR4 are protected against these effects. Thus, alcohol-induced TLR4 signalling can induce

epigenetic modifications to histones, thereby modulating the expression of reward and plasticity related genes which influences alcohol-related behaviour.

1.8.5 TLR4-CREB

Neural plasticity is required for the development of alcohol addiction. Of pivotal importance to addiction-related plasticity is CREB. Alcohol-induced activation of TLR4 increases CREB in the prefrontal cortex and nucleus accumbens (Aurelian et al., 2016; Montesinos et al., 2016). This alcohol-induced TLR4-CREB signalling has been implicated in both the rewarding and anti-rewarding effects of alcohol by inducing alterations to BDNF and glutamatergic pathways (Aurelian et al., 2016; Montesinos et al., 2016). Further, TLR4s downstream signalling molecules such as interferon activate CREB to limit inflammation (Liu et al., 2004) which has the potential to limit reward-related neuroimmune signalling.

1.8.6 TLR4-neurodegeneration

Long-term exposure to alcohol results in demyelination, synaptic loss, and a reduction in white matter. These processes are mediated by glial cells and alcohol-induced excitotoxicity. Chronic exposure to alcohol increases the expression and release of inflammatory mediators and changes microglia and astrocytes to an immunologically active state via TLR4 (Alfonso-Loeches et al., 2010; Blanco et al., 2005). For example, alcohol-induced TLR4 signalling increases COX-2 and iNOS, which subsequently elevate reactive oxygen species. Reactive oxygen species activate the caspase pathway within neurons culminating in apoptosis and neurodegeneration (Alfonso-Loeches et al., 2012; 2010). The inflammatory response additionally causes dysregulation of the autophagy pathway causing the accumulation of ubquitinated proteins that cannot be removed owing to an inability to form specific phagosomes.

The build-up of these proteins additionally activates apoptotic pathways inducing neurodegeneration (Pla et al., 2016; 2014).

The alcohol-induced TLR4-inflammatory response has also been linked to the down-regulation of proteins involved in the synthesis of myelin and the alteration in the composition of myelin sheaths collectively causing myelin aberrations (Montesinos et al., 2015; Pascual et al., 2014; Alfonso-Loeches et al., 2010). These alterations coincide with decreased performance in behavioural tests assessing cognitive function, memory, learning and reward processing. Importantly, alcohol does not activate glia or the neuroimmune system in TLR4$^{-/-}$ mice (Montesinos et al., 2015; Pascual et al., 2011; Alfonso-Loeches et al., 2010). These mice are therefore protected against neurodegeneration and demyelination and exhibit improved performance in behavioural tests compared to wildtype mice. Neurodegeneration and demyelination create a brain that is less plastic. The reduced plasticity may assist in the retention of anxiety and reward-sensitivity following chronic alcohol exposure as mice can no longer interpret and adapt to new stimuli and are thus solely focused on obtaining alcohol.

1.9 Integrating TLR4 into reward and anti-reward behaviour

The preceding sections have highlighted that alcohol activates TLR4 signalling, inducing an inflammatory response. Acute exposure results in a transient, sub-inflammatory rise in TLR4's immune mediators. These immune molecules can act as neuromodulators, altering the function of neurons and the behavioural response towards alcohol. Prolonged access to alcohol, induces a robust increase in TLR4's immune mediators and can influence alcohol-related behaviour by inducing epigenetic modifications to neuroplasticity and neurotransmitter-related genes, increase activation of immune and plasticity associated transcription factors, cause neuronal

dysregulation via alterations in myelin and distract astrocytes from maintaining glutamate homeostasis. Collectively, these processes highlight the pivotal importance of alcohol-induced TLR4 signalling in the neurobiological basis of reward and anti-reward. From a behavioural perspective however, we are only beginning to appreciate the translational importance of TLR4 in mediating the effects of alcohol-induced reward and anti-reward.

1.9.1 TLR4-induced alcohol reward behaviour

1.9.1.1 Acute

Acute reward behaviour is typically assessed using a variety of experimental approaches. The most commonly used are conditioned place preference and alcohol two-bottle choice which primarily assess the “wanting” and the “liking” component of alcohol-induced reward respectively. Longer two-bottle choice tests can also examine the “wanting” component of alcohol reward to some degree.

The past five years has seen an influx of manuscripts assessing the role of alcohol-induced TLR4 signalling on acute reward behaviour. However, there is a lack of consensus surrounding the role of TLR4 in the behavioural manifestation of “liking” and “wanting”. This is likely attributable to different behavioural models, background strains of mice, inappropriate statistical analysis and method of investigating TLR4 (genetic knockout or pharmacological antagonists). Studies have shown that:

1. Genetic knockout of TLR4s co-receptor, CD14 reduces the preference but not intake of alcohol during a 24 h two-bottle choice test compared to wildtype mice. However, there was no difference between $CD14^{-/-}$ and wildtype mice during a 2 h two-bottle choice test (Blednov et al., 2017; 2011b).

2. Mice deficient in TLR4-NFκB signalling (C3/HeJ mice) demonstrate reduced intake and preference for alcohol during a 24 h and 3 h two-bottle choice test compared to genetically similar mice (C3H/HeOuJ) (Mayfield et al., 2016).
3. $TLR2^{-/-}$ mice display reduced 24 h and 2 h intake and preference for alcohol. Further these mice also display enhanced quinine intake compared to wildtype mice indicating a potentially altered olfactory or gustatory sense (Blednov et al., 2017).
4. $MyD88^{-/-}$ mice consume less alcohol during a 2 h but not 24 h test compared to wildtype mice. Further, male but not female $MyD88^{-/-}$ mice exhibit reduced saccharin preference compared to wildtype mice indicating an alteration to the "liking" component of reward (Blednov et al., 2017).
5. 24 h and 2 h alcohol and saccharin intake and preference are similar in wildtype (C57BL/6J) and $TLR4^{-/-}$ (C57BL/6J background) mice (Montesinos et al., 2016; Pascual et al., 2011). Further, wildtype (Balb/c) and $TLR4^{-/-}$ (Balb/c background) mice exhibit similar 8 h saccharin and quinine intake and preference but reduced 8 h alcohol preference (see appendix).
6. Pharmacological inhibition of TLR4 via (+)-Naloxone (30 or 60mg/kg) reduced alcohol intake during a 24 h but not 2 h alcohol preference test (Harris et al., 2017).

Collectively, the results from voluntary drinking paradigms, suggest CD14, TLR2 and MyD88 are involved in the acute preference and intake of alcohol indicating these receptors and signalling pathways are crucial to the "liking" and to some extent the "wanting" component of alcohol-reward. However, the role of TLR4 is ambiguous with studies demonstrating either a reduction or no effect on alcohol intake (potentially indicating compensatory mechanisms). This suggests TLR4 may modify aspects of the "liking" component of reward. However, no study has examined TLR4's role in the

"wanting" component of reward (using conditioned paradigms) or demonstrated a link between alcohol, TLR4 and the mechanisms underlying "liking" component of reward such as the μ opioid receptor or endocannabinoids. Consequently, additional studies are required to clarify TLR4's, MyD88's and TRIF's role in the molecular underpinnings and behavioural manifestations of the "liking" and "wanting" components of alcohol reward.

1.9.1.2 Priming

The TLR4-signalling and the mesolimbic reward pathway are primed/sensitised following alcohol exposure. Upon re-exposure to alcohol or other drugs of abuse, the expression of immune mediators and reward-related molecules (dopamine and opioid peptides) is exacerbated inducing a heightened state of reward. This effect is particularly apparent in adolescents that consume high levels of alcohol (binge drinking). Binge drinking during adolescence primes the reward and TLR4-related pathways to over-respond in adulthood (see Chapter 4). Consequently, these individuals are extremely susceptible to "liking" and "wanting" alcohol later in life.

There are limited studies examining the effects of TLR4-induced signalling on mediating this reward-priming effect from a behavioural perspective. Associative studies have shown adolescent alcohol exposure potentiates *Tlr4* mRNA expression in brain regions associated with the "wanting" component of reward in adult. However, the reward-related effects of this are presently unknown. Montesinos et al., (2016) however, demonstrated alcohol exposure during adolescence potentiated the "wanting" of cocaine in adulthood, an effect absent in $TLR4^{-/-}$ mice. In addition, $TLR4^{-/-}$ mice are protected against adolescent alcohol-induced anxiety and attenuated cognitive performances in adulthood. These author's hypothesised this effect was mediated by alterations in a TLR4-NMDA interaction within the prefrontal cortex.

Interestingly, adolescent but not adult morphine (a TLR4 agonist) pre-exposure increased the expression of *Tlr4* mRNA and $TLR4^{+}$ $CD11b^{+}$ cells (microglia) in the nucleus accumbens, an effect that coincided with a potentiation of morphine conditioned place preference later in life (Schwarz & Bilbo, 2013). Attenuating microglia activation decreased the rise in *Tlr4* mRNA and decreased morphine-induced conditioned place preference (Schwarz & Bilbo, 2013). Collectively, these studies imply that activation of TLR4 signalling during adolescence can create a priming effect that potentiates drug "wanting" behaviour later in life. However, studies using $TLR4^{-/-}$ mice are inherently confounded owing to the pivotal role of TLR4 in neurodevelopment (Okun et al., 2011; Chapter 4). Thus, no study has determined whether transiently attenuating TLR4 (thereby allowing normal development to continue) during alcohol exposure in adolescence can prevent priming and the potentiation of alcohol "liking", "wanting" or anti-reward behaviour later in life. Further no study has considered which TLR4 signalling pathway mediates priming, or how TLR4 priming alters the neuronal system.

1.9.1.3 Chronic

Long-term alcohol intake desensitises the reward pathway, shifting the balance of alcohol-induced reward from a predominately "like" to a "want" driven phenomena (Berridge & Robinson, 2016). Studies examining the long-term consequences of alcohol typically use a model termed 'chronic intermittent access' to induce dependence. In brief, mice receive alcohol vapour or are exposed to a bottle containing alcohol on alternating days. On intervening days, mice receive water. This exposure model lasts for a minimum of two to six weeks. Mice are then tested for alcohol preference using two-bottle choice. At this stage, most researchers assume their mice are dependent and consequently describe this test as a measure of "wanting" behaviour. As previously mentioned, animals can exhibit stable drug intake without

exhibiting signs of addiction and dependence. Further, these do not test for alterations in the “liking” response (assessed via saccharine preference test following chronic alcohol consumption). Thus, these studies are somewhat limited in their conclusion and consequently, caution must be used when interpreting any effects (or lack thereof) attributable to TLR4 on reward behaviour following chronic exposure.

In spite of these caveats, studies have demonstrated, chronic alcohol exposure increases the expression of TLR4 and its downstream transcription factors and signalling molecules in key brain regions associated with reward such as the ventral tegmental area (for example, June et al., 2015; Vetreno et al., 2013). Consequently, a large body of evidence has focused on understanding the role of TLR4 in chronic alcohol-reward behaviour. Again, there is a lack of consensus surrounding the role of TLR4 in the behavioural manifestation of “liking” and “wanting” following chronic alcohol intake. For example:

1. Transcriptome profiling revealed an over-representation of the TLR4 signalling pathways following chronic alcohol exposure in brain regions associated with reward in mice (Mayfield et al., 2016).
2. A long-term intermittent access model of alcohol exposure determined $TLR2^{-/-}$, $TLR4^{-/-}$ and wildtype (C57BL/6J) mice exhibit similar levels of intake and preference for alcohol (Blednov et al., 2017; Pascual et al., 2011).
3. $TLR4^{-/-}$ mice retain conditioned taste aversion following chronic access to alcohol (indicating a potential reduction of alcohol “liking”) (Pascual et al., 2011).
4. Pharmacological inhibition of TLR4 via T5432126 significantly reduced alcohol intake and preference following chronic intermittent access and alcohol vapour exposure – an effect which additionally reduced microglia reactivity in the amygdala (Bajo et al., 2016).

5. (+)-Naloxone attenuated alcohol intake following chronic intermittent access (Harris et al., 2017).
6. siRNA knock down of TLR4 in the VTA and CeA but not the ventral palladium decreased operant self-administration of alcohol (June et al., 2015; Liu et al., 2011).

Collectively, these studies highlight that pharmacological blockade but not genetic knockout of TLR4 influences the “wanting” component of reward following long-term administration of alcohol. However, the involvement of TLR4s on the “liking” component of reward, the relative influence of either the MyD88 or TRIF pathway in “wanting” or “liking” and a link between alcohol-TLR4 and wanting/liking remains to be elucidated.

1.9.2 TLR4-induced alcohol anti-reward behaviour

In addition to tolerance and desensitisation of the reward pathway following long-term alcohol use, the anti-reward pathway is recruited in order to restore homeostasis to the brain. Chronic alcohol exposure increases the expression of TLR4-related immune mediators in brain regions governing anti-reward (Knapp et al. 2016; Crews et al., 2013). Behaviourally, alcohol-induced TLR4 signalling increases anxiety-like behaviour as assessed by the elevated plus maze, open field maze and the light-dark box. Further, these changes were associated with alterations in epigenetic processes including down regulation of histone acetyltransferase and acetylation of histone H3 and H4 and an upregulation of NFκB (Pascual et al., 2011). Importantly, $TLR4^{-/-}$ mice were protected against alcohol-induced anxiety and epigenetic modifications (Pascual et al., 2011) highlighting the importance of this receptor in some aspects of anti-reward behaviour.

Another variable influencing anti-reward is reduced plasticity following chronic alcohol exposure. TLR4$^{-/-}$ mice are protected against short and impairment in long-term memory, conditioned aversion and reversal learning behaviour following chronic alcohol exposure (Pascual et al., 2011). These behaviours rely on the ability to form new memories and engage higher order functions that are dependent upon synaptic plasticity and epigenetic processes.

The engagement of anti-reward behaviour is dependent upon repeated cycles of alcohol drinking. However, given that antagonising TLR4 by pharmacological and genetic means reduces alcohol consumption it is difficult to gauge whether behaviours exhibited by these mice are simply due to a reduction in alcohol intake and thus anti-reward processes are less engaged, or whether TLR4 is actually crucial for the anti-reward process independent of alcohol intake. Additionally, these studies, do not explore which TLR4 signalling pathway is involved in anti-reward behaviour nor do they provide a link between TLR4 and neuronal drivers of anti-reward behaviour.

1.9.3 Summary

The introductory chapter of this thesis aimed to highlight TLR4's pivotal role in the molecular causes and the behavioural translation of reward and anti-reward behaviour. However, there are numerous limitations and gaps in the current field that prevent researchers to conclusively discern TLR4's role in alcohol-induced reward and anti-reward behaviours. For example:

1. Which TLR4 signalling pathway, MyD88 or TRIF is activated following acute and chronic alcohol exposure? And do they remain primed following adolescent alcohol use?
2. Are these signalling pathways of equal importance to reward and anti-reward behaviour?

3. Is the TLR4 pathway (MyD88 or TRIF) involved in the “wanting” component of alcohol reward following acute and chronic exposure? And are they involved with the sensitisation of the “wanting” component of reward following adolescent alcohol use?
4. Is the TLR4 pathway (MyD88 or TRIF) involved in the “liking” component of alcohol reward following chronic alcohol exposure? And are they involved with the sensitisation of the “liking” component of reward following adolescent alcohol use?
5. Does the TLR4 pathway mediate neuronal and neuroimmune priming following alcohol exposure?
6. How does alcohol-induced TLR4 signalling influence traditional neuronal mediators of reward and anti-reward?

These questions predominately focus on discerning which TLR4 signalling pathway is responsible for reward and anti-reward behaviour. As such, the overarching aim of this thesis project was to identify whether both TLR4 signalling pathways (MyD88 and TRIF) were active during and following acute and chronic alcohol exposure and to determine the effects of attenuating the TLR4-TRIF pathway on reward and anti-reward behaviour. Specifically, chapters 2 and 3 explore the interaction between circadian rhythm, TLR4 and reward following acute alcohol exposure. These studies test whether the efficacy of (+)-Naltrexone, a potential TLR4-TRIF biased antagonist, on acute alcohol drinking (“liking”) and seeking (“wanting”) behaviour is dependent on light-cycle. Chapters 4 and 5 examine the effects of TLR4 on adolescent alcohol-induced priming/sensitisation and later life reward and anti-reward behaviours. These experiments demonstrate that administration of (+)-Naltrexone before or after adolescent alcohol exposure prevents alcohol drinking (“liking”) but not seeking (“wanting”) behaviour in adulthood. Chapters 6 and 7 examined (+)-Naltrexone’s effect

on reward and anti-reward behaviours following chronic alcohol use. These studies determined (+)-Naltrexone had limited efficacy in modifying reward or anxiety-like behaviour following long-term alcohol use. The precise experimental questions, aims and hypothesis pertinent to each set of experiments can be found in chapters 2, 4 and 6.

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Chapter 2: Circadian rhythms, reward and TLR4

2.1 Circadian rhythm

The earth's daily rotation creates periods of light and darkness imbuing a diurnal rhythm upon life. Therefore, to maximise host fitness, organisms ranging from simple bacteria to complex mammals, oscillate their behaviour, biochemistry and physiology to meet the specific demands of that time-of-day (Lowrey & Takahashi, 2004; Harmer et al., 2001). This oscillatory pattern of expression is termed circadian rhythm. The circadian rhythm is an entrainable 24-hour rhythm that responds to external cues (zeitgiebers, ZT) including food, water, light, drugs of abuse (including alcohol) and immune responses (Hutko et al., 2008; Marpegán et al., 2005; Spanagel et al., 2005; Gauvin et al., 1997; Stephan & Zucker 1972). However, the circadian rhythm can persist in the absence of zeitgiebers, suggesting inherent biological mechanisms underlie the daily rhythm in physiology (Partch et al., 2014; Dardente & Cermakian, 2007).

2.2 How is circadian rhythm controlled?

Within organisms, the circadian rhythm is organised in a hierarchical manner consisting of a system level (cell extrinsic) and molecular level (cell intrinsic) oscillators (Reppert & Weaver, 2002). The suprachiasmatic nuclei (SCN) within the hypothalamus is the main brain region involved in the regulation of circadian rhythm at the system level (Reppert & Weaver, 2002). The SCN consists of approximately 10,000 – 20,000 neurons which function in a concerted manner generating a rhythmic network whose activity oscillates around a 24 h period (Mohawk & Takahashi, 2011; Abrahamson & Moore, 2001; Welsh et al., 1995). The SCN receives retinal input, entraining neurons to light cues and consequently linking the external world to the internal environment (Abrahamson & Moore, 2001). Brain regions isolated from the SCN often cease to

exhibit the circadian rhythm. Hence, these regions receive sympathetic and parasympathetic input to entrain and regulate a 24 h rhythm. For example, SCN innervates the adrenals to release glucocorticoids supressing and de-repressing the immune system at different times of the day (Chung et al., 2011; Abe et al., 1979) and sympathetic innervation to the liver results in daily rhythms in insulin and plasma glucose (Yamamoto et al., 1987).

Cells can also oscillate independently from SCN input suggesting intrinsic molecular mechanisms generate a 24 h rhythm. Cellular rhythm is created by a regulatory feedback loop consisting of interconnected transcription factors and kinases that are expressed in a repetitive and predictable manner forming the cellular clock. The cellular clock consists of two activator (CLOCK and BMAL1) and two repressor proteins (PER and CRY). In brevity, the cellular clock begins when CLOCK and BMAL1 dimerise and bind to enhancer boxes (E-box; DNA response elements) in the promoter region of CRY and PER, initiating their transcription. PER and CRY accumulate, dimerise and supress the transcription of CLOCK and BMAL1. The subsequent decline in CLOCK and BMAL1 decreases the transcription of PER and CRY. Therefore, the decreased expression of the repressor proteins enables the transcription of the activator proteins again (see Partch et al., (2014) for review). This cycle occurs over a 24 h period and in doing so imparts a repetitive rhythm (a time-of-day effect) on the mammalian transcriptome – with 2 to 10 per cent of all genes being regulated by this process (Miller et al., 2007; Akhtar et al., 2002; Duffield et al., 2002).

2.3 Rhythmic oscillations in reward

2.3.1 Circadian rhythms in reward pathways

The nucleus accumbens and ventral tegmental area (VTA) are innervated by, and express key components of the intrinsic and extrinsic clock. For example, the SCN

innervates the VTA via glutamatergic afferents from the medial prefrontal cortex regulating diurnal reward behaviour (Baltazar et al., 2014; Baltazar et al., 2013); and dopamine transporter, dopamine D1 and D2 receptors, monoamine oxidase and tyrosine hydroxylase – key proteins regulating dopamine synthesis and the "wanting" component of reward, contain BMAL1 and CLOCK binding sites in their promoter regions (Hampp et al., 2008; Sleipness et al., 2008; Sleipness et al., 2007; McClung et al., 2005; Akhisaroglu et al., 2005). This suggests the circadian clock controls aspects of dopaminergic activity including neurotransmitter synthesis, release, degradation and postsynaptic actions. No study has determined whether the μ opioid receptor or any of its endogenous agonists (associated with the "liking" component of reward) are under the control of clock proteins in the nucleus accumbens or VTA. However, aspects of the μ opioid receptor signalling pathway exhibit time-of-day effects (Pačesová et al., 2015). This suggests either cell extrinsic or intrinsic mechanisms may additionally influence the mediators underlying the "liking" component of reward.

The preceding section highlighted that elements of the reward pathway are influenced by circadian rhythm. These findings translate to time-of-day differences in the activity of the reward pathway. Under basal conditions c-FOS (a marker of neuronal activation) peaks and nadirs within the nucleus accumbens, prefrontal cortex and VTA midway through the active (dark) phase and the inactive (light) phase in rodents respectively (Baltazar et al., 2013). This daily fluctuation occurs within dopaminergic and non-dopaminergic cells. However, particular emphasis has been placed on dopaminergic fluctuations. For example, the expression of tyrosine hydroxylase, monoamine oxidase and extracellular concentration of dopamine, its metabolites, and the rate of clearance are elevated during the active and diminished during the inactive phases (Baltazar et al., 2013; Webb et al., 2009; Paulson & Robinson, 1994). However, the firing rate of

VTA dopaminergic neurons does not differ between active and inactive phases indicating that the time-of-day differences are due to the synthesis, release and binding of dopamine and not the electrophysiological properties of the neurons themselves (Luo & Aston-Jones, 2009; Luo et al., 2008; Miller et al., 1983). Interestingly, non-TH glutamatic acid decarboxylase expressing neurons (GABAergic) in the VTA exhibit increased firing during the dark cycle (Luo & Aston-Jones, 2009). Little research has examined the time-of-day dependent effects on opioidergic cells. Morphine- (a μ opioid receptor agonist) induced c-FOS expression does not fluctuate according to time-of-day in the SCN. However, its downstream signalling molecules ERK1/2 and GSK3β exhibit a daily rhythm in their expression (Pačesová et al., 2015).Therefore, while overall neuronal activity does not differ, specific parts of the opioid receptor signalling pathway may be more sensitive or tolerant reflecting the time-of-day. This suggests there may be specific times at which an individual is more sensitive or averse to rewarding stimuli.

Collectively, the reward pathway in rodents exhibits peaks and troughs in activity during the active and inactive phases respectively. From an evolutionary perspective having a heightened reward pathway during the active phase is beneficial allowing an individual to better discriminate between rewarding and aversive stimuli, thus enabling motivational value to be assigned to objects pertinent for survival such as food, water and sex. This same mechanism however, may now exacerbate the rewarding properties conferred by drugs of abuse, such as alcohol.

2.3.2 Behavioural consequences of circadian rhythm in reward mechanisms

Given the diurnal variation in the expression of dopamine, it suggests that there are times at which individuals are uniquely sensitive towards drugs of abuse. In rodents, the self-administration of cocaine, opioids, barbiturates and phencyclidine typically

peaks during the active period (dark) and nadirs during the mid-to-late inactive period (light) (Perreau-Lenz et al., 2012; Lynch et al., 2008; Baird & Gauvin, 2000; Gauvin et al., 1997; Lukas et al., 1984; Deneau et al., 1969). Further, self-administered electrical brain stimulation of reward pathways peaks during the active phase (Terman & Terman, 1975; Terman & Terman, 1970). This technique is independent of metabolism suggesting that time-of-day effects in reward behaviour are not simply due to altered pharmacodynamics. However, these time-of-day results are far from uniform. For example, cocaine-induced conditioned place preference is greatest early in the inactive (light) phase and the lowest during active (dark) phase (Kurtuncu et al., 2004; Abarca et al., 2002). This suggests that time-of-day effects are dependent on the type of drug and the behavioural measure assessed.

Like most drugs of abuse, alcohol intake peaks during active phase and nadirs during the inactive phase (Gauvin et al., 1997). However, supporting experiments to eliminate the confounding variables such as thirst, taste and aversion have not been performed. Therefore, it is unclear whether the time-of-day dependent effects are due solely to alcohol's rewarding properties ("liking" and "wanting"). Associative evidence suggests saccharin (an innate inducer of the "liking" component of reward) intake peaks during the dark phase (Perreau-Lenz et al., 2009) – indicating that the time-of-day effects observed with alcohol may also be due to rhythmic fluctuation in "liking". No study has examined the "wanting" component of alcohol reward with respect to the time-of-day.

Aside from reward as a driver of alcohol intake, thirst and energy demand must also be considered. The circadian clock entrains to, and is entrained by food and water intake (see Eckel-Mahan & Sassone-Corsi, (2013) for review). When food and water is freely available, rodents usually eat and drink during the active (dark) phase. This effect is entrained by light cues and is thus mediated by the SCN and hypothalamus.

The hypothalamus subsequently interacts with peripheral organs such as the liver and pancreas to regulate metabolism via the release of hormones such as leptin and ghrelin (Waddington Lamont et al., 2007). If food availability is restricted, the activity of these organs shift to coincide with food intake (Mistlberger & Antle, 2011). Therefore, studies assessing alcohol intake must consider whether the effects of a treatment aimed at attenuating alcohol intake is related to alterations in thirst, hunger or reward.

Collectively, the behavioural outcomes following acute drug exposure are dependent on time-of-day. In contrast, the long-term use of drugs are not dependent on time-of-day rather, they alter the expression of CLOCK, BMAL1, PER and CRY (Prosser et al., 2014; Glass et al., 2012; Melendez et al., 2011; Chen et al., 2004; Tornatzky & Miczek, 1999). Therefore, long-term alcohol or drug use functions as a zeitgieber entraining the circadian rhythm to when alcohol is usually consumed (Stowie et al., 2015; Brager et al., 2010). However, unlike other zeitgiebers that are predictable, for example, light, alcohol intake during periods of addiction is unpredictable and unstable (American Psychiatric Association, 2013; Koob & Le Moal, 1997). This in turn dysregulates numerous neurobiological processes which are dependent upon rhythmic oscillations and can lead to immune dysfunction and heightened levels of circulating stress hormones (Voigt et al., 2013).

2.4 Rhythmic oscillations in the immune system

2.4.1 Peripheral immune system

In addition to the effects on the reward pathway, the activity of the immune system is dependent on time-of-day (Bass & Lazar, 2016). These oscillations are driven by sympathetic and parasympathetic effects from the SCN (for example, glucocorticoid release) and the molecular clock mechanisms that regulate the; movement of innate and adaptive immune cells; expression of pattern recognition receptors and their

signalling pathways; transcription and translation of cytokines and complement proteins; and specific immune activities (for example, phagocytosis) (Guerrero-Vargas et al., 2014; Gibbs et al., 2012; Silver et al., 2012; Spengler & Kuropatwinski, 2012; Barriga et al., 2001). The time-of-day dependent effects are regulated primarily at the transcription-translation stage. For example, BMAL1-CLOCK bind to E-boxes in the promoters of chemokine genes such as CCL2 and CCL8 (Nguyen et al., 2013); CLOCK can directly interact with the p65 subunit of NFκB enhancing its activity (Spengler & Kuropatwinski, 2012); REV-ERBs, a negative regulator of the molecular clock, mediates the recruitment of nuclear receptor co-repressor and histone deacetylase to promoter regions, down regulating gene expression (Lam et al., 2013); and glucocorticoid receptor binds to NFκB and AP-1 repressing their activities (Dickmeis et al., 2013; Coutinho & Chapman, 2011). These regulatory processes act in a concerted manner, temporally gating specific parts of the immune response to distinct times of the day. For example, the highest number of leukocytes peaks at ZT5 and the expression of TLR9 nadirs at ZT6 (Gibbs et al., 2012; Scheiermann et al., 2012; Silver et al., 2012). These regulatory processes limit various aspects of the immune system to specific times of the day to prevent synchrony thereby decreasing the likelihood of inducing a “cytokine storm” such as those seen during sepsis (Curtis et al., 2014).

Given daily oscillation in immune processes, the degree of immune response and the subsequent susceptibility to immune challenges, such as LPS-induced septic shock, vary according to time-of-day (Shackelford & Feigin 1973; Halberg et al., 1960). It is important to note that while each particular aspect of the immune system peaks and nadirs at different circadian phases, in general the immune system and the subsequent lethality induced by LPS can be partitioned into two phases (Curtis et al., 2014). As rodents’ transitions to their active period (ZT9 – ZT15), a phase in which the risk of

infection or injury is the greatest, the immune system is in a state of heightened sensitivity with increased number of immune cells and inflammatory cytokines and enzymes. As the individual transitions to the inactive, rest stage (ZT21 –ZT3) where the likelihood of infection and injury is lessened, there is a reduction in inflammatory related processes and an increase in the expression anti-inflammatory and repair molecules (see Curtis et al., (2014) for review).

Like drugs of abuse, immune activation can modulate circadian oscillations. For example, LPS can disrupt the phase, period and amplitude of the molecular clock shifting the phase of optimal immune response, and can disrupt interaction between cellular clock components and immune-related transcription factors; limiting the anti-inflammatory actions of clock proteins (for example; Wang et al., 2016; Okada et al., 2008). Consequently, it is hypothesised that there would be a state of heightened inflammation irrespective of time of day.

2.4.2 Neuroimmune system

The circadian oscillation in immune function has primarily been characterised in the periphery. However, almost all cells of the neuroimmune system are influenced by cell extrinsic clocks and express the molecular machinery necessary for the cell intrinsic clocks (Fonken et al., 2015; Prolo, 2005). Consequently, neuroimmune cells exhibit circadian rhythms *in vivo* and *in vitro*. For example, the protein and mRNA expression of GLAST (an astrocyte-specific glutamate transporter) peaks during the middle of the active phase and nadirs at the beginning of the inactive phase (an effect which is regulated alcohol intake) (Spanagel et al., 2004); the expression of GFAP exhibits rhythmic oscillations in the SCN with astrocytes appearing more star-shaped during the active phase compared to the inactive phase (Vialle & Servière, 1993); and SCN astrocytes increase secretion of ATP (a DAMP) in the latter half of the active cycle

compared to the inactive cycle (Womac et al., 2009). Further, microglia display rhythmic fluctuations in basal inflammatory gene expression (IL-1β, TNFα and IL-6) with the magnitude of an immune response towards LPS and subsequent behavioural changes dependent on the light cycle - with the greatest effect observed during the inactive phase (Fonken et al., 2015). Interestingly, *ex vivo* microglia isolated from older rats exhibit a flattened circadian-dependent expression in cytokine levels and diminished circadian rhythms compared to young rats (Fonken et al., 2016). However, caution must be used when examining *ex vivo* microglia as they no longer receive the feedback from the SCN resulting in dysfunction and loss of synchronisation and rhythm.

2.4.3 TLR4

While the activity of the neuroimmune cells fluctuates according to time-of-day, it is unclear whether an immune response, specifically, a TLR4-based immune response is dependent on circadian rhythm. Fonken et al., (2015) demonstrated LPS induces a potentiated immune response during the inactive period (ZT6) compared to the active period (ZT16) with increases in IL-1β, IL-6 and TNFα, and decreased IL-10 protein and mRNA. No other study has examined a TLR4-based immune response in the central nervous system. Rather, most studies have examined the time-of-day effects of TLR4 in the periphery. For example, Keller et al., (2009) demonstrated that 8 per cent of the macrophage transcriptome oscillates throughout the day with genes involved in the regulation and expression of TLR4 and its downstream signalling pathways (MyD88 and TRIF) varying substantially. In general, the expression of the TLR4 signalling pathway was greater during the beginning of the active (night) compared to the inactive phase. Further research supports these findings as LPS-induced production of inflammatory cytokines such as IL-6, IL-12, CCL2 and CCL5 from macrophages and recruitment of leukocytes is greater at the beginning of the active (ZT12) compared to

inactive phase (ZT0) (Gibbs et al., 2012). The potentiated immune response during the active period was attributed to the increased interaction between NFκB and CLOCK during the active phase. Collectively, the results from the periphery contrast those in the central nervous system with exaggerated levels of TLR4-induced cytokines during the active phase compared to the inactive phase. Further studies are needed to determine whether there are time-of-day differences between the MyD88 and TRIF pathways in the periphery and the brain.

2.5 Study 1 aims and hypothesis

Given the expression of reward and TLR4 pathway both exhibit time-of-day dependent effects, and that TLR4 activation potentially alters acute reward behaviour, it raises the possibility that an alcohol-induced TLR4 signal exhibits a greater influence on the reward pathway reflecting the time-of-day. Consequently, drugs targeting the TLR4 pathway may be more or less effective at attenuating reward processing during specific times of the day. Therefore, the aims of this study were to determine:

1. whether the “liking” and “wanting” components of alcohol reward vary according to the time-of-day;
2. whether the TLR4 signalling and reward pathway exhibits peaks and nadirs in expression which reflect the time-of-day;
    a. whether the TRIF or MyD88 pathway exhibit time-of-day effects; and
3. whether the efficacy of a TLR4-TRIF antagonist on attenuating the “liking” and “wanting” component of alcohol reward is dependent on the time of day.

Given that a large body of work demonstrates the expression of peripheral TLR4 and molecular mediators of reward is greatest during the active phase, it was hypothesised that attenuating TLR4 during this phase would have the greatest effect on reward-like behaviour in mice.

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Chapter 3: The efficacy of (+)-Naltrexone on alcohol preference and seeking behaviour is dependent on light-cycle.

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3.1 Abstract

Circadian rhythm affects drug-induced reward behaviour and the innate immune system. Peaks in reward-associated behaviour and immune responses typically occur during the active (dark) phase of rodents. While the role of the immune system, specifically, Toll-like receptor 4 (TLR4, an innate immune receptor) in drug-induced reward is becoming increasingly appreciated, it is unclear whether its effects vary according to light-cycle. Therefore, the aim of this study was to characterise the effects of the phase of the light-cycle and the state of the innate immune system on alcohol reward behaviour and subsequently determine whether the efficacy of targeting the immune component of drug reward depends upon the light-cycle.

This study demonstrates that mice exhibit greater alcohol-induced conditioned place preference and alcohol two-bottle choice preference during the dark cycle. This effect overlapped with elevations in reward-, thirst- and immune-related genes. Administration of (+)-Naltrexone, a biased TLR4 antagonist, reduced immune-related gene expression and alcohol preference with its effects most pronounced during the dark cycle. However, (+)-Naltrexone, like other TLR4 antagonists exhibited off-target side effects, with a significant reduction in overall saccharin intake – an effect likely attributable to a reduction in *tyrosine hydroxylase* (*Th*) mRNA expression levels. Collectively, the study highlights a link between a time-of-day dependent influence of TLR4 on natural and alcohol reward-like behaviour in mice.

Key words: Toll-like receptor 4, TRIF, alcohol, circadian, tyrosine hydroxylase, interferon, time-of-day

3.2 Introduction

Alcohol is the most widely consumed drug globally (WHO, 2015). The initial consumption of alcohol is characterised by its rewarding, hedonic properties. These properties assist in the development of repetitive/habitual use, which can lead to misuse, loss of control of intake, and addiction (Koob & Le Moal, 2001). The rewarding properties of alcohol are attributable to its actions on the brain's mesolimbic system (Imperato & Di Chiara, 1986). Specifically, dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) are thought to mediate reward and attach salience (Koob & Volkow, 2009; Wise, 2004). Alcohol activates these neurons via multiple pathways for example, alcohol; causes the release of GABA, or directly activates the GABAA receptors in VTA; increases opioid peptides in the VTA and NAcc; and alters glutamate signalling which innervates the NAcc's dopaminergic projections (see Nestler, (2005) for review). Collectively, these processes control dopaminergic neurotransmission thereby influencing reward, specifically, the likability of, and the motivation to consume alcohol (Robinson et al., 2013).

The extent to which alcohol initially activates the mesolimbic system is dependent upon the time-of-day (circadian rhythm, and associated diurnal or nocturnal behaviours). Within mammals, circadian rhythm is generated and maintained, at a circuit level via, the suprachiasmatic nucleus (SCN), and on a cellular level, by a feedback loop involving a group of transcription factors (Reppert & Weaver, 2002). The SCN functions as the master regulator, linking the external (via the retinal-hypothalamic tract) and internal environments (Moore & Lenn, 1972; Stephan & Zucker, 1972). Consequently, the SCN sends neural and endocrine signals to regulate the function of organs and cells according to the time-of-day (for example Moore & Eichler, 1972). However, most cells can generate their own rhythm via an auto-regulatory feedback loop involving

transcription factors. These transcription factors include two activator (CLOCK and BMAL1) and two repressor proteins (PER and CRY). In brevity, the cellular clock begins when CLOCK and BMAL1 dimerise and bind to enhancer box (E-box; DNA response elements) in the promoter region of CRY and PER, initiating their transcription. PER and CRY accumulate, dimerise and suppress the transcription of CLOCK and BMAL1. The subsequent decline in CLOCK and BMAL1 decreases the transcription of PER and CRY. This rhythmic interaction generates the cellular circadian rhythm over a 24 h period (Partch et al., 2014; Dardente & Cermakian, 2007), which is estimated to influence approximately 2 – 10 per cent of the mammalian transcriptome (Miller et al., 2007; Akhtar et al., 2002; Duffield et al., 2002).

The circadian influence on the reward pathway varies according to brain region and cell type. In the NAcc, dopaminergic and non-dopaminergic cell activity peaks during the active (dark) phase of rodents (Baltazar et al., 2013). This is attributable to transient elevations in transcription and translation of genes pertinent to the function of the mesolimbic system, such as tyrosine hydroxylase and the dopamine transporter (Chung et al., 2014; Ferris et al., 2014). Behaviourally, this manifests as a heightened sensitivity towards rewarding experiences to alcohol and other drugs of abuse during the active phase and a lower sensitivity during the inactive phase. For example, alcohol intake, alcohol preference and self-intracranial electrical stimulation of the reward pathway are greatest during the active (dark) phase relative to the inactive (light) phase (Perreau-Lenz et al., 2012; Gauvin et al., 1997; Terman & Terman, 1975). However, each drug of abuse appears to be unique, as cocaine-induced conditioned place preference is greatest during the light cycle (Kurtuncu et al., 2004).

Alcohol-induced reward-like behaviours are additionally influenced by the neuroimmune system (Crews et al., 2017; Lacagnina et al., 2016; Cui et al., 2014).

Particular emphasis has been placed on Toll-like receptor 4 (TLR4), a pattern recognition receptor as a key mediator of reward induced by alcohol and other drugs of abuse (Bachtell et al., 2015). Activation of TLR4 results in the induction of two signalling pathways (MyD88 or TRIF) that culminates in the expression of classical pro-inflammatory cytokines or type-one interferons respectively (Akira & Takeda, 2004). Alcohol and other drugs of abuse activate TLR4 (either directly or indirectly) resulting in the induction of inflammatory mediators (Fernandez-Lizarbe et al., 2013; 2009). It is hypothesised that inflammatory mediators act on neighbouring neurons within the mesolimbic system (tetrapartite synapse), culminating in altered neuronal function and potentially influencing the presentation of reward-like behaviours (Lacagnina et al., 2016; Jacobsen et al., 2014).

Translationally, the effects of TLR4 on alcohol reward-like behaviour are mixed with studies demonstrating either attenuation or no effect on alcohol intake and preference (Harris et al., 2017; Aurelian et al., 2016; Bajo et al., 2016; June et al., 2015; Liu et al., 2011; Pascual et al., 2011). These discrepancies are likely due to methodological differences between the studies including brain region examined and method of drinking. Interestingly, despite TLR4 being implicated in alcohol drinking behaviour, few studies have considered which TLR4-signaling pathway (MyD88 or TRIF) is driving the alterations in reward behaviour (Blednov et al., 2017; Harris et al., 2017), and they have not considered the time-of-day associated rhythmicity of TLR4 expression and the subsequent impact this may have on behaviour.

Similar to the cellular and molecular components of the mesolimbic system, the expression of the TLR4-signaling pathway oscillates according to the time-of-day (Bass and Lazar, 2016). In the periphery, peak expression of TLR4-related signalling and inflammatory molecules are observed at the onset of their active (dark, nocturnal)

phase and nadirs at the beginning of the inactive (light, diurnal) phase (Keller et al., 2009). Within the brain however, the opposite response is observed. Isolated hippocampal microglial cells exhibit peak TLR4-related gene expression during the light phase compared to the dark phase (Fonken et al., 2015). However, it is unclear whether the MyD88 or TRIF pathway fluctuates according to circadian rhythm within the brain and periphery and whether these signalling pathways are involved in alcohol-induced reward-like behaviour. Therefore, this study sought to determine whether light cycle (dark vs light) differences exist in the expression of MyD88, TRIF and their downstream signalling molecules in naïve mice and mice following alcohol exposure. This study also investigated whether the efficacy of a biased TLR4 antagonist on attenuating reward-like behaviour is dependent on the light-cycle. The results presented suggest that the preference for rewarding and aversive compounds peaks and nadirs during the dark cycle with reward-, thirst-, hunger-, and immune-related genes following a similar pattern. We demonstrate that attenuating TLR4 via (+)-Naltrexone reduces alcohol drinking and conditioned place preference (key indicators of reward) with the degree of attenuation greater during the dark cycle. However, we found that (+)-Naltrexone additionally reduces saccharin preference. These effects coincide with a reduction in *Tlr4* and *Ifnb* and *Th* mRNA in the nucleus accumbens. Collectively these results suggest TLR4 may play a role in dopamine synthesis and natural reward-like behaviour.

3.3 Methods

3.3.1 Animals

Male (8 – 10-week-old) Balb/c mice, obtained from the University of Adelaide Laboratory Animal Services (Adelaide, SA, Australia) were used for the following experiments. Mice were housed in light/dark (12:12 hours) and temperature controlled rooms (23±3°C) with food and water available ad libitum. The light cycle began at 7am

(ZT0) and concluded at 7pm (ZT12). Following seven days of acclimatisation, mice were handled by the experimenter for five days prior to experimentation. Mice were weighed daily throughout the handling and experimental periods. All animal care and experiments complied with the principles of the Australian Code of Practice for the care and use of animals for scientific purposes and were approved by the University of Adelaide's Animal Ethics Committee.

3.3.2 Drugs

Saccharin and quinine were purchased from Sigma Aldrich (St Louis, MO, USA). Ethanol (99.5%) (herein referred to as alcohol) was purchased from Chemsupply (Gliman, SA, Australia). Oral gavages of alcohol were dosed at 1.5g/kg and 3.2g/kg (25 per cent v/v) for conditioned place preference and molecular studies respectively. Saline oral gavages were volume-matched. The dose of alcohol used in conditioned place preference was based upon the effective dose 50 from an unpublished conditioned place preference dose response curve. 3.2g/kg was derived from the mean 2 h intake of alcohol from mice on the first day of drinking in the dark tests.

(+)-Naltrexone, a TLR4-TRIF antagonist was synthesised and kindly supplied by Dr Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute of Alcohol Abuse and Alcoholism, Bethesda, MD, USA). (+)-Naltrexone was administered via intraperitoneal injections with doses ranging from 1 to 75 mg/kg (dose volume 10 ml/kg). Saline intraperitoneal injections were volume-matched.

3.3.3 Rationale of behavioural tests

This study implemented a range of paradigms to assess alcohol reward. Specifically, this study was designed to assess two components of reward, likability and seeking

behaviour (motivation to obtain alcohol “wanting”). The likability of alcohol was assessed using the two-bottle choice paradigm. Despite its relative coarseness in obtaining accurate drinking information, the two-bottle choice test can inform researchers about the general avidity of alcohol (Tabakoff & Hoffman, 2000). For example, the consumption of low concentration alcohol is largely driven by taste. By contrast, the consumption of alcohol at higher concentrations are attributable to its actions on the mesolimbic pathway, as increasing the concentration of alcohol imparts an increasingly bitter and aversive taste (Spanagel, 2000; Tabakoff & Hoffman, 2000). Consequently, when testing the likability of alcohol, a range of concentrations must be assessed.

The two-bottle choice test is limited in its assessment of the motivational properties of alcohol (Tabakoff & Hoffman, 2000). To infer this component of alcohol, conditioned place preference was utilised. Conditioned place preference is a paradigm in which mice learn to associate alcohol in one particular environment. If alcohol is hedonic (reinforcing), the mouse will choose to spend more time in that environment over another when given free access to both. By contrast, if the mouse finds alcohol aversive, it will spend less time in the environment. Thus, the motivation to seek alcohol is illustrated by the time spent in the paired environment in the absence of receiving alcohol (Bardo & Bevins, 2000).

To control for taste, and basal hedonic tone, the preference for quinine and saccharin were assessed. Quinine, a bitter compound is thought to reflect the higher concentrations of alcohol while saccharin, a sweet compound is thought to reflect the lower concentrations of alcohol. Additionally, saccharin is innately reinforcing, thus if mice exhibit deficits in basal hedonic behaviour, this will become evident during the test.

3.3.4 Experimental design

Testing began at ZT2 and ZT14 for mice undergoing tests during the light (inactive) and dark (active) phase respectively (figure 1). The behavioural experiments ranged from 2 – 24 h. For studies evaluating (+)-Naltrexone (and saline), mice were injected 30 min prior to undergoing behavioural testing (ZT1:30 and ZT13:30 for light and dark phases respectively) (figure 1).

3.3.5 Alcohol drinking tests

Three alcohol-drinking paradigms were used for the following experiments: 24 h and 8 h two-bottle choice and drinking in the dark.

3.3.5.1 Alcohol two-bottle choice

Alcohol drinking and preference was assessed using an 8 or 24 h two-bottle choice paradigm. Following 14 days of acclimatisation and handling, mice were placed into individual cages. After a further week of acclimatisation, mice were presented with two bottles containing water 2 h after the beginning of the light or dark cycle (ZT2 or ZT14 respectively) for 8 or 24 h. Two bottles of water were initially presented to mice in order to control for novelty-induced drinking. For the 8 h test, the bottles were removed at ZT10 or ZT22, weighed and replaced with a single bottle of water randomised to either the left or right side of the cage for the remaining 16 h. For the 24 h two-bottle choice test, after the test period had elapsed the bottles were removed, weighed and replaced with two new bottles.

Following five days of drinking water from two bottles, mice were offered one bottle containing water and the other 3, 6, 9, 12, 15, 18, 21 or 42 per cent of alcohol (v/v). The concentration and bottle position was randomised daily to prevent the immediate acquisition of alcohol drinking and side preferences respectively. After 8 h bottles were

removed weighed and replaced by a water bottle randomly allocated to either side of the cage lid. For mice in the longer test, the bottles were replaced with two new bottles (one containing alcohol the other water) after 24 h.

The amount of alcohol consumed was determined by the difference in bottle weights before and after drinking sessions. This enabled the calculation of the amount of alcohol consumed per kilogram bodyweight (grams/kilogram) and the preference ratio (alcohol intake ÷ (total water + alcohol intake)) for each mouse and averaged for each group per concentration of alcohol. An empty cage with two bottles was used to determine the rate of evaporation and spillage. The rate of evaporation was subtracted from the final weight of test bottles.

3.3.5.2 Saccharin and quinine two-bottle choice

Mice were also tested using the same 8 h paradigm above for saccharin (1, 15, 30, 45 and 60 mM) and quinine (0.001, 0.01, 0.1, 1 and 5 mM) preference. However, instead of the bottle of alcohol, mice received a bottle of saccharin or quinine and a bottle of water.

For studies evaluating the dose of (+)-Naltrexone, the protocol for the 8 h two-bottle choice protocol was followed. However, the concentration of the alcohol, saccharin and quinine were fixed at 12 per cent, 15 mM, 0.1 mM respectively.

3.3.5.3 Drinking in the dark (alcohol and saccharin)

Drinking in the dark, a limited access alcohol intake test was additionally used. Following two weeks of acclimatisation and handling, mice were individually housed. After one week of further acclimatisation, the single bottle of water was replaced with a bottle of 20 per cent (v/v) alcohol 2 h into the beginning of the light or dark cycle (ZT2

and ZT14). After 2 h, the bottle of alcohol was removed weighed and replaced with a bottle of water. This was repeated for the following two days. On the fourth day, mice were offered the bottle for 4 h.

Mice were additionally tested for saccharin (15 mM) consumption using a 2 h limited access paradigm as outlined above. However, this test was not repeated for 4 consecutive days.

3.3.6 Conditioned place preference

Conditioned place preference was used to infer alcohol-seeking behaviour (Bardo & Bevins, 2000).

3.3.6.1 Apparatus

The conditioning apparatus consisted of two conditioning chambers (10.9 (length) x 9.3 (width) x 35 (height) cm) separated by a neutral chamber (16.6 x 4.8 x 35 cm). The neutral chamber contained black walls with grey flooring. The conditioning chambers differed in tactile and visual cues. The flooring of the conditioning chambers were either black plexiglass perforated holes (5 mm apart) or black plexiglass grids (5 mm apart). The walls of each chamber were white or black. The combination of wall colour to floor texture was randomised for each cohort to prevent any inherent biases mice have for a specific texture x colour combination.

During conditioning, a sliding partition restricted access to only one chamber. Movement and time spent in each chamber was recorded using Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA).

3.3.6.2 Procedure

Day 1: Pretest. Mice were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

Day 2 to 9: Conditioning. Mice received an oral gavage of alcohol (1.5 g/kg) and placed within their conditioning chamber for 30 min on days 1, 3, 5 and 7. On days 2, 4, 6 and 8, mice received an oral gavage of saline and placed within the unconditioned chamber for 30 min. Mice received a total of four conditioning sessions with each drug (alcohol or saline).

Day 10: Test. Mice received an oral gavage of saline, were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

To infer whether the conditioning was successful, the time spent in the conditioned chamber during the post-test was subtracted from the time spent in the conditioned chamber during the pre-test.

3.3.7 Blood alcohol concentration assay

Serum alcohol concentration was measured using a commercial kit (ADH-NAD Reagent Multiple Test Vial; Sigma-Aldrich) and performed as per the manufacturer instructions. In brief, it estimates alcohol induced reduction of nicotinamide adenine dinucleotide (NAD+) to NADH in the presence of alcohol dehydrogenase. The reaction is observed by recording the absorbance of 340 nM by the solution. For two-bottle choice tests blood was acquired immediately after behavioral testing by creating a small incision into the tail of the mouse. To determine blood alcohol concentration following conditioned place preference, a separate cohort of mice underwent the conditioned place preference procedure. However, 30 minutes after the last alcohol

conditioning session tail blood was collected. Blood was subsequently collected and spun down (1500g) at 4^{o}C for 10 mins, thereby separating serum from the residual pellet.

3.3.8 Molecular analysis

3.3.8.1 Gavage model

The dose and duration of alcohol administered for the molecular studies were designed to model the drinking in the dark tests. 3.2g/kg was derived from the mean 2 h intake of alcohol from all cohorts of mice on the first day of drinking in the dark tests.

In brief, mice were injected for four consecutive days with either (+)-Naltrexone or saline followed by a gavage of alcohol or saline 30 min later. The injections of (+)-Naltrexone or saline commenced at either ZT1 or ZT13. The gavages of alcohol occurred at ZT1:30 or ZT13:30. On the final day of testing mice were culled at ZT2 and ZT14.

3.3.9 RNA Isolation and qPCR

Brain regions were isolated by placing the brain into an acrylic matrix (Able Scientific, Canning Vale, WA, AUS) and subsequently cutting them into 1 to 2 mm thick sections. The nucleus accumbens and the hypothalamus was subsequently microdissected using micropunches (Kai Medical, Seki City, Japan) and submerged in RNAlater® ICE (ThermoFisher Scientific, Waltham, MA, USA) prior to performing RNA isolation. RNA was isolated using Maxwell® 16 LEV simply RNA Tissue Kit (Promega, Madison, WI, USA) as per manufacturer instructions. RNA was quantified using spectrophotometric analysis, with the quality of RNA verified by the OD260/280 ratio. 900 ng of RNA was reverse transcribed into cDNA using iScript^{TM} cDNA reverse transcription kit (BioRad, Hercules, CA, USA) as per manufacturer instructions.

Gene expression was assessed using iTaq^{TM} Universal SYBR® Green Supermix (as per manufacturer instructions). Real time PCR was performed using the CFX96 Touch^{TM} Real-Time PCR Detection System (BioRad). All primers were synthesised by Integrated DNA Technologies Pte. Ltd. (Baulkham Hills, NSW, Australia) with their sequences outlined in the supplementary material (Table 1).

The relative difference in expression level of each of the genes of interest were normalised to the CT of GAPDH for both the test and control sample. The ΔCT of the test sample was normalised to the ΔCT a control sample (equal amount of cDNA from all samples), and then expressed as a ratio ($2^{-\Delta\Delta CT}$).

3.3.10 Statistical analysis

Experiment 1: The effect of light-cycle on the intake and preference for alcohol, saccharin and quinine was analysed using a two-way ANOVA with Tukey post hoc (figure 2). The effect of light-cycle on conditioned place preference and relative conditioned place preference was analysed using a two-way ANOVA with Tukey post hoc and a paired two-tail t-test respectively (figure 3).

Experiment 2: The effect of light-cycle on hunger-, reward-, thirst- and TLR4-related gene expression was analysed using a paired two-tail t-test (figures 4 and 5).

Experiment 3: The effects of light-cycle and the dose of (+)-Naltrexone on alcohol, saccharin and quinine intake and preference was analysed using two-way ANOVA with Tukey post hoc (figure 6). The effects of light-cycle and (+)-Naltrexone on the intake and preference for varying concentrations of alcohol, saccharin and quinine was analysed using a three-way ANOVA with Tukey post hoc (figure 7). The effects of light-cycle and (+)-Naltrexone on conditioned place preference and relative conditioned

place preference was assessed using three-way ANOVA with Tukey post hoc and two-way ANOVA with Tukey post hoc respectively (figure 8 and s5).

Experiment 4: The effect of light-cycle and (+)-Naltrexone on hunger-, reward-, thirst- and TLR4-related gene expression was analysed using a two-way ANOVA with Bonferonni post hoc (figures 9 and 10).

All summary values presented as mean ± standard error of mean (SEM). p-values ≤ 0.05 were considered statistically significant.

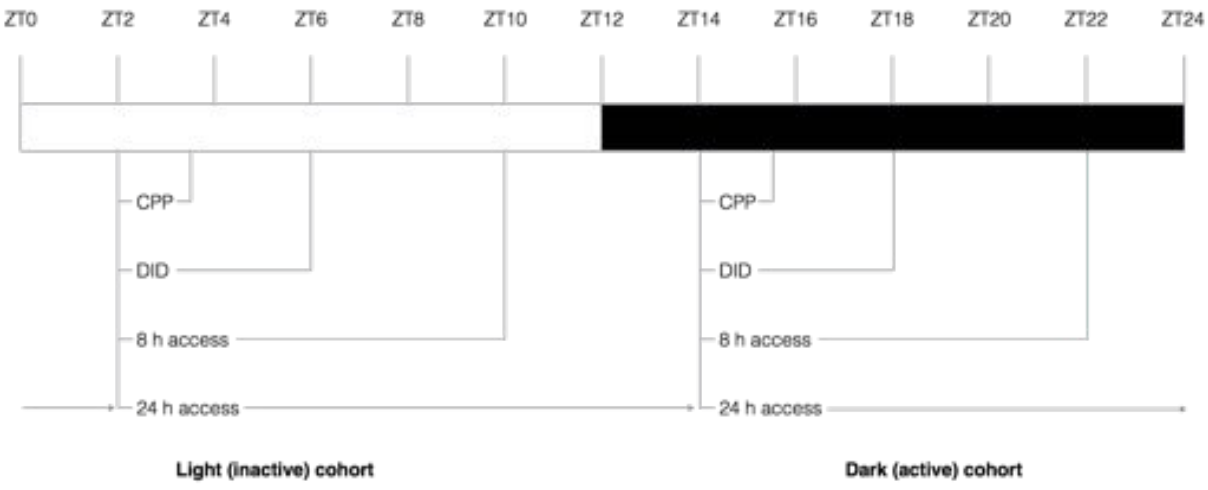

Figure 1 Experimental timeline. All behavioural testing began 2 h into the light or dark phase. Conditioned place preference occurred between ZT2 to ZT3 and ZT14 to ZT15 for mice in the light or dark phase respectively. Drinking in the dark (days 1 – 3) occurred between ZT2 to ZT4 and ZT14 to ZT16 for the light and dark cohorts respectively. On the final day of testing, the test concluded at ZT6 and ZT18 for the light and dark cohorts respectively.

3.4 Results

3.4.1 Experiment 1: Are there light-cycle differences in reward-like behaviour?

To determine whether alcohol reward-like behaviour was dependent on light-cycle, mice underwent the two-bottle choice paradigm for 8 h during the light (ZT2 – ZT10) or dark cycle (ZT14 – ZT22) (figure 2a – b). A two-way ANOVA determined a significant effect of light-cycle on alcohol intake and preference (effect of light-cycle, $F_{(1, 9)} = 5.21$, $p = 0.048$ and $F_{(1, 9)} = 9.16$, $p = 0.014$, respectively). Post hoc analysis determined that mice exposed to alcohol during the dark cycle exhibited significantly greater intake and preference for alcohol compared to those in the light cycle at 21 and 42 per cent.

To determine whether the light-cycle dependent effect on alcohol intake and preference was due to an increased reward or decreased aversion, the preference for two compounds: saccharin, a sweet-tasting and rewarding compound; and quinine, a bitter-tasting and aversive compound, was assessed (figures 2c – f). Similar to alcohol, saccharin intake and preference was dependent on light-cycle (effect of light-cycle, $F_{(1, 9)} = 15.53$, $p = 0.0034$ and $F_{(1, 9)} = 8.32$, $p = 0.015$, respectively) with mice in the dark cycle exhibiting potentiated intake and preference compared to the light cycle at 30 and 60mM (post hoc analysis). However, the behavioural response to quinine was inconsistent. There was a significant effect of light-cycle for intake and the preference ratio (intake ratio) (effect of light-cycle, $F_{(1, 9)} = 4.72$, $p = 0.052$ and $F_{(1, 9)} = 20.31$, $p = 0.0009$, respectively). Overall, mice in the dark cycle exhibited greater intake but a reduced intake ratio for quinine. For all preceding tests the concentration of the solution (alcohol, saccharin or quinine) was a significant variable. The statistical values can be found in the supplementary material.

The light-cycle dependent effects observed in the alcohol, saccharin and quinine drinking tests may be attributable to thirst rather than reward. Indeed, a light-cycle

dependent effect was found for water intake, with mice in the dark cycle exhibiting greater intake (supplementary material, figure s1, $p = 0.0057$). Thus, to control for this confounding variable, conditioned place preference, a reward/memory paradigm which is independent of thirst was used (figure 3a – b). The change in conditioned-chamber time was significantly affected by conditioning drug (effect of drug, $F_{(1, 9)} = 50.45$, $p = 0.004$). However, there was no effect of light-cycle or an interactive effect (effect of light-cycle, $F_{(1, 9)} = 2.41$, $p = 0.17$; and interaction, $F_{(1, 9)} = 0.95$, $p = 0.37$, respectively). However, if the relative change in conditioned place preference is considered (alcohol chamber time – saline chamber time), a significant light-cycle effect emerges. Mice in dark cycle exhibited a greater change in conditioned preference than those in the light cycle (effect of light-cycle, t= 2.17 df = 16, $p = 0.047$). Therefore, for the remaining experiments, relative conditioned chamber time was evaluated.

3.4.2 Experiment 2: Are there light-cycle differences in the molecular basis of reward-like behaviour?

The light-cycle dependent variations in preference and intake are potentially explained by the circadian oscillations in reward-, hunger- and thirst-related genes. Thus, the expression of these genes in the nucleus accumbens and hypothalamus was compared between light (ZT2) and dark (ZT14) cycles (figure 4a – b). A two-tail t-test determined significant light-dark cycle differences in the expression of: *Drd2*, *Oprm1*, *Avp* and *Ghrl* (effect of light-cycle, t = 1.77 df = 4, $p = 0.11$ and t = 3.96 df = 4, $p = 0.0033$; t = 3.18 df = 4, $p = 0.0097$ *and* t = 3.67 df = 4, $p = 0.021$, respectively). This effect was not ubiquitous among reward- and hunger-related genes, as light-cycle had no effect on the expression of *Drd1*, *Th*, *Lepr* and *Rxfp1* (effect of light-cycle, t = 1.77 df = 9, $p = 0.11$; t = 1.00 df = 4, $p = 0.071$; t = 2.45 df = 4, $p = 0.69$ and t = 0.65 df = 4, $p = 0.55$, respectively).

Given the emerging role of the neuroimmune system in the manifestation of reward-like behaviour, the expression of the TLR4-signaling pathway was additionally assessed (figures 5a – b). Light cycle significantly influenced the expression of *Tlr4* mRNA in the nucleus accumbens but not hypothalamus (effect of light-cycle, t = 3.9 df = 4, p = 0.019; and t = 2.97 df = 4, p = 0.069, respectively). By contrast, the expression of *Ccl2* and *Ifnb* mRNA was significantly influenced by light-cycle in the nucleus accumbens (effect of light-cycle, t = 2.27 df = 4, p = 0.05; and t = 2.49 df = 4, p = 0.047, respectively) and hypothalamus (effect of light-cycle, t = 2.57 df = 4, p = 0.049 and t = 2.85 df = 4, p = 0.049, respectively). The effect of light-cycle was more pronounced in the hypothalamus with *Md2*, *Trif* and *Il1b* exhibiting light-cycle dependent effects as well (effect of light-cycle, t = 6.76 df = 4, p = 0.0025; t = 6.18 df = 4, p = 0.0035 and t = 4.0 df = 4, p = 0.043 respectively). No differences were observed in either the nucleus accumbens or hypothalamus for *Cd14*, *Myd88*, *Il10*, or *Hmgb1* mRNA (see supplementary material for results of the statistical analysis).

3.4.3 Experiment 3: Does the efficacy of (+)-Naltrexone on attenuating the reward-like behaviour depend on the light-cycle?

Since light-cycle differences were observed in reward-like behaviour and TLR4-related gene expression, questions arose as to whether TLR4 was causatively involved in these effects, and if it was, were these events mediated by the TRIF- or MyD88-dependent pathway and would the efficacy of a TLR4-based intervention be dependent upon light-cycle. Given the expression of *Ifnb* mRNA was elevated in the nucleus accumbens and hypothalamus, it was hypothesised that the TRIF-pathway may be mediating these effects. Thus, (+)-Naltrexone, a biased TLR4-TRIF antagonist, was used in the following experiments. Interestingly, there was not an effect of (+)-Naltrexone on serum alcohol concentration following 2, 8 or 24 h two-bottle choice paradigms (effect of pretreatment, $F_{(1, 5)}$ = 0.070, p = 0.80, $F_{(1, 5)}$ = 1.59, p = 0.24 and

$F_{(1, 5)}$ = 3.76, p = 0.088 respectively) (figure 4s a – c). However, post hoc analysis determined a significant difference in serum alcohol concentration between saline and (+)-Naltrexone treated mice during the dark cycle in the 8 but not 2 or 24 h tests. Further, there was a significant effect of (+)-Naltrexone on serum alcohol concentration following conditioned place preference (effect of pretreatment, $F_{(1, 5)}$ = 29.93, p < 0.0001). Again, post hoc analysis determined there was a significant difference between (+)-Naltrexone and saline mice during the dark cycle. Therefore, caution must be used when interpreting these studies as (+)-Naltrexone may modify alcohol metabolism following a bolus gavage.

For the following experiments characterising the light-cycle effects on the behavioural pharmacology of (+)-Naltrexone, a significant effect of light-cycle was observed for alcohol, saccharin and quinine intake and preference (figure 6a – f). Like earlier experiments, mice in the dark cycle exhibited significantly greater intake and preference for alcohol and saccharin (supplementary data).

Overall, there was a significant effect of (+)-Naltrexone's dose on the intake and preference for 12 per cent alcohol (effect of dose, $F_{(6, 48)}$ = 15.72, p < 0.0001 and $F_{(6, 48)}$ = 7.57, p < 0.0001, respectively) (figure 6a and b) with a significant interactive effect observed for alcohol intake but not preference (interaction, $F_{(6, 48)}$ = 4.98, p = 0.0005 and $F_{(6, 48)}$ = 0.63, p = 0.70, respectively). Post hoc analysis determined that mice in the dark cycle exhibited a significant reduction in intake and preference from 45 – 75 mg/kg doses of (+)-Naltrexone relative to saline. In comparison, mice in the light cycle exhibited a reduction in preference but not intake at doses of 60 – 75 mg/kg.

The preference but not intake of saccharin was significantly modified by (+)-Naltrexone's dose (effect of dose, $F_{(6, 48)}$ = 3.82, p = 0.0034 and $F_{(6, 48)}$ = 0.56, p = 0.76

respectively) (figure 6c – d). Further, both intake and preference demonstrated interactive effects between (+)-Naltrexone and light-cycle (interaction, $F_{(6, 48)} = 2.01$, $p = 0.024$ and $F_{(6, 48)} = 3.65$ $p < 0.0046$, respectively). Post hoc analysis further demonstrated a significant reduction in saccharin preference in the light-cycle between 30 and 75 mg/kg dose of (+)-Naltrexone - an effect absent in the dark cycle. By contrast, quinine intake but not the intake ratio was significantly affected by (+)-Naltrexone (effect of dose, $F_{(6, 48)} = 3.05$, $p = 0.013$ and $F_{(6, 48)} = 0.79$, $p = 0.58$, respectively). There were no interactive effects for intake or the intake ratio (interaction, $F_{(6, 48)} = 1.7$, $p = 0.15$ and $F_{(6, 48)} = 1.2$, $p = 0.28$, respectively). Collectively, the results suggest (+)-Naltrexone attenuates the intake and preference of alcohol. However, this action of (+)-Naltrexone may be due to non-specific effects as saccharin and quinine intake were significantly perturbed as well.

To further the findings of light-cycle-dependent effects of (+)-Naltrexone on alcohol intake and preference, 60 mg/kg dose of (+)-Naltrexone was administered to mice and their preference for differing concentrations of alcohol was examined. These tests are important, given different mechanisms are thought to govern the responses to low and high doses of alcohol (Kiefer, 1995).

Light-cycle and concentration were found to be significant variables influencing preference and intake for alcohol, saccharin and quinine ($p < 0.05$) (see supplementary data). There was a significant effect of pretreatment on the intake and preference for alcohol (effect of pretreatment, $F_{(1, 344)} = 4.95$, $p = 0.027$ and $F_{(1, 344)} = 13.58$, $p = 0.00027$) (figure 7a – b). No interactive effects were found for intake or preference. However, post hoc analysis determined (+)-Naltrexone significantly reduced alcohol preference at 12, 21 and 42 per cent alcohol compared to saline. There were no post

hoc differences between (+)-Naltrexone and saline during the light phase for intake or preference of alcohol.

There was a main effect of pretreatment on saccharin intake but not preference (effect of pretreatment, $F_{(1, 220)} = 8.95$, $p = 0.0031$ and $F_{(1, 220)} = 0.25$, $p = 0.62$, respectively) (figure 7c – d). A significant interactive effect was observed for saccharin intake (light-cycle x treatment, $F_{(4, 220)} = 9.11$, $p = 0.0026$). However, this effect was not observed for saccharin preference ($F_{(1, 220)} = 1.37$, $p = 0.24$). Further, there were no post hoc differences between the groups for intake or preference. By contrast, quinine intake and the intake ratio were unaffected by pretreatment (effect of pretreatment, $F_{(1, 220)} = 4.315$, $p = 0.09$ and $F_{(1, 220)} = 2.01$, $p = 0.16$, respectively) (figure 7e – f). There were no interactive effects or post hoc differences for quinine intake and the intake ratio.

To provide further evidence indicating TLR4-TRIF involvement in reward/reinforcing behaviour, conditioned place preference was assessed (figure 8). Pretreatment significantly modified relative alcohol-induced conditioned place preference (effect of pretreatment, $F_{(1, 7)} = 20.52$, $p = 0.0027$). Post hoc analysis determined (+)-Naltrexone significantly decreased relative alcohol-induced conditioned place preference time compared saline during the dark only. There was no effect of light-cycle, nor an interaction between light-cycle and pretreatment (effect of light-cycle, $F_{(1, 7)} = 0.0011$, $p = 0.92$; and interaction, $F_{(1, 7)} = 1.62$, $p = 0.24$).

(+)-Naltrexone was additionally screened against a 24 h two-bottle choice and drinking in the dark paradigm. Both paradigms found a significant effect of (+)-Naltrexone with post hoc analysis demonstrating a significant effect of (+)-Naltrexone on alcohol intake during the dark but not light cycle. See supplementary materials for figures and precise statistical information.

3.4.4 Experiment 4: Does the efficacy of (+)-Naltrexone on attenuating the TLR4 pathway depend on the light-cycle?

qPCR was used to identify potential mechanisms underpinning the behavioural changes induced by alcohol and (+)-Naltrexone. There were significant effects of light-cycle, pretreatment ((+)-Naltrexone vs. saline) and drug (alcohol vs. saline) for *Tlr4* and *Ifnb* mRNA expression in the nucleus accumbens (*Tlr4,* effect of light cycle, $F_{(1, 16)}$ = 11.79, p = 0.0034; pretreatment, $F_{(1, 16)}$ = 7, p = 0.022; and drug, $F_{(1, 16)}$ = 6.49, p = 0.021) (*Ifnb* light-cycle, $F_{(1, 16)}$ = 9.09 *p* = 0.0083; pretreatment, $F_{(1, 16)}$ = 8.26, *p* = 0.010; and drug, $F_{(1, 16)}$ = 13.69, *p* = 0.0019). Within the hypothalamus only *Ifnb* exhibited a significant effect of light-cycle ($F_{(1, 16)}$ = 8.92, *p* = 0.0087), pretreatment ($F_{(1, 16)}$ = 13.63, *p* = 0.020) and drug ($F_{(1, 16)}$ = 9.54, *p* = 0.007). *Tlr4* expression was significantly influenced by light-cycle ($F_{(1, 16)}$ = 9.45, *p* = 0.0073), drug ($F_{(1, 16)}$ = 20.14, *p* = 0.004) but not pretreatment ($F_{(1, 16)}$ = 3.71, *p* = 0.072). Post hoc analysis furthered these findings as (+)-Naltrexone attenuated alcohol-induced increases in *Tlr4* and *Ifnb* mRNA expression in the dark but not light cycle.

Interestingly, (+)-Naltrexone did not affect the expression of *Trif* in the nucleus accumbens or hypothalamus (NAcc, effect of light-cycle, $F_{(1, 16)}$ = 0.71, *p* = 0.41; pretreatment, $F_{(1, 16)}$ = 4.46, *p* = 0.053; and drug, $F_{(1, 16)}$ = 0.71, *p* = 0.41) (hypo light-cycle, $F_{(1, 16)}$ = 23.23, *p* = 0.0002; pretreatment, $F_{(1, 16)}$ = 3.21, *p* = 0.092; and drug, $F_{(1, 16)}$ = 1.36, *p* = 0.26). All remaining TLR4-related genes did not exhibit a significant effect of all three variables (statistical information are available in the supplementary material, see figures s5 – 6).

Interestingly, while there was an effect of pretreatment on the expression of *Lepr* ($F_{(1, 16)}$ = 6.44, *p* = 0.022) and *Rxfp1* mRNA ($F_{(1, 16)}$ = 6.01, *p* = 0.026) there was no effect of drug nor light-cycle (see supplementary). However, there was a significant effect of

light-cycle ($F_{(1, 16)}$ = 13.15 p = 0.0023), pretreatment ($F_{(1, 16)}$ = 17.76, p = 0.0007) but not drug ($F_{(1, 16)}$ = 2.38, p = 0.14) on the expression of *Th* mRNA (figure 10). Post hoc analysis determined a (+)-Naltrexone significantly reduced alcohol-potentiated *Th* mRNA expression compared to saline in the dark but not light cycle.

3.4.5 Figures

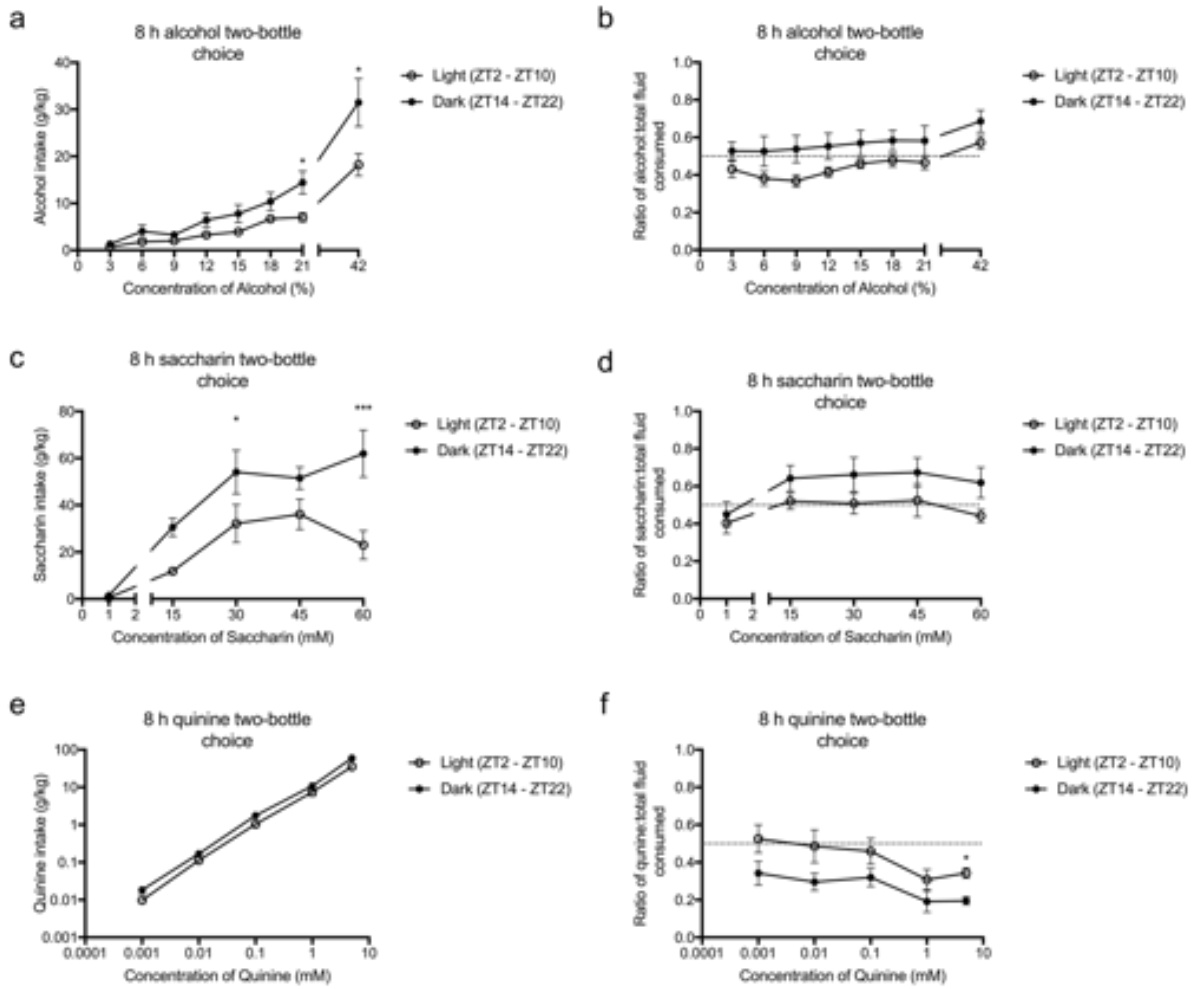

Figure 2 Circadian timing affects the intake and preference of alcohol (a - b) and saccharin (c - d) but not quinine (e - f). There was a main effect of light-cycle on the intake and preference for alcohol (3 – 42%) and saccharin (1 – 60mM) and the intake ratio of quinine (0.001 – 5mM). However, the intake of quinine was independent of light cycle. Post hoc analysis determined significant differences between light and dark at 21 – 42 per cent alcohol (a), 30 – 60mM of saccharin (c) and 5mM quinine (f). All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10, *$p < 0.05$; **$p < 0.01$, *** $p < 0.001$.

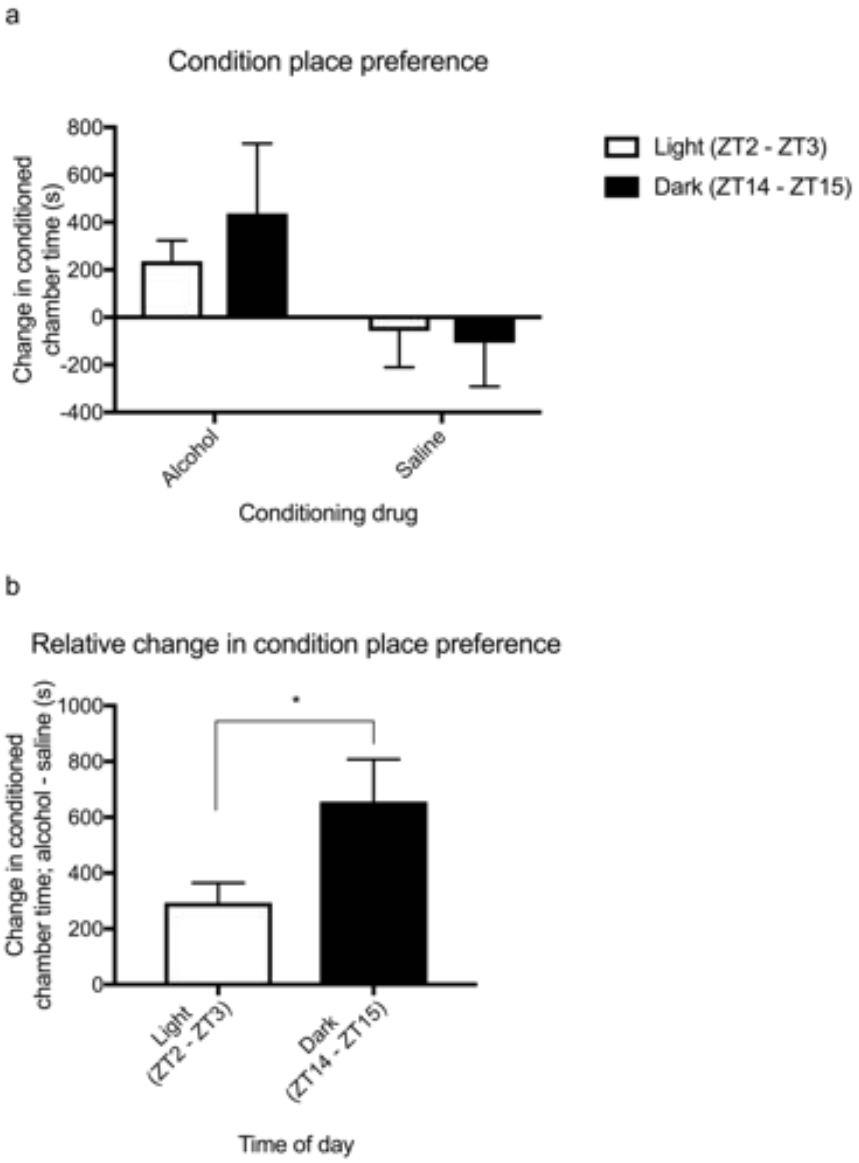

Figure 3 Circadian timing alters the relative preference for an alcohol-induced conditioned place preference. Light-cycle did not alter alcohol-induced conditioned place preference (a). However, when the relative change in conditioned chamber time was assessed, there was significantly greater preference towards alcohol during the dark compared to the light cycle (b). All data was analysed using a two-way ANOVA with Tukey post hoc (a) and a paired two-tail t-test (b). Summary values represented as mean±SEM; n=8, *$p < 0.05$.

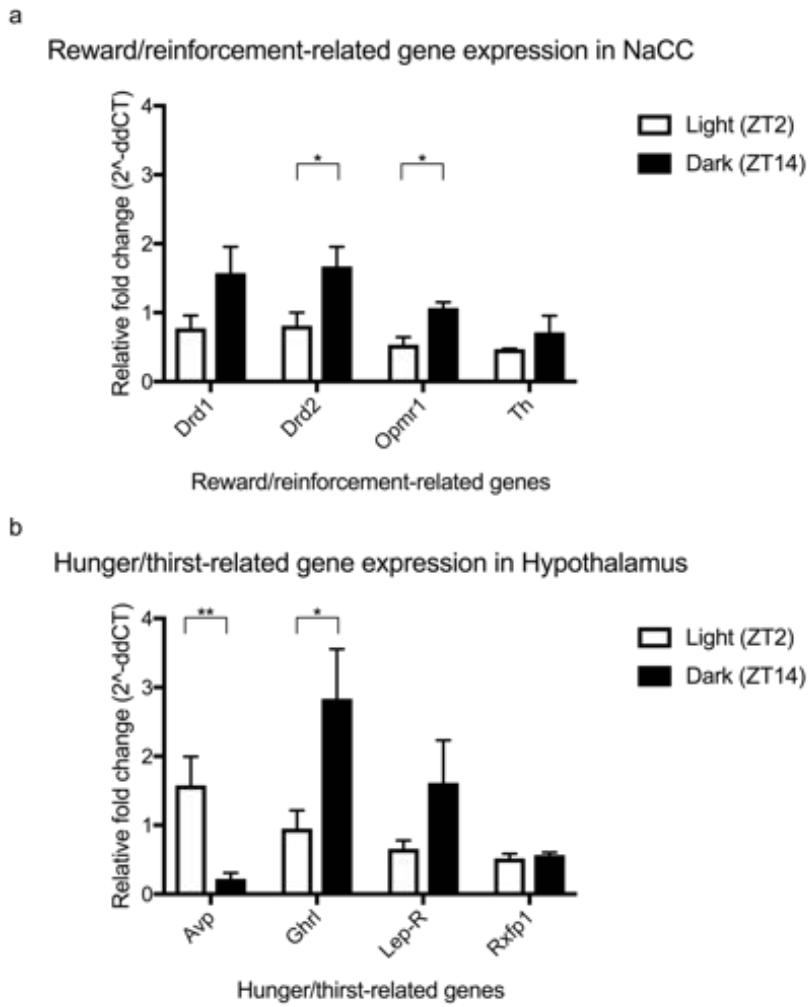

Figure 4. **Circadian timing effects the expression of genes relating to reward (a), thirst and hunger (b).** The expression of *Drd2*, *Oprm1* and *Ghrl* were significantly elevated during the dark cycle compared to the light cycle. By contrast, the expression of *Avp*, was significantly elevated during the light compared to the dark cycle. The expression of *Drd1*, *Th*, *Lepr* and *Rxfp1* was unaffected by light-cycle. All data was analysed using a paired two-tail t-test. Summary values represented as mean±SEM; n=3, *$p < 0.05$; **$p < 0.01$.

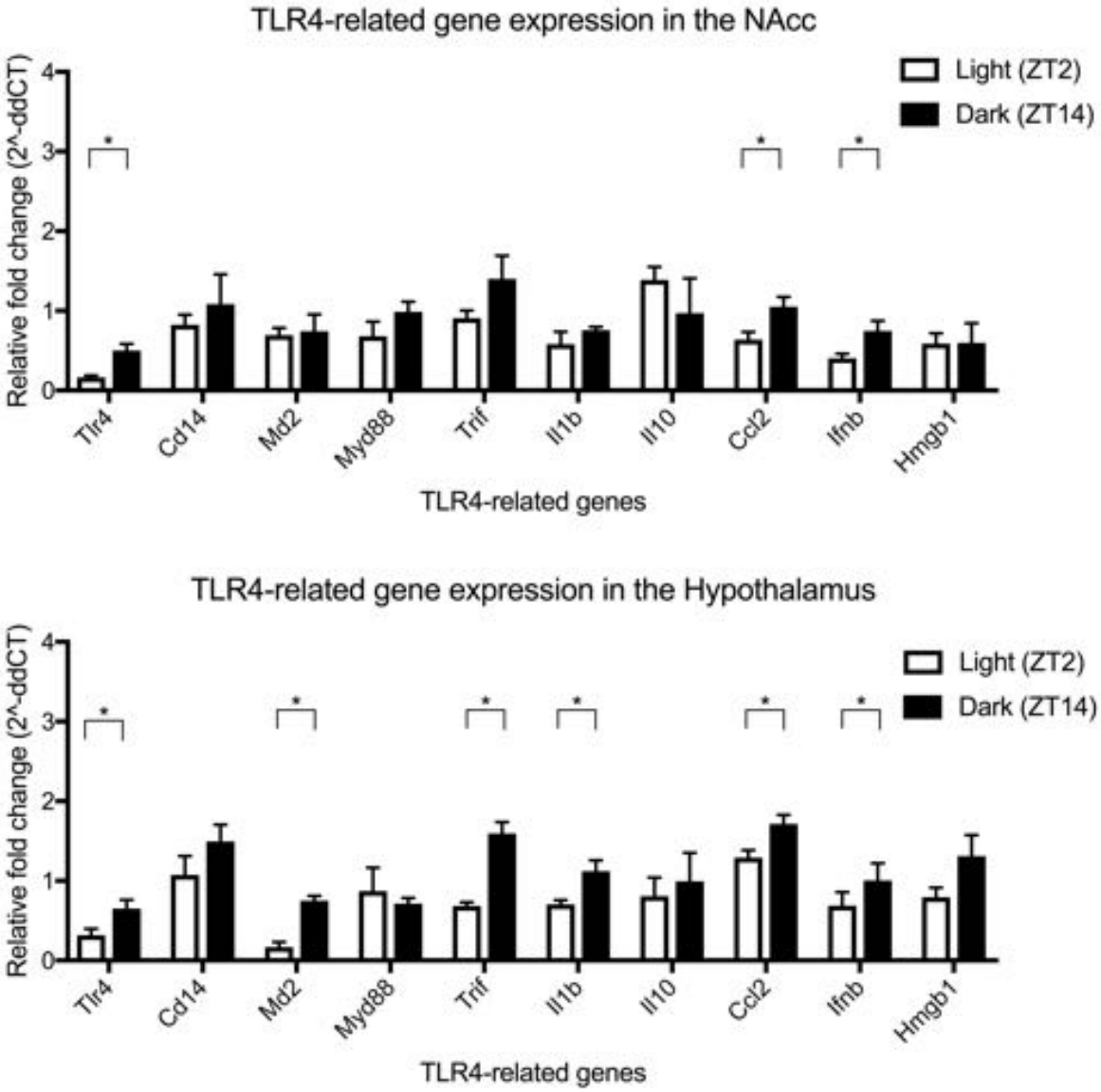

Figure 5 Circadian timing effects the expression of TLR4-related genes in the nucleus accumbens (a) and hypothalamus (b). The expression of *Tlr4*, *Ccl2* and *Ifnb* was significantly greater during the dark cycle compared to the light cycle in the nucleus accumbens. By contrast, the hypothalamus exhibited a more pronounced effect of circadian timing with greater expression of *Tlr4*, *Md2*, *Trif*, *Il1b*, *Ccl2* and *Ifnb* during the dark cycle compared to the light cycle. All data was analysed using a paired two-tail t-test. Summary values represented as mean±SEM; n=3-4, *$p < 0.05$.

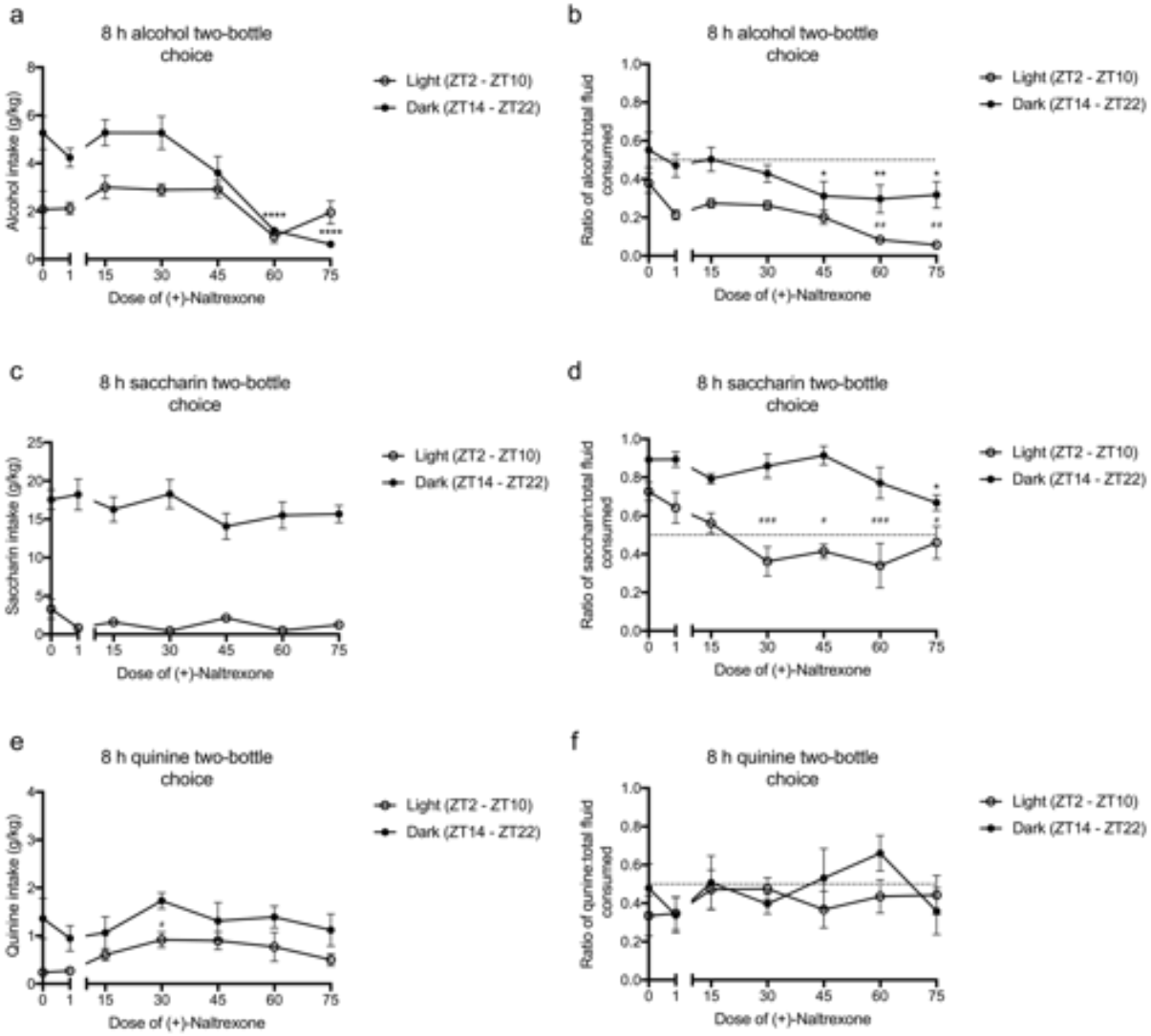

Figure 6 Circadian timing influences the efficacy of (+)-Naltrexone on the intake and preference for alcohol (12%) (a – b), saccharin (30 mM) (c – d) and quinine (0.1 mM) (e – f). (+)-Naltrexone decreased the intake and preference for alcohol with a greater effect observed during the dark cycle compared to the light cycle as inferred by post hoc differences. (+)-Naltrexone did not affect the intake of saccharin, however, the drug significantly decreased the preference for saccharin between 30 - 75mgkg and 75mg/kg in the light and dark cycles respectively. The response to quinine was the opposite, with (+)-Naltrexone altering intake but not intake ratio. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8-10, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared to saline (dark); #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ compared to saline (light).

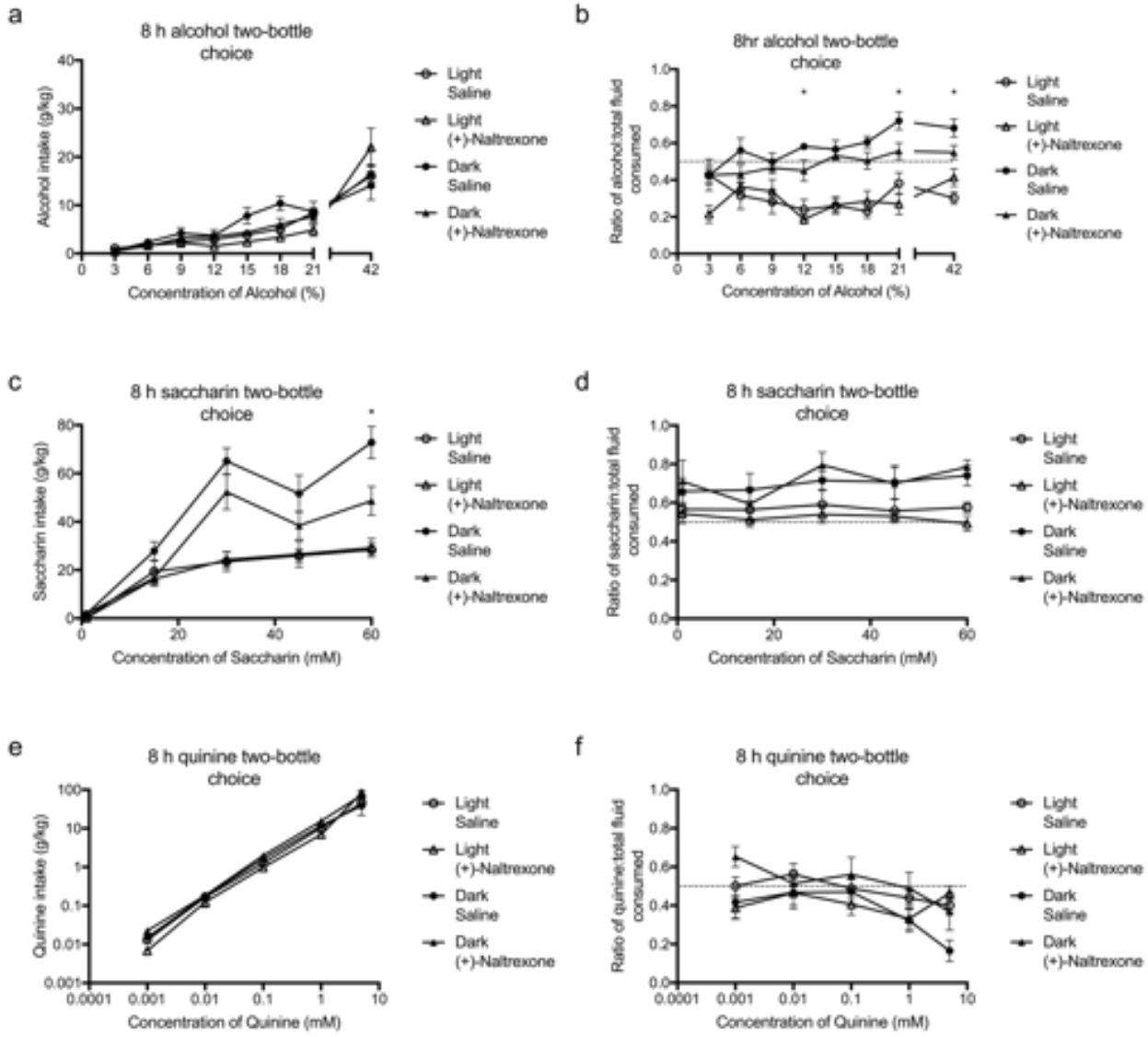

Figure 7 Circadian timing influences the efficacy of (+)-Naltrexone (60 mg/kg) on decreasing the intake and preference for alcohol (a – b) and saccharin (c – d) but not quinine (e – f). There was a main effect of pretreatment on the intake (a) and preference (b) for alcohol (3 – 42%) with (+)-Naltrexone exhibiting a greater effect during the dark cycle (post hoc analysis). Similarly, there was a main effect of pretreatment on intake (c) but not preference (d) for saccharin (1 – 60mM). Pretreatment had no effect on quinine intake (e) or intake ratio (f) (0.001 – 10mM). All data was analysed using a three-way ANOVA with Tukey post hoc Summary values represented as mean±SEM; n=10.

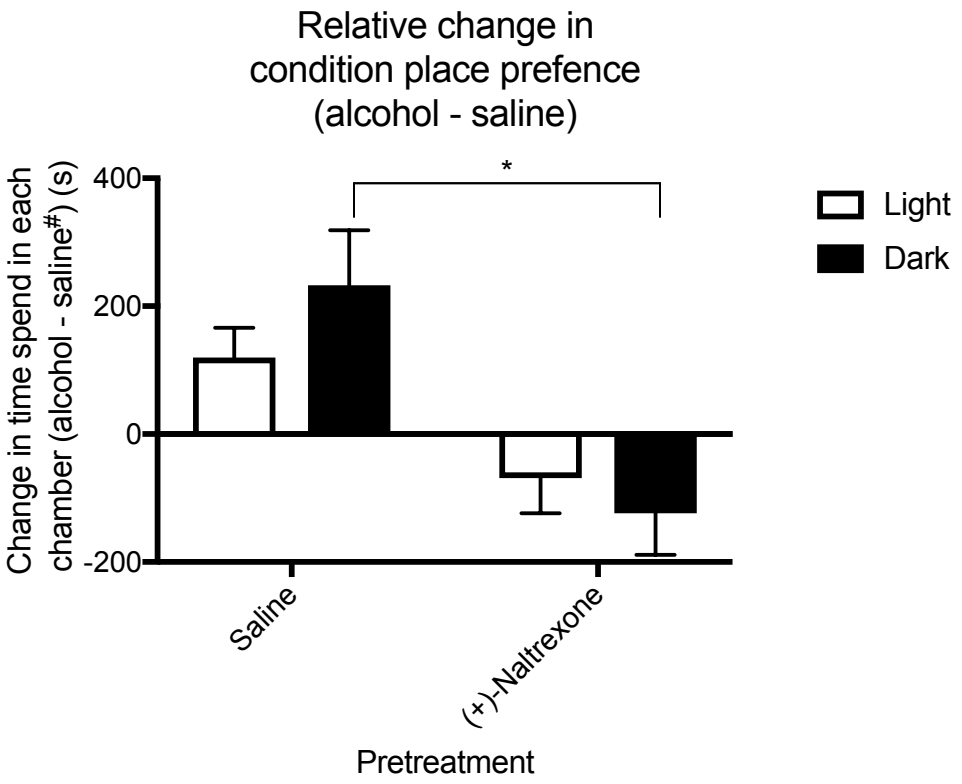

Figure 8 Circadian timing influences efficacy of (+)-Naltrexone on *relative* change in conditioned chamber time. There was a significant effect of pretreatment on alcohol-induced conditioned place preference time with mice in the dark cycle exhibiting a significant reduction between saline and (+)-Naltrexone in conditioned place preference time. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8, *$p < 0.05$. saline# = saline conditioned mice.

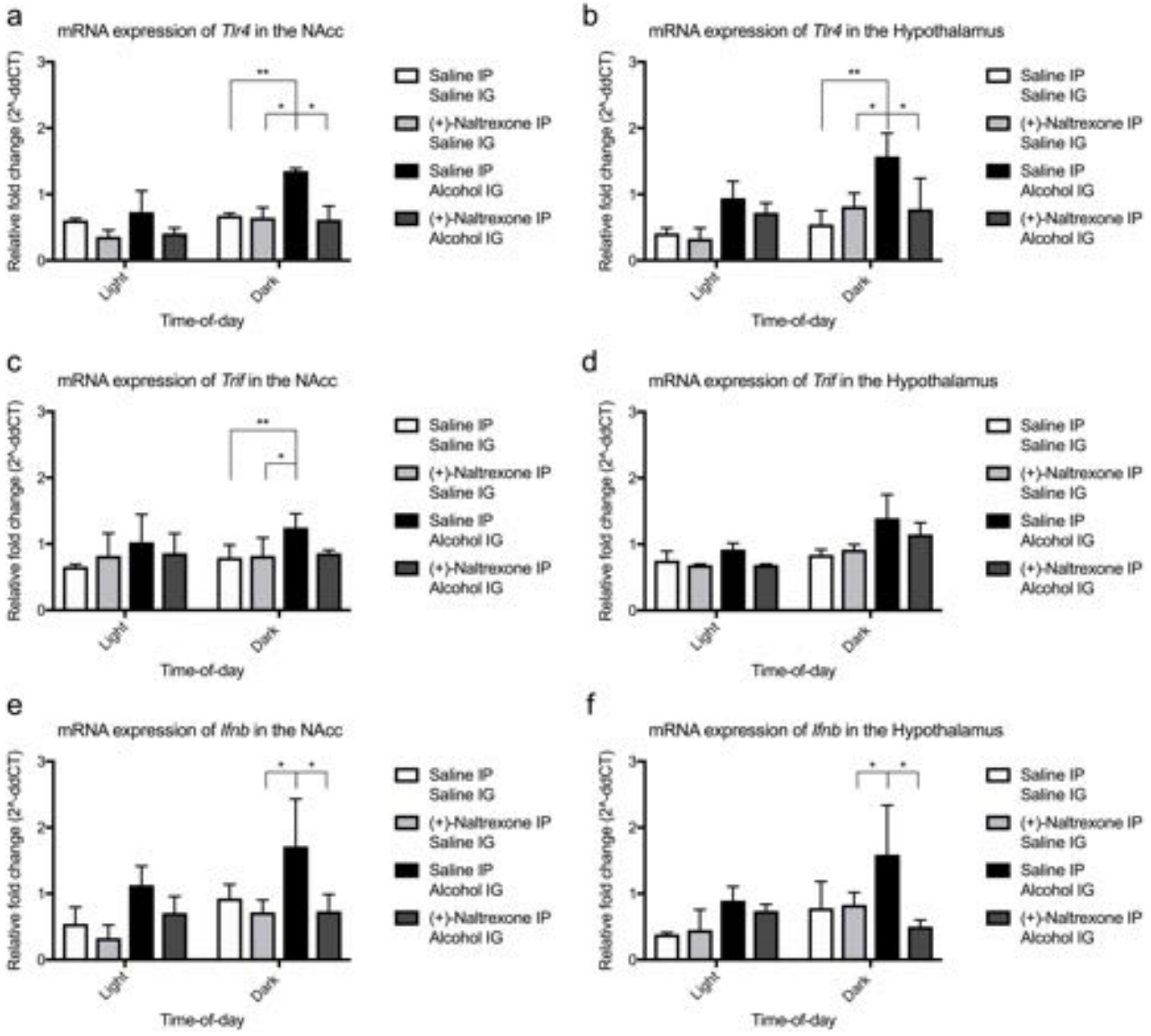

Figure 9 Circadian timing influences efficacy of (+)-Naltrexone on decreasing the mRNA expression of the TLR4-signaling pathway. There was a significant effect of pretreatment on the expression of *Tlr4* and *Ifnb* in the nucleus accumbens and *Ifnb* in the hypothalamus. Post hoc analyses determined differences between the groups were observed in the dark cycle only. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=3, *$p < 0.05$, **$p < 0.01$.

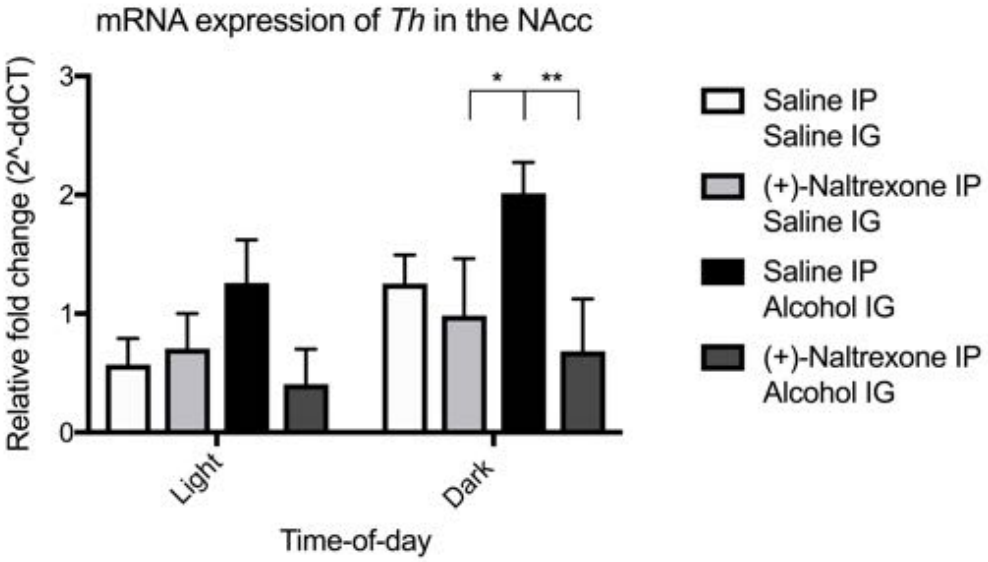

Figure 10 Circadian timing influences the efficacy of (+)-Naltrexone on decreasing the mRNA expression of tyrosine hydroxylase. (+)-Naltrexone significantly reduced the expression of *Th* with its greatest effect observed during the dark cycle. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=3, *$p < 0.05$, **$p < 0.01$.

3.5 Discussion

The current study demonstrates that the intake and preference for alcohol, saccharin and quinine fluctuate according to the time-of-day. The preference for alcohol and saccharin peaked during the dark phase, while quinine preference was greatest during the light phase. This effect coincided with elevations in reward-, thirst- and immune-related genes. This study further highlighted that the efficacy of (+)-Naltrexone, a biased TLR4 antagonist, on attenuating alcohol-induced immune signalling and alcohol preference is dependent on the light-cycle, with the greatest effect again observed in the dark cycle. However, (+)-Naltrexone additionally reduced saccharin intake and preference. These effects are potentially attributable to (+)-Naltrexone's down regulation of *Th* mRNA. Given T5342126, a TLR4-MD2 disruptor, additionally attenuates alcohol and saccharin intake (Bajo et al., 2016), the studies collectively indicate a pivotal link between TLR4 and natural reward-like behaviours.

The effects of the circadian influence on reward and drug seeking behaviour have recently received renewed interest (see Parekh et al., 2015; Perreau-Lenz & Spanagel, 2015; Webb et al., 2015 for review). Earlier studies indicated rodents have a higher preference and intake of alcohol during the dark cycle (Gauvin et al., 1997). Interestingly, the time of heightened sensitivity towards drugs of abuse appears to be unique to each class, as cocaine exhibits its greatest rewarding effects during the day (Kurtuncu et al., 2004). Results presented in this study reinforce the importance of the light-cycle with respect to alcohol reward-like behaviour. Despite higher water intake during the dark cycle, mice in the dark cycle exhibited greater preference and intake of alcohol compared to those in the light cycle. These findings were furthered as mice exhibited relatively higher conditioned place preference towards alcohol during the dark cycle compared to the light. The increased intake and preference for alcohol however, are potentially attributable to either an increase in the rewarding- or a

decrease in the aversive properties of alcohol. To control for this possibility, the intake of saccharin; a sweet non-calorific, non-alcoholic rewarding solution, and quinine; an aversive, bitter solution, was measured. Mice displayed light-cycle-dependent differences in saccharin intake and preference, with the greatest preference observed during the dark cycle. By contrast, the lowest intake ratio for quinine was during the dark cycle. As mice in the dark cycle exhibited enhanced and reduced preference towards saccharin and quinine, respectively. It is difficult to determine whether the increased preference of alcohol was due to increased reward or reduced aversion. Further, one cannot rule out the possibility of alcohol as an energy source acting as a motivator for increases in preference and intake.

Previous studies have identified circadian differences in nucleus accumbens and ventral tegmental area (key reward-related regions) in terms of gene and protein expression and the activity of dopaminergic- and non-dopaminergic neurons (Hampp et al., 2008; Sleipness et al., 2008; Sleipness et al., 2007). Our results are in accordance with these findings; the expression of dopamine and opioid receptors and tyrosine hydroxylase mRNA in the nucleus accumbens was increased during the dark cycle. The elevated levels of reward-related genes (if translated into protein) may enhance an individual's sensitivity towards alcohol (Mendez & Morales-Mulia, 2008; Gianoulakis, 2001*)*. In addition, we observed light-cycle dependent expression in genes related to thirst and hunger in the hypothalamus. The fluctuations in vasopressin and leptin mRNA may additionally drive the intake of alcohol (Pickering et al., 2007; Wurst et al., 2007). The day-night differences in reward-related gene expression are attributable to multiple circuit-level and molecular mechanisms. For example, the SCN innervates the reward pathway via glutamatergic afferents from the medial prefrontal cortex regulating reward behaviour (Baltazar et al., 2014; Baltazar et al., 2013); and dopamine transporter, dopamine D1 and D2 receptors, monoamine oxidase and

tyrosine hydroxylase – key proteins regulating dopamine synthesis and reward, contain BMAL1 and CLOCK binding sites in their promoter regions (Webb et al., 2009; Hampp et al., 2008; Sleipness et al., 2008; Sleipness et al., 2007; McClung et al., 2005; Akhisaroglu et al., 2005). This suggests the circadian clock controls aspects of dopaminergic activity including neurotransmitter synthesis, release, degradation and postsynaptic actions. No study has determined whether the μ opioid receptor or any of its endogenous agonists (associated with the "liking" component of reward) are under the control of clock proteins in the nucleus accumbens or VTA.

Given the emerging role of the immune system in reward-like behaviour, the expression of the TLR4 pathway was additionally examined. Interestingly, a light-cycle dependent effect was observed for some, but not all TLR4-related genes in the nucleus accumbens and hypothalamus. Both these regions exhibited increases in *Tlr4*, *Ifnb* and *Ccl2* mRNA during the dark cycle. However, the hypothalamus reported additional light-dark differences in *Md2*, *Trif* and *Il1b* expression. These findings are in contrast to Fonken et al., (2015) who observed that isolated microglia exhibit peaks in inflammatory gene expression during the light cycle. There are however, numerous differences in terms of study design between the present study and Fonken et al., (2015), which may explain these differences (genes and cells examined, *in vivo* vs *ex vivo* tissue and methods of analysis). The present findings are similar to studies examining circadian influence on peripheral immune cells. For example, peripheral macrophages exhibit an increase in TLR4-related mRNA during the dark (active) cycle (Keller et al., 2009). Nevertheless, these data point to regional specific circadian control of brain innate immune reactivity. Like the reward-related gene expression, the oscillations in TLR4-related gene expression are driven by sympathetic and parasympathetic effects from the SCN and the molecular clock mechanisms. For example, BMAL1-CLOCK binds to E-boxes in the promoters of chemokine genes

(Nguyen et al., 2013); CLOCK can directly interact with p65 subunit of NFκB enhancing its activity (Spengler & Kuropatwinski, 2012); and glucocorticoid receptors bind to NFκB and AP-1 repressing their activities (Dickmeis et al., 2013; Coutinho & Chapman 2011). These regulatory processes act in a concerted manner, temporally gating specific parts of the immune response to distinct times of the day.

The role of TLR4 in regulating cocaine- and opioid-induced reward is well established (Northcutt et al., 2015; Hutchinson et al., 2012). However, for TLR4's impact on alcohol pharmacodynamics, there are conflicting evidence with studies demonstrating either no effect or a reduction in alcohol drinking and reward-like behaviour (Blednov et al., 2017; Harris et al., 2017; Aurelian et al., 2016; Bajo et al., 2016; June et al., 2015; Liu et al., 2011; Pascual et al., 2011). The differential results are likely attributable to differences in brain regions examined, models of alcohol exposure and species examined. However, only two of the preceding studies have considered whether the differences (or lack thereof) are attributable to activation of different TLR4-signaling pathways (TRIF or MyD88) (Blednov et al., 2017; Harris et al., 2017). Given the light-cycle differences in the expression of *Ifnb* mRNA, and previous work establishing a causal relationship between interferon signalling and excessive alcohol use (Duncan et al., 2016; Manzardo et al., 2016; Johnson et al., 2015), (+)-Naltrexone, a biased TLR4-TRIF antagonist was used to explore the role of TLR4-TRIF signalling on light-cycle dependent differences in alcohol drinking and reward behaviour.

(+)-Naltrexone significantly attenuated alcohol intake and preference across a range of doses, alcohol concentrations and testing times. However, the response was mixed. While there were significant effects regarding the dose of (+)-Naltrexone on intake and preference, post hoc analysis determined the differences were most pronounced during the dark cycle. Similarly, the reduction in relative alcohol-induced conditioned

place preference by (+)-Naltrexone was only statistically significant during the dark cycle. These two paradigms would infer that (+)-Naltrexone attenuates alcohol-induced reward-like behaviour with the greatest effect during the dark cycle. However, the results are confounded. (+)-Naltrexone significantly modified saccharin (but not quinine) intake and preference. Therefore, (+)-Naltrexone may act as an antagonist towards all rewarding compounds, rather than one specific to alcohol. While this finding contrasts Northcutt et al., (2015), they are congruent with the actions of other pharmacological TLR4 antagonists such as T5342126 (Bajo et al., 2016). Hence, there appears to be a critical circadian-TLR4 signalling involvement in the rewarding properties of multiple diverse agents.

The findings presented in this manuscript add to the growing body of evidence aimed at elucidating the precise function of each of the TLR4-signaling pathways in alcohol-reward behaviour. Interestingly, our findings largely contrast those by Harris et al., (2017) who demonstrated a lack of effect of (+)-Naloxone, a chemically-related compound and TLR4-TRIF antagonist, on alcohol drinking behaviour in naïve mice. However, Harris et al., (2017) observed a significant effect of (+)-Naloxone in paradigms designed to mimic excessive drinking. This would suggest TLR4-TRIF is involved in the chronic but not acute effects of alcohol. In addition to the TRIF pathway, TLR4 signals via MyD88 raising the possibility that the acute effects of alcohol are mediated by this pathway as well. Recent evidence has shown naïve MyD88$^{-/-}$ mice exhibit potentiated alcohol intake compared to wildtype mice (Blednov et al., 2017). On the surface, this may suggest MyD88 is a negative regulator of alcohol-reward behaviour. However, MyD88$^{-/-}$ mice also display reduced saccharin intake (Blednov et al., 2017) and opioid-induced reward (Hutchinson et al., 2012), suggesting that MyD88$^{-/-}$ mice may find alcohol less rewarding than wildtype mice and therefore must consume greater quantities to achieve the same pharmacological effect. Collectively, these

studies and ours highlights the growing appreciation that the individual TLR4-signalling pathways play a unique role in alcohol reward.

To identify potential mechanisms underlying (+)-Naltrexone's ability to attenuate alcohol-reward like behaviour, genes relating to reward and the immune system within the nucleus accumbens and hypothalamus were examined. Alcohol increased the expression of genes related to the TLR4 pathway. Specifically, a rise in *Tlr4*, *Cd14*, *Md2*, *Trif*, *Myd88*, *Ccl2*, *Hmgb1* and *Ifnb* mRNA expression was observed. This indicates an acute moderate dose of alcohol upregulates markers of the MyD88 and the TRIF pathway. Only *Tlr4* and *Ifnb*, however, reported additional light-cycle and pretreatment effects. There was a significant decrease in the expression of *Tlr4* and *Ifnb* mRNA following (+)-Naltrexone. However, like the behavioural tests, only significant post hoc differences were found in the dark cycle. Again, highlighting that the largest effect of (+)-Naltrexone occurred during the dark cycle. The dark cycle effect may be due to a floor effect. That is, because TLR4 expression is relatively lower during the light cycle, an antagonist may be unable to reduce the signalling and expression further. By contrast, when the expression is comparatively higher (during the dark cycle), the antagonist now appears to exert an effect. This extends to conclusions about TLR4s involvement in reward-like behaviour. During the light cycle, TLR4 expression was low and therefore, TLR4 may exert a smaller effect on reward behaviour compared to during the dark when its expression was the highest.

No genes associated with hunger or thirst were significantly altered by alcohol in the hypothalamus. By contrast, alcohol significantly potentiated the expression of *Oprm1* and *Th* mRNA in the nucleus accumbens. These genes are pivotally involved in the manifestation of reward-like behaviour (Alves et al., 2015; Charbogne et al., 2014; Webb et al., 2009). However, only the expression of *Th* mRNA was significantly altered

by light-cycle and pretreatment – suggesting a link between TLR4 and tyrosine hydroxylase (TH) (figure 10). This study is not the first to highlight a potential link between TLR4 and TH as Aurelian et al., (2016) determined TLR4 activation induces the expression of TH in VTA dopaminergic neurons via a PKA/pCREB signal. Work from the present study builds upon this connection suggesting that either IFNβ or CCL2 may underlie this link given both inflammatory mediators demonstrated a significant effect of pretreatment. Interestingly, all three inflammatory mediators (IFNβ, CCL2 and TLR4) can signal through PKA and CREB (Akira &Takeda, 2004), leading to altered transcription of *Th* mRNA. Importantly the downregulation of *Th* mRNA following (+)-Naltrexone potentially explains the broad effects (decreased saccharin preference) observed with TLR4 antagonists. Tyrosine hydroxylase is the rate limiting enzyme of catecholamine synthesis, catalysing the conversion of tyrosine to L-DOPA, a precursor molecule for dopamine (see Daubner et al., (2011) for review). Consequently, reducing its transcription using (+)-Naltrexone may reduce basal dopamine level. Thus, mice experience reduced rewarding sensations upon consuming saccharin and alcohol. Collectively, the results highlight the importance of TLR4 in regulating basal dopamine synthesis and implicates the TLR4 system in the rewarding properties of multiple diverse agents.

In summary, the results highlighted above suggest the preference for rewarding and aversive stimuli peak and nadir during the dark cycle respectively. This effect coincides with elevations in genes relating to dopaminergic and opioidergic transmission and the TLR4-signalling pathway. Attenuating the TRIF component of the TLR4-signalling pathway significantly reduced alcohol preference, with a greater effect during the dark cycle. Saccharin preference was additionally reduced by TLR4 blockade– an effect potentially attributable to a reduction in *Th* mRNA. Given that antagonism of TLR4 reduced alcohol- and saccharin preference and tyrosine hydroxylase mRNA, TLR4

may play a role in the dopamine synthesis and natural reward-like behaviour. Further research is required to establish how these preclinical studies translate to the human condition, and whether future pharmacological targeting of neuroimmune systems generally (Ray et al., 2017) or TLR4 specifically, may need to be timed specifically to a light cycle. Moreover, these data point to a significant impact on the brain of time-of-day on long term impact of alcohol exposure.

3.6 Acknowledgements

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The authors declare no competing financial interests.

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3.8 Supplementary material

Table 1 Primer sequence used in qPCR

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Ccl2 – Chemokine (C-C motif) ligand 2	ACACTGGTTCCTGACTCCTCT	ACCTGAGGACTGATGGTGGT
Cd14 – Cluster of differentiation 14 antigen	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT
Drd1 – Dopamine receptor D1 transcript variant 1	GTTGAGTCCAGGGGTTTTGGG	ACTTTTCGGGGATGCTGCC
Drd2 – Dopamine receptor D2	GTGAACAGGCGGAGAATGGA	TGGGAGGGATGGGGCTATAC
Gapdh – Glyceraldehyde-3-phosphate dehydrogenase transcript variant 1	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Ghrl – Grehlin transcript variant 1	ATCGTCCTCACCACCAAGAC	CTTGGATTCCTTTCTCTGGGCTT
Hmgb1 – High mobility group box 1, transcript variant 1	CCATTGGTGATGTTGCAAAG	CTTTTTCGCTGCATCAGGTT
Ifnb – Interferon beta 1, fibroblast	TGGGAGATGTCCTCAACTGC	CCAGGCGTAGCTGTTGTACT
Lepr – Leptin receptor transcript variant 3	TCCAAAAGAGAACGGACACTC T	TGTATGGACTGTTGGGAAGTTG
Il1b – Interleukin 1 beta	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Il10 – Interleukin 10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Md2 – Lymphocyte antigen 96 transcript variant 1, 2	CGCTGCTTTCTCCCATATTGA	CCTCAGTCTTATGCAGGGTTCA

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Myd88 – Myeloid differentiation primary response gene 88	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCA
Oprm1 – Opioid receptor, mu 1, transcript variant 1C, 1M, 1U	TCCGACTCATGTTGAAAAACCC	CCTTCCCCGGATTCCTGTCT
Rxfp1 – Relaxin/insulin-like family peptide receptor 1	CGAGCTGTCCCATCAGTTTCT	AGACGCTCACGGAGTGAATC
Tlr4 – Toll-like receptor 4	GCCTTTCAGGGAATTAAGCTCC	GATCAACCGATGGACGTGTAAA
Trif – Toll-like receptor adaptor molecule 1	AACCTCCACATCCCCTGTTTT	GCCCTGGCATGGATAACCA
Th –Tyrosine Hydroxylase	CCTTCCGTGTGTTTCAGTGC	TCAGCCAACATGGGTACGTG

3.8.1 Statistics for in-text figures

Figure 2 Circadian timing affects the intake and preference of alcohol (a - b), saccharin (c - d) but not quinine (e - f) and the conditioned preference towards alcohol (g – h). All data analysed using a two-way ANOVA with Tukey post hoc.

(a) *Alcohol intake*

Effect of concentration, $F_{(8, 72)} = 68.34$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 9)} = 5.21$, $p = 0.048$

Interaction: concentration x light-cycle, $F_{(8, 72)} = 2.02$, $p = 0.056$

(b) *Alcohol preference*

Effect of concentration, $F_{(8, 72)} = 2.2$, $p = 0.037$

Effect of light-cycle, $F_{(1, 9)} = 9.16$, $p = 0.014$

Interaction: concentration x light-cycle, $F_{(8, 72)} = 0.30$, $p = 0.96$

(c) *Saccharin intake*

Effect of concentration, $F_{(4, 36)} = 18.94$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 9)} = 15.53$, $p = 0.0034$

Interaction: concentration x light-cycle, $F_{(4, 36)} = 2.98$, $p = 0.0318$

(d) *Saccharin preference*

Effect of concentration, $F_{(4, 36)} = 1.74$, $p = 0.16$

Effect of light-cycle, $F_{(1, 9)} = 8.32$, $p = 0.015$

Interaction: concentration x light-cycle, $F_{(4, 36)} = 0.40$, $p = 0.81$

(e) *Quinine intake*

Effect of concentration, $F_{(4, 36)} = 180.4$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 9)} = 12.09$, $p = 0.0052$

Interaction: concentration x light-cycle, $F_{(4, 36)} = 6.14$, $p = 0.0005$

(f) *Quinine preference*

Effect of concentration, $F_{(4, 36)} = 2.94$, $p = 0.031$

Effect of light-cycle, $F_{(1, 9)} = 20.31$, $p = 0.0009$

Interaction: concentration x light-cycle, $F_{(4, 36)} = 0.35$, $p = 0.84$

Figure 5 Circadian timing affects the expression of genes relating to reward (a), thirst and hunger (b) and the TLR4 pathway (c – d). All data analysed using a two-way ANOVA with Tukey post hoc.

(c) *Nucleus accumbens*

Effect of light cycle, *Cd14*, t = 0.67 df = 4, $p = 0.54$

Effect of light cycle, *Md2*, t = 0.22 df =4, $p = 0.84$

Effect of light cycle, *Myd88*, t = 1.37 df = 4, $p = 0.24$

Effect of light cycle, *Trif*, t =1.62 df = 4, $p = 0.18$

Effect of light cycle, *Il1b*, t = 0.12 df = 4, $p = 0.90$

Effect of light cycle, *Il10*, t = 0.89 df=4, $p = 0.42$

Effect of light cycle, *Hmgb1*, t = 0.033 df = 4, $p = 0.98$

(d) *Hypothalamus*

Effect of light cycle, *Cd14*, t = 1.31 df = 4, $p = 0.26$

Effect of light cycle, *Myd88*, t = 0.52 df = 4, $p = 0.63$

Effect of light cycle, *Ccl2*, t = 1.16 df = 4, $p = 0.33$

Effect of light cycle, *Il10*, t = 1.78 df = 4, $p = 0.68$

Effect of light cycle, *Hmgb1*, t = 1.6 df = 4, $p = 0.24$

Figure 6 Circadian timing influences the efficacy of (+)-Naltrexone on decreasing the intake and preference for alcohol (a – b) and saccharin (c – d) but not quinine (e – f). All data analysed using a two-way ANOVA with Tukey post hoc.

(a) *Alcohol intake*

Effect of dose of (+)-Naltrexone, $F_{(6, 48)} = 15.72$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 8)} = 15.12$, $p = 0.0046$

Interaction: dose of (+)-Naltrexone x light-cycle, $F_{(6, 48)} = 4.99$, $p = 0.0005$

(b) *Alcohol preference*

Effect of concentration, $F_{(6, 48)} = 7.57$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 8)} = 40.85$, $p = 0.0002$

Interaction: dose of (+)-Naltrexone x light-cycle, $F_{(6, 48)} = 0.64$, $p = 0.70$

(c) *Saccharin intake*

Effect of dose of (+)-Naltrexone, $F_{(6, 48)} = 2.56$, $p = 0.076$

Effect of light-cycle, $F_{(1, 8)} = 64.85$, $p < 0.0001$

Interaction: dose of (+)-Naltrexone x light-cycle, $F_{(6, 48)} = 0.86$, $p = 0.53$

(d) *Saccharin preference*

Effect of dose of (+)-Naltrexone, $F_{(6, 48)} = 3.82$, $p = 0.0034$

Effect of light-cycle, $F_{(1, 8)} = 39.16$, $p = 0.0002$

Interaction: dose of (+)-Naltrexone x light-cycle, $F_{(6, 48)} = 2.68$, $p = 0.024$

(e) *Quinine intake*

Effect of dose of (+)-Naltrexone, $F_{(6, 48)} = 3.05$, $p = 0.013$

Effect of light-cycle, $F_{(1, 8)} = 11.09$, $p = 0.010$

Interaction: dose of (+)-Naltrexone x light-cycle, $F_{(6, 48)} = 1.67$, $p = 0.15$

(f) *Quinine preference*

Effect of dose of (+)-Naltrexone, $F_{(6, 48)} = 0.79$, $p = 0.58$

Effect of light-cycle, $F_{(1, 8)} = 1.08$, $p = 0.33$

Interaction: dose of (+)-Naltrexone x light-cycle, $F_{(6, 48)} = 1.29$, $p = 0.28$

Figure 7 Circadian timing influences the efficacy of (+)-Naltrexone (60 mg/kg) on decreasing and the intake and preference for alcohol (a – b) and saccharin (c – d) but not quinine (e – f) and the conditioned preference for alcohol (g – h). All data analysed using a three-way ANOVA with Tukey post hoc.

(a) *Alcohol intake*

Effect of concentration, $F_{(7, 320)} = 61.53$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 320)} = 4.12$, $p = 0.043$

Effect of pretreatment, $F_{(1, 320)} = 4.95$, $p = 0.026$

Interaction: concentration x light-cycle, $F_{(7, 320)} = 3.05$, $p = 0.0040$

Interaction: concentration x pretreatment, $F_{(7, 320)} = 0.60$, $p = 0.76$

Interaction: light-cycle x pretreatment, $F_{(1, 320)} = 2.33$, $p = 0.13$

Interaction: concentration x light-cycle x pretreatment, $F_{(7, 320)} = 2.82$, $p = 0.0073$

(b) *Alcohol preference*

Effect of concentration, $F_{(7, 320)} = 3.72$, $p = 0.0007$

Effect of light-cycle, $F_{(1, 320)} = 311.2$, $p < 0.0001$

Effect of pretreatment, $F_{(1, 320)} = 25.68$, $p < 0.0001$

Interaction: concentration x light-cycle, $F_{(7, 320)} = 2.52$, $p = 0.016$

Interaction: concentration x pretreatment, $F_{(7, 320)} = 2.31$, $p = 0.026$

Interaction: light-cycle x pretreatment, $F_{(1, 320)} = 11.17$, $p = 0.0009$

Interaction: concentration x light-cycle x pretreatment, $F_{(7, 320)} = 0.79$, $p = 0.60$

(c) *Saccharin intake*

Effect of concentration, $F_{(4, 220)} = 97.07$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 220)} = 75.11$, $p < 0.0001$

Effect of pretreatment, $F_{(1, 220)} = 8.95$, $p = 0.0031$

Interaction: concentration x light-cycle, $F_{(4, 220)} = 12.39$, $p < 0.0001$

Interaction: concentration x pretreatment, $F_{(4, 220)} = 1.43$, $p = 0.23$

Interaction: light-cycle x pretreatment, $F_{(1, 220)} = 9.11$, $p = 0.0028$

Interaction: concentration x light-cycle x pretreatment, $F_{(4, 220)} = 1.83$, $p = 0.13$

(d) *Saccharin preference*

Effect of concentration, $F_{(4, 220)} = 0.85$, $p = 0.49$

Effect of light-cycle, $F_{(1, 220)} = 31.38$, $p < 0.0001$

Effect of pretreatment, $F_{(1, 220)} = 0.25$, $p = 0.62$

Interaction: concentration x light-cycle, $F_{(4, 220)} = 0.68$, $p = 0.61$

Interaction: concentration x pretreatment, $F_{(4, 220)} = 0.24$, $p = 0.92$

Interaction: light-cycle x pretreatment, $F_{(1, 220)} = 1.37$, $p = 0.24$

Interaction: concentration x light-cycle x pretreatment, $F_{(4, 220)} = 0.27$, $p = 0.89$

(e) *Quinine intake*

Effect of concentration, $F_{(4, 220)} = 45.38$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 220)} = 0.016$, $p = 0.90$

Effect of pretreatment, $F_{(1, 220)} = 4.32$, $p = 0.039$

Interaction: concentration x light-cycle, $F_{(4, 220)} = 0.38$, $p = 0.83$

Interaction: concentration x pretreatment, $F_{(4, 220)} = 4.27$, $p = 0.0024$

Interaction: light-cycle x pretreatment, $F_{(1, 220)} = 0.018$, $p = 0.89$

Interaction: concentration x light-cycle x pretreatment, $F_{(4, 220)} = 0.062$, $p = 0.99$

(f) *Quinine preference*

Effect of concentration, $F_{(4, 220)} = 4.39$, $p = 0.0020$

Effect of light-cycle, $F_{(1, 220)} = 0.0058$, $p = 0.94$

Effect of pretreatment, $F_{(1, 220)} = 2.02$, $p = 0.16$

Interaction: concentration x light-cycle, $F_{(4, 220)} = 2.49$, $p = 0.0443$

Interaction: concentration x pretreatment, $F_{(4, 220)} = 0.89$, $p = 0.47$

Interaction: light-cycle x pretreatment, $F_{(1, 220)} = 14$, $p = 0.0002$

Interaction: concentration x light-cycle x pretreatment, $F_{(4, 220)} = 0.51$, $p = 0.73$

Figure 8 Circadian timing influences efficacy of (+)-Naltrexone on relative change in conditioned chamber time. All data analysed using a two-way ANOVA with Tukey post hoc.

Relative conditioned place preference

Effect of pretreatment, $F_{(1, 7)} = 20.52$, $p = 0.0027$

Effect of light-cycle, $F_{(1, 7)} = 0.0011$, $p = 0.92$

Interaction: pretreatment x light-cycle, $F_{(1, 7)} = 1.62$, $p = 0.24$)

3.8.2 Supplementary figures

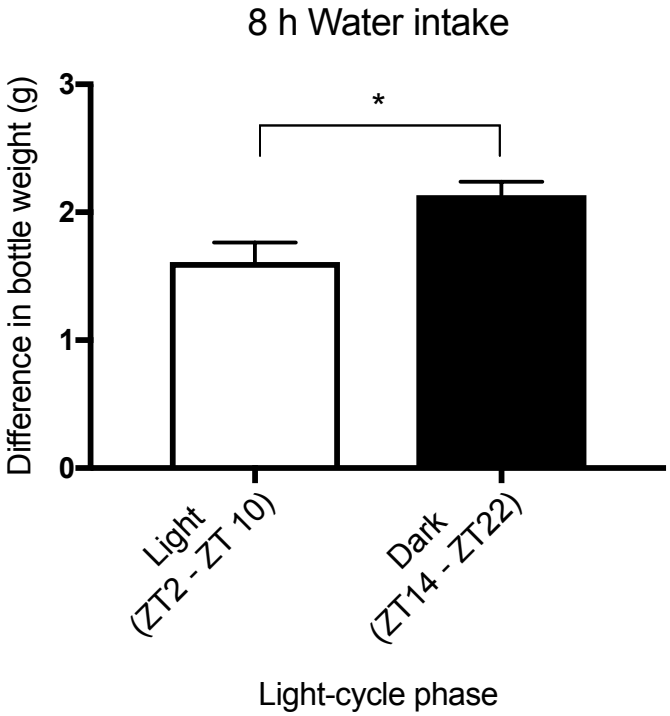

Figure s1 Circadian timing affects the intake of water. Mice in the dark cycle consumed significantly more water compared to mice in the light cycle. All data was analysed using a paired two-tail t-test. Summary values represented mean±SEM; n=49, *$p < 0.05$.

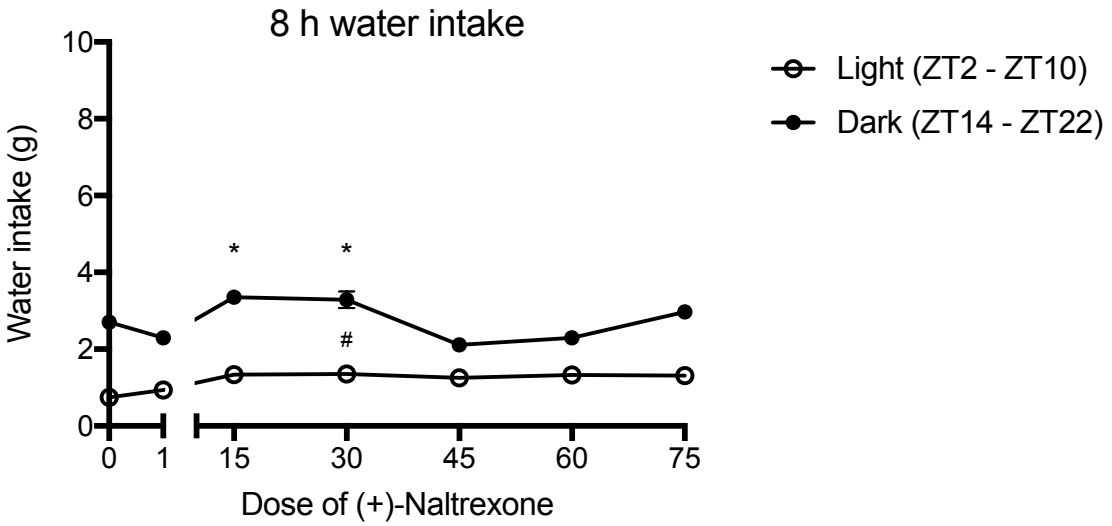

Figure s2 Circadian timing and the dose of (+)-Naltrexone significantly modify water intake. Mice receiving (+)-Naltrexone in the dark cycle consumed significantly more water compared to mice in the light cycle**.** All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=9, *p < 0.05 compared to saline (dark); # p < 0.05 compared to saline (light).

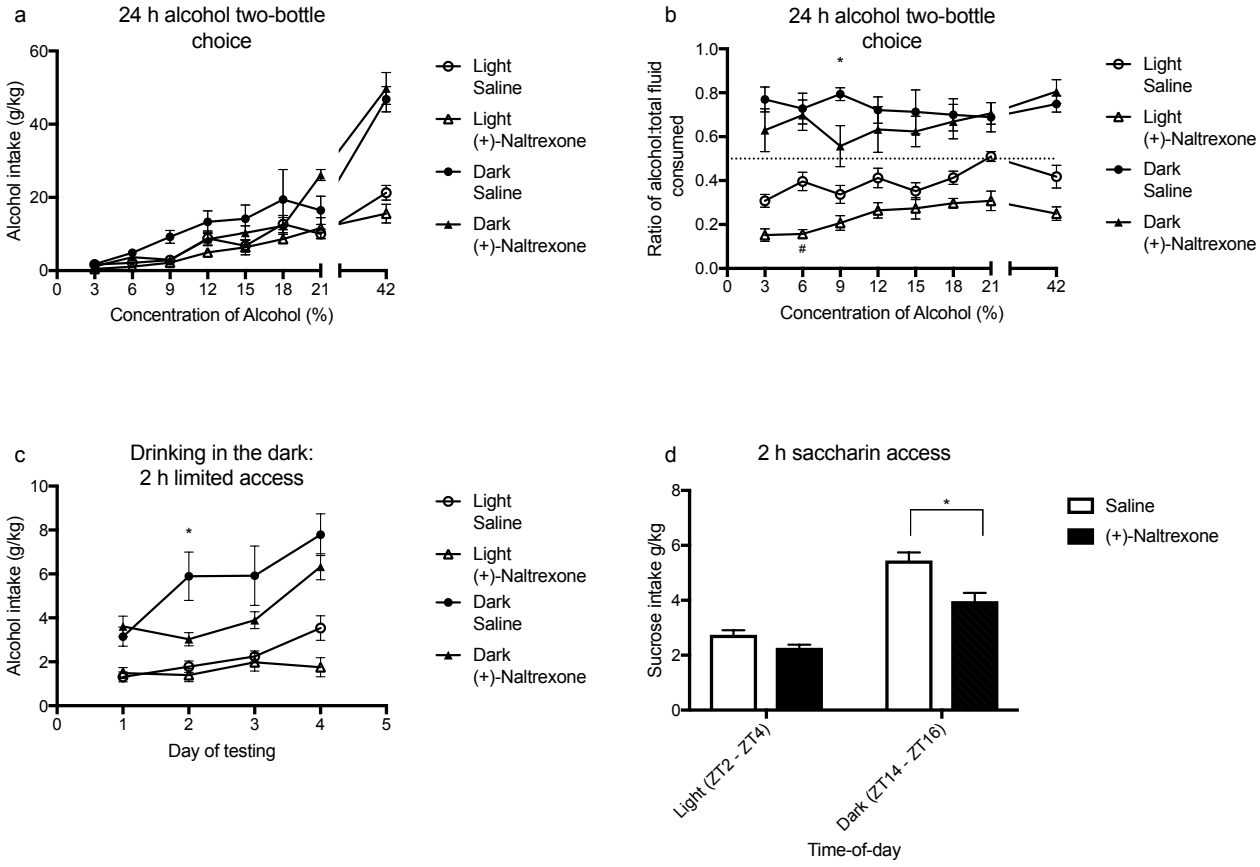

Figure s3 The efficacy of (+)-Naltrexone (60 mg/kg) on decreasing 24 h intake and preference (a –b) of alcohol (20%), 2-4 h intake of alcohol (c) and saccharin (15mM) (d) is greatest during the dark cycle. All data was analysed using a three-way ANOVA with Tukey HSD post hoc (a – c) and a two-way ANOVA with Tukey post hoc (d). There was a significant effect of pretreatment on the intake and preference of alcohol during the 24 h two-bottle choice tests. Similarly, the drinking in the dark and 2 h saccharin access tests exhibited a significant effect of pretreatment. Post hoc analysis determined (+)-Naltrexone significantly attenuated intake compared to saline during the dark but not light cycle in both paradigms. Summary values represented as mean±SEM; n=11–12, $p < 0.05$ compared to saline (dark); # $p < 0.05$ compared to saline (light).

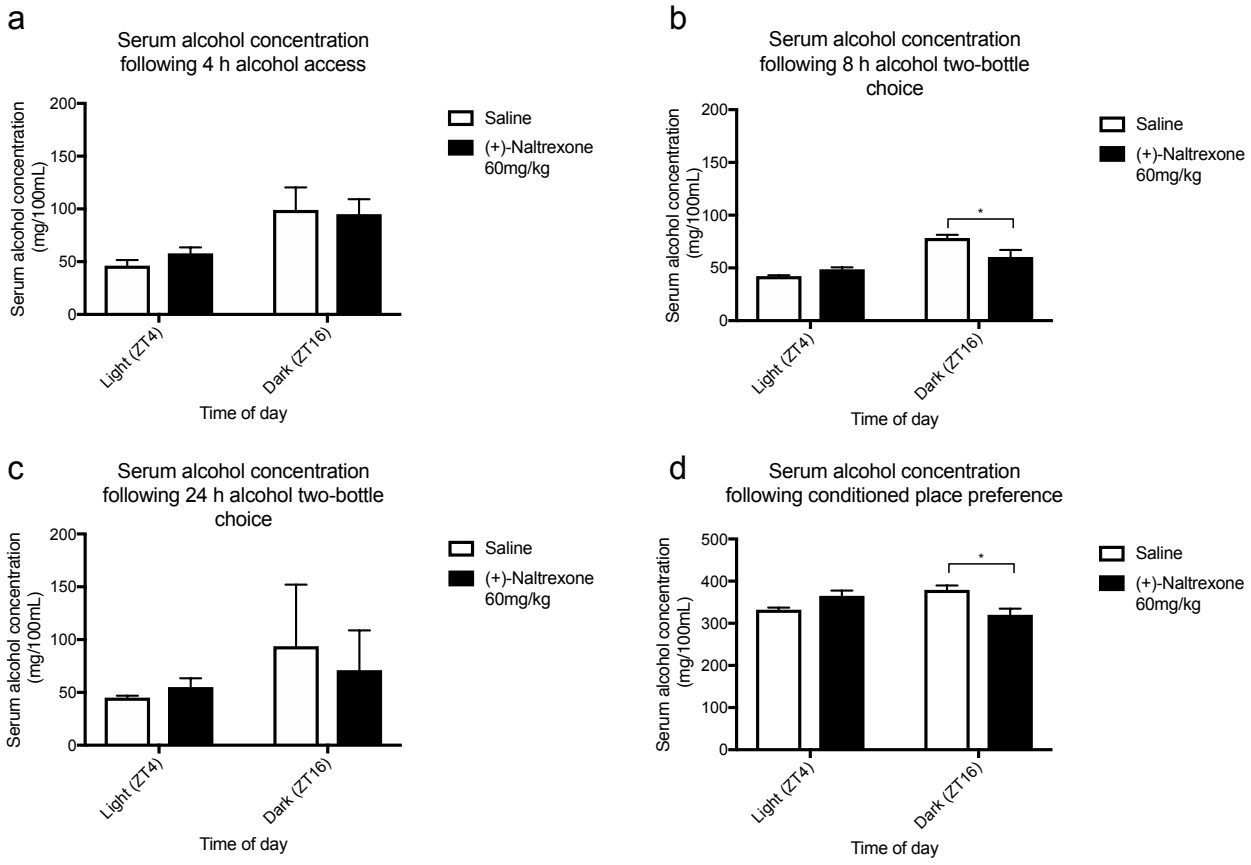

Figure s4 Serum alcohol concentration from saline and (+)-Naltrexone-treated mice (60 mg/kg) following 2 h (a), 8 h (b) and 24 h (c) alcohol drinking tests and conditioned place preference (d). All data was analysed using a two-way ANOVA with Tukey post hoc (a – d). Summary values represented as mean±SEM; n=6.

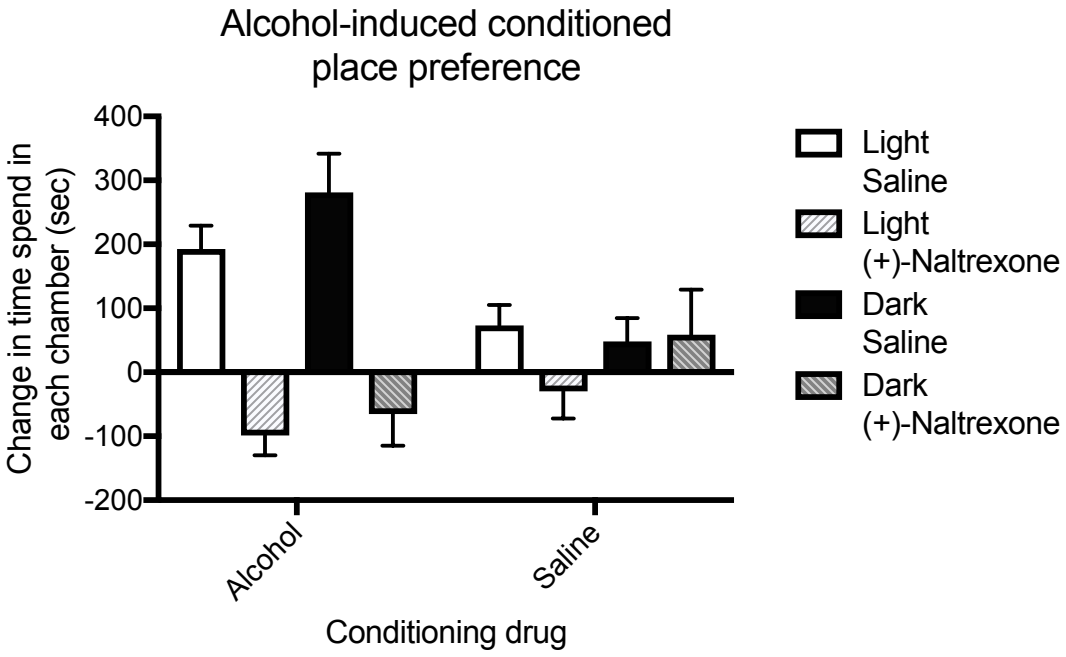

Figure s5. Circadian timing influences efficacy of (+)-Naltrexone (60 mg/kg) on change in conditioned chamber time. All data was analysed using a three-way ANOVA with Tukey post hoc (a – d). Summary values represented as mean±SEM; n=8, *$p < 0.05$.

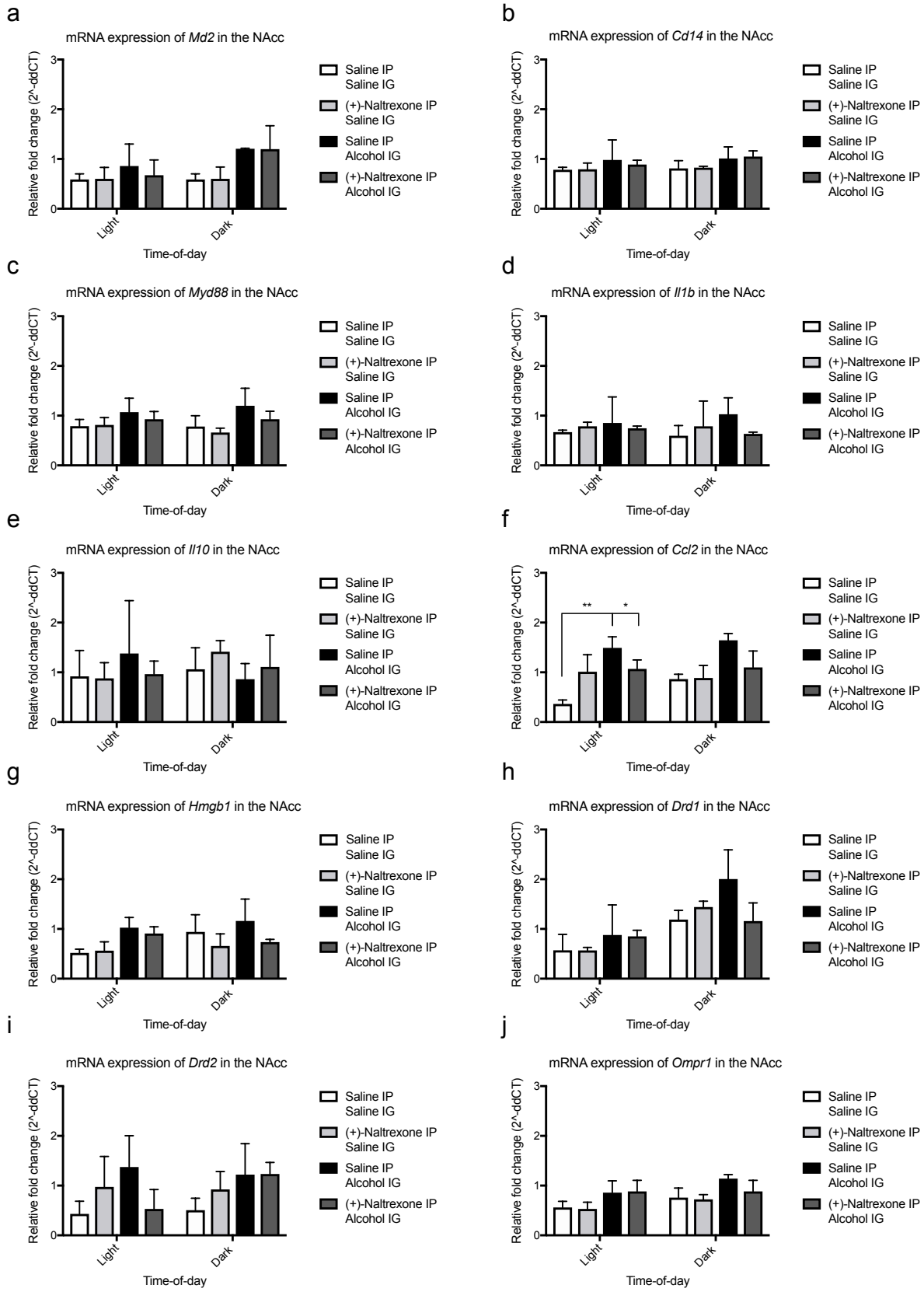

Figure s6 Effect of alcohol, saline (I.G), (+)-Naltrexone (60 mg/kg) on the expression of TLR4 and reward-related genes in the Nucleus Accumbens. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=3, *$p < 0.05$; **$p < 0.01$.

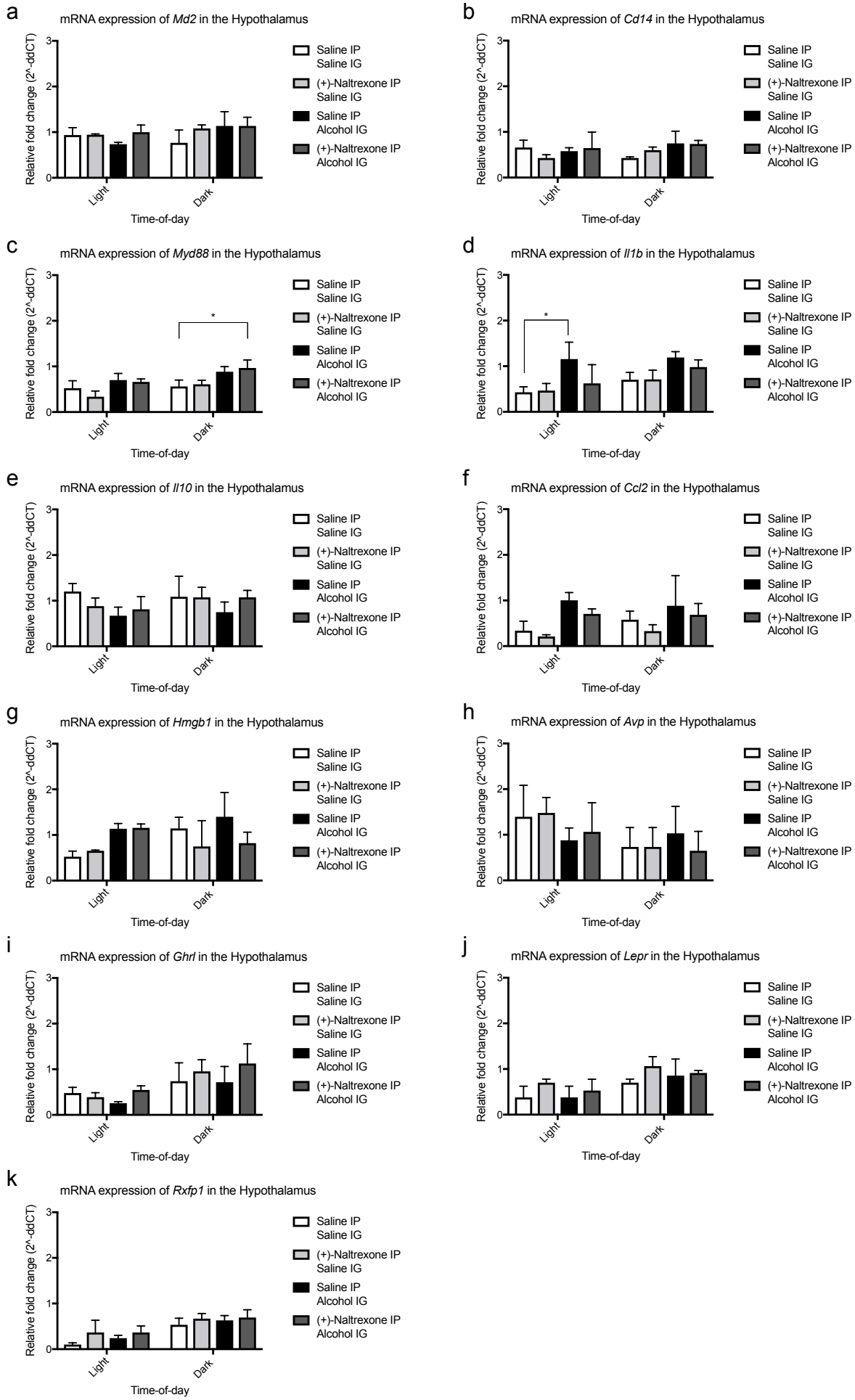
a
mRNA expression of Md2 in the Hypothalamus
b
mRNA expression of Cd14 in the Hypothalamus
c
mRNA expression of Myd88 in the Hypothalamus
d
mRNA expression of Il1b in the Hypothalamus
e
mRNA expression of Il10 in the Hypothalamus
f
mRNA expression of Ccl2 in the Hypothalamus
g
mRNA expression of Hmgb1 in the Hypothalamus
h
mRNA expression of Avp in the Hypothalamus
i
mRNA expression of Ghrl in the Hypothalamus
j
mRNA expression of Lepr in the Hypothalamus
k
mRNA expression of Rxfp1 in the Hypothalamus
Relative fold change (2^-ddCT)
Time-of-day
Light
Dark
Saline IP
Saline IG
(+)-Naltrexone IP
Saline IG
Saline IP
Alcohol IG
(+)-Naltrexone IP
Alcohol IG
*

Figure s7 Effect of alcohol, saline and (+)-Naltrexone (60 mg/kg) on the expression of TLR4 and hunger/thirst-related genes in the hypothalamus. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=3, *$p < 0.05$; **$p < 0.01$.

3.8.3 Supplementary material statistics

Figure s1. Light-cycle dependent water intake.

Paired two-tail t-test

Effect of light cycle, t=2.83 df=96, $p = 0.0057$

Figure s2 Circadian timing and the dose of (+)-Naltrexone significantly modify water intake. All data analysed using a two-way ANOVA with Tukey post hoc.

Effect of dose of (+)-Naltrexone, $F_{(6, 48)} = 12.01$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 8)} = 99.62$, $p < 0.0001$

Interaction: dose x light-cycle, $F_{(6, 48)} = 5.72$, $p = 0.0002$

Figure s3 Circadian timing influences the efficacy of (+)-Naltrexone on decreasing 24 h intake and preference (a –b) of alcohol, 2-4 h intake of alcohol (c) and saccharin (d). All data analysed using a two-way three-way ANOVA with Tukey post hoc (a – c) and two-way ANOVA with Tukey post hoc (d).

(a) *24 h intake*

Effect of concentration, $F_{(7, 288)} = 69.58$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 288)} = 78.51$, $p < 0.0001$

Effect of pretreatment, $F_{(1, 288)} = 3.66$, $p = 0.050$

Interaction: concentration x light-cycle, $F_{(7, 288} = 14.53$, $p <0.0001$

Interaction: concentration x pretreatment, $F_{(7, 288} = 1.82$, $p = 0.071$

Interaction: light-cycle x pretreatment, $F_{(1, 288)} = 0.089$, $p = 0.77$

Interaction: concentration x light-cycle x pretreatment, $F_{(7, 288} = 1.13$, $p = 0.34$

(b) *24 h preference*

Effect of concentration, $F_{(7, 288)} = 1.37$, $p = 0.22$

Effect of light-cycle, $F_{(1, 288)} = 356.1$, $p < 0.0001$

Effect of pretreatment, $F_{(1, 288)} = 29.93$, $p < 0.0001$

Interaction: concentration x light-cycle, $F_{(7, 288)} = 1.25$, $p = 0.27$

Interaction: concentration x pretreatment, $F_{(7, 288)} = 0.55$, $p = 0.79$

Interaction: light-cycle x pretreatment, $F_{(1, 288)} = 4.60$, $p = 0.033$

Interaction: concentration x light-cycle x pretreatment, $F_{(7, 288)} = 1.13$, $p = 0.35$

(c) *2 – 4 h limited access to alcohol*

Effect of day of testing, $F_{(3, 144)} = 11.77$, $p < 0.0001$

Effect of light-cycle, $F_{(1, 144)} = 97.97$, $p < 0.0001$

Effect of pretreatment, $F_{(1, 144)} = 11.19$, $p = 0.0011$

Interaction: day of testing x light-cycle, $F_{(3, 144)} = 2.77$, $p = 0.044$

Interaction: day of testing x treatment, $F_{(3, 144)} = 2.29$, $p = 0.08$

Interaction: light-cycle x pretreatment, $F_{(1, 144)} = 2.23$, $p = 0.14$

Interaction: day of testing x light-cycle x pretreatment, $F_{(3, 144)} = 1.39$, $p = 0.25$

(d) *2 h saccharin intake*

Effect of light-cycle, $F_{(1, 9)} = 68.31$, $p < 0.0001$

Effect of treatment, $F_{(1, 9)} = 21.31$, $p = 0.0013$

Interaction: light-cycle x treatment, $F_{(1, 9)} = 5.34$, $p = 0.046$

Figure s4 Serum ethanol concentration following 2 h (a), 8 h (b) and 24 h (c) alcohol drinking tests and conditioned place preference (d). Summary values represented as mean±SEM; n=6, *$p < 0.05$; **$p < 0.01$. All data analysed using a two-way ANOVA with Tukey post hoc.

(a) *2 h*

Effect of light-cycle, $F_{(1, 5)} = 35.06$, $p = 0.0004$

Effect of pretreatment,, $F_{(1, 5)} = 0.070$, $p = 0.80$

Interaction (light-cycle x pretreatment), $F_{(1, 5)} = 0.33$, $p = 0.58$

(b) *8 h*

Effect of light-cycle, $F_{(1, 5)} = 95.86$, $p < 0.0001$

Effect of pretreatment,, $F_{(1, 5)} = 1.59$, $p = 0.24$

Interaction (light-cycle x pretreatment), $F_{(1, 5)} = 0.039$, $p = 0.85$

(c) *24 h*

Effect of light-cycle, $F_{(1, 5)} = 0.42$, $p = 0.54$

Effect of pretreatment,, $F_{(1, 5)} = 3.76$, $p = 0.088$

Interaction (light-cycle x pretreatment), $F_{(1, 5)} = 16.27$, $p = 0.0038$

(d) *Conditioned place preference*

Effect of light-cycle, $F_{(1, 5)} = 356.1$, $p < 0.0001$

Effect of pretreatment,, $F_{(1, 5)} = 29.93$, $p < 0.0001$

Interaction (light-cycle x pretreatment), $F_{(1, 5)} = 29.93$, $p < 0.0001$

Figure s5 Circadian timing influences efficacy of (+)-Naltrexone on change in conditioned chamber time. All data analysed using a two-way or three-way ANOVA with Tukey post hoc.

Conditioned place preference

Effect of pretreatment, $F_{(1, 56)} = 26.65$, $p < 0.0001$

Effect of conditioning drug, $F_{(1, 56)} = 2.15$, $p = 0.15$

Effect of light-cycle, $F_{(1, 56)} = 2.74$, $p = 0.10$

Interaction: conditioning drug x pretreatment, $F_{(1, 56)} = 14.26$ $p = 0.0004$

Interaction: conditioning drug x light-cycle, $F_{(1, 56)} = 0.51$, $p = 0.48$

Interaction: pretreatment x light-cycle, $F_{(1, 56)} = 0.51$, $p = 0.48$

Interaction: conditioning drug x pretreatment x light-cycle, $F_{(1, 56)} = 0.954$, $p = 0.33$

Figure s6 Effect of alcohol and (+)-Naltrexone on the expression of TLR4 and reward-related genes in the Nucleus Accumbens. All data analysed using **t**wo-way ANOVA with Bonferonni post hoc.

(a) *Md2* light-cycle ($F_{(1, 24)} = 3.51$, $p = 0.08$), drug ($F_{(1, 24)} = 11.3$, $p = 0.04$), pretreatment ($F_{(1, 24)} = 0.133$, $p = 0.32$). No significant interactions.

(b) *Cd14*, light-cycle ($F_{(1, 24)} = 0.66$ $p = 0.43$), drug ($F_{(1, 24)} = 5.4$, $p = 0.033$), pretreatment ($F_{(1, 24)} = 0.92$, $p = 0.48$). No significant interactions.

(c) *Myd88*, light-cycle ($F_{(1, 24)} = 0.0072$ $p = 0.93$), drug ($F_{(1, 24)} = 10.11$, $p = 0.0058$), pretreatment ($F_{(1, 24)} = 2.21$, $p = 0.16$). No significant interactions.

(d) *Il1b*, light-cycle ($F_{(1, 24)}$ = 0.0006 p = 0.98), drug ($F_{(1, 24)}$ = 0.79, p = 0.37), pretreatment ($F_{(1, 24)}$ = 0.16, p = 0.69). No significant interactions.

(e) *Il10*, light-cycle ($F_{(1, 24)}$ = 0.12 p = 0.73), pretreatment ($F_{(1, 24)}$ = 0.027, p = 0.87), drug ($F_{(1, 24)}$ = 0.0024, p = 0.96). No significant interactions.

(f) *Ccl2*, light-cycle ($F_{(1, 24)}$ = 0.433 p = 0.51), pretreatment ($F_{(1, 24)}$ = 0.12, p = 0.91), drug ($F_{(1, 24)}$ = 29.7, p < 0.0001). There were significant interactions between light-cycle and preatreatment ($F_{(1, 24)}$ = 10.17, p = 0.0057) light-cycle, pretreatment and drug ($F_{(1, 24)}$ = 6.07, p = 0.025). No other significant interactions.

(g) *Hmgb1*, light-cycle ($F_{(1, 24)}$ = 1.47 p = 0.24), pretreatment ($F_{(1, 24)}$ = 3.88, p = 0.066), drug ($F_{(1, 24)}$ = 8.49, p = 0.01). No significant interactions.

(h) *Drd1*, light-cycle ($F_{(1, 24)}$ = 25.22 p = 0.001), pretreatment ($F_{(1, 24)}$ = 1.14, p = 0.30), drug ($F_{(1, 24)}$ = 3.7, p = 0.072). No significant interactions.

(i) *Drd2*, light-cycle ($F_{(1, 24)}$ = 0.62 p = 0.44), pretreatment ($F_{(1, 24)}$ = 0.032, p = 0.86), drug ($F_{(1, 24)}$ = 4.27, p = 0.55). There was a significant interactions between drug and pretreatment ($F_{(1, 24)}$ = 05.92 p = 0.027). No other significant interactions.

(j) *Oprm1*, light-cycle ($F_{(1, 24)}$ = 5.63 p = 0.031), drug ($F_{(1, 24)}$ = 17.78, p = 0.0007), pretreatment ($F_{(1, 24)}$ = 1.09, p = 0.31). No significant interactions.

Figure s7 Effect of alcohol and (+)-Naltrexone on the expression of TLR4 and hunger/thirst-related genes in the hypothalamus. All data analysed using **t**wo-way ANOVA with Bonferonni post hoc.

(a) *Md2*, light-cycle ($F_{(1, 24)} = 2.79$, $p = 0.11$), drug ($F_{(1, 24)} = 0.89$, $p = 0.36$), pretreatment ($F_{(1, 24)} = 3.82$, $p = 0.069$). No significant interactions.

(b) *Cd14*, light-cycle ($F_{(1, 24)} = 0.52$, $p = 0.48$), drug ($F_{(1, 24)} = 4.29$, $p = 0.055$), pretreatment ($F_{(1, 24)} = 0.0001$, $p = 0.99$). No significant interactions.

(c) *Myd88*, light-cycle ($F_{(1, 24)} = 13.94$, $p = 0.0018$), drug ($F_{(1, 24)} = 30.61$, $p = 0 < 0.001$), pretreatment ($F_{(1, 24)} = 0.21$, $p = 0.65$). No significant interactions.

(d) *Il1b*, light-cycle ($F_{(1, 24)} = 5.59$, $p = 0.031$), drug ($F_{(1, 24)} = 17.92$, $p = 0.006$), pretreatment ($F_{(1, 24)} = 3.22$, $p = 0.092$). No significant interactions.

(e) *Il10*, light-cycle ($F_{(1, 24)} = 1.06$, $p = 0.32$), drug ($F_{(1, 24)} = 5.27$, $p = 0.035$), pretreatment ($F_{(1, 24)} = 0.11$, $p = 0.74$). No significant interactions.

(f) *Ccl2*, light-cycle ($F_{(1, 24)} = 0.22$, $p = 0.64$), drug ($F_{(1, 24)} = 3.58$, $p = 0.077$), pretreatment ($F_{(1, 24)} = 15.65$, $p = 0.0011$). No significant interactions.

(g) *Hmgb1*, light-cycle ($F_{(1, 24)} = 1.65$, $p = 0.22$), drug ($F_{(1, 24)} = 8.29$, $p = 0.011$), pretreatment ($F_{(1, 24)} = 2.68$, $p = 0.12$). There was a significant interactions between light-cycle and pretreatment ($F_{(1, 24)} = 5.04$, $p = 0.039$). No other significant interactionss.

(h) *Avp*, light-cycle ($F_{(1, 24)}$ = 4.23, p = 0.056), drug ($F_{(1, 24)}$ = 041, p = 0.84), pretreatment ($F_{(1, 24)}$ = 3.61, p = 0.076). No significant interactions.

(i) *Grhl*, light-cycle ($F_{(1, 24)}$ = 18.36, p = 0.006), drug ($F_{(1, 24)}$ = 1.33, p = 0.27), pretreatment ($F_{(1, 24)}$ = 2.28, p = 0.15). No significant interactions.

(j) *Lepr*, light-cycle ($F_{(1, 24)}$ = 19.38, p = 0.004), drug ($F_{(1, 24)}$ = 0.22, p = 0.64), pretreatment ($F_{(1, 24)}$ = 6.437, p = 0.022). No significant interactions.

(k) *Rxfp1*, light-cycle ($F_{(1, 24)}$ = 36.89, p < 0.0001), drug ($F_{(1, 24)}$ = 11.20, p = 0.29), pretreatment ($F_{(1, 24)}$ = 6.01, p = 0.026). No significant interactions.

3.9 Summary

The preceding manuscript (chapter 3) highlighted the importance of TLR4's circadian rhythm in the expression of the "liking" and "wanting" components of alcohol-induced reward (Table 3). In brief, there were significant time-of-day effects in behavioural tests assessing the "liking" and "wanting" components of alcohol (Figure 2 and 3) with peak reward behaviours observed during the dark cycle (active period). Similarly, there were light-cycle dependent effects in the expression of genes pertaining to both the "liking" and "wanting" component of reward (*Drd1* and *Oprm1* for example) and the TLR4 pathway. Again, peak expression was generally observed during the dark cycle. Interestingly, the hypothalamus exhibited more light-cycle-dependent effects in terms of TLR4 pathway expression compared to the nucleus accumbens. The reason underlying this is remains to be fully elucidated, but may relate to the relative number or phenotype of neuroimmune cells within each area (Lawson et al., 1990) or that the SCN is located within hypothalamus and may therefore influence this area more than the nucleus accumbens. Genes representing the TRIF pathway (*Trif* and *Ifnb*) exhibited similar light-cycle dependent effects compared to genes representing the MyD88 pathway (*Il1b, Tnfa* and *Ccl2*) suggesting both pathways have elements which fluctuate according to the time-of-day. Antagonising the TLR4-TRIF pathway with (+)-Naltrexone reduced the intake and preference of alcohol during 2 and 8 h two-bottle choice preference tests; indicating a potential reduction in the "liking" component of alcohol-reward. The effect of (+)-Naltrexone was not specific to alcohol-induced "liking" as saccharin (an innate like-inducing solution) intake and preference was additionally reduced. The decreased alcohol intake may additionally be attributable to an altered aversive or gustatory response as quinine intake was modified by (+)-Naltrexone. However, when quinine was assessed over a broader concentration range, the effect of (+)-Naltrexone was lost. Given quinine reflects the aversive bitter component of concentrated alcohol solutions, it suggests the TLR4-TRIF pathway may be involved

in modifying the aversive component of alcohol in addition to reducing the “liking”. The “wanting” component of reward, as inferred by conditioned place preference and the 24 h two bottle choice, was significantly reduced following (+)-Naltrexone. The reduced “wanting” of alcohol is potentially attributable to the decreased expression of *Th* mRNA suggesting dopamine synthesis is reduced and thus, alcohol is imbued with less motivational significance. However, the mechanism underlying the reduced “liking” component remains elusive and may be attributable to acute electrophysiological modifications to opioidergic or cannabinoid neurons as well as decreases in gene and protein expression. Further experiments are therefore required to elucidate this finding. The effects of (+)-Naltrexone on attenuating reward-behaviour were generally more pronounced during the active (dark) phase (post hoc analysis) coinciding with the highest mRNA expression in the *Tlr4* signalling pathway and reward pathway activity. The greater effect of (+)-Naltrexone may simply be due to the greater preference for alcohol and saccharin observed during the active period. Therefore, antagonising this behaviour when it’s at its peak may result in the largest reduction in behaviour rather than examining a true “circadian-dependent phenomenon”. Alternatively, the diurnal variation in drinking behaviour may also suggest TLR4’s influence on the reward pathway is more pronounced during the active phase when its expression is highest. Thus, attenuating the TLR4-TRIF pathway during this phase has the greatest effect on reducing alcohol, saccharin and quinine intake and preference.

Collectively, this study highlights the importance of TLR4-TRIF in the acute actions of the “liking” and “wanting” components of reward and the importance of considering circadian timing when examining the efficacy of TLR4 antagonists.

Table 2 Summary of behavioural results (post hoc differences) from chapter 3

	Effective dose(s) of (+)-Naltrexone			
	Light-cycle			
Behaviour	**Light**		**Dark**	
	Intake	*Preference*	*Intake*	*Preference*
Water intake	↑ 30 mg/kg	N/A	↑ 15 – 30 mg/kg	N/A
2 h alcohol intake	—	N/A	↓ 60 mg/kg	N/A
8 h alcohol two bottle choice	—	↓ 60 – 75 mg/kg	↓ 60 – 75 mg/kg	↓ 45 – 75 mg/kg
2 h saccharin intake	—	N/A	↓ 60 mg/kg	N/A
8 h saccharin two bottle choice	—	↓ 30 – 75 mg/kg	—	↓ 75 mg/kg
8 h quinine two bottle choice	↑ 30 mg/kg	—	—	—
24 h alcohol two bottle choice	—	—	—	↓ 60 mg/kg
Conditioned place preference	N/A	—	N/A	↓ 60 mg/kg

N/A, not applicable; ↑, increased intake/preference relative to saline; ↓ decreased intake/preference relative to saline; — no difference relative to saline

3.10 References

Lawson, L. J., Perry, V. H., Dri, P., & Gordon, S., 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience, 39(1), 151–170.

Chapter 4: Adolescence, reward priming and TLR4

Following exposure to alcohol, the TLR4-signalling and reward pathway can remain in a primed or sensitised state (Vetreno & Crews, 2012; McClain et al., 2011; Pascual et al., 2009). This manifests as alterations in the expression of cytokines, neuroplastic and reward-related genes and protein (for example, Montesinos et al., 2016; Boutros et al., 2015; Centanni et al., 2014; Alaux-Cantin et al., 2013 Vetreno et al., 2013). These elevations increase the subsequent sensitivity towards alcohol causing a heightened “liking” and “wanting” sensation (see Doremus-Fitzwater & Spear, 2016; Spear, 2016 for review). Age is a key variable influencing the rate at which priming occurs. For example, adolescence, a period characterised by profound neurodevelopmental changes, are particularly sensitive to perturbations in immunological and neuronal homeostasis and therefore readily undergo priming (Spear & Swartzwelder, 2014; Bilbo & Schwarz, 2012). By contrast, in adulthood, the brain has reached developmental maturity and is therefore more resilient to perturbations, requiring higher doses or longer stimulation times before exhibiting a priming effect.

4.1 Adolescence

Adolescence is an evolutionary conserved period demarcating childhood from adulthood. In humans and rodents, adolescence begins following a surge of gonadal hormones, and typically occurs between 12 – 25 years and 21 – 42 postnatal days, respectively. However, the absolute boundaries of adolescence in both humans and rodents is relatively imprecise and there is little consensus regarding the definitive end of this period (Adriani et al., 2004; Adriani & Laviola, 2004; Tirelli et al., 2003; Spear, 2000; Petersen & Leffert, 1995). Both species exhibit similar neurodevelopmental patterns characterised by hormonal, physiological, neural and behaviour alterations. This confers a set of conserved behaviours evident in both species such as increased

peer-oriented social interactions (including bonding and aggression) (for example, Csikszentmihalyi et al., 2014; Primus & Kellogg, 1989) and novelty seeking/risk taking (for example, Steinberg, 2010; Adriani et al., 1998).

The potentiated novelty seeking behaviour is attributed to neurodevelopmental alterations in the limbic system predisposing adolescent's to be overly sensitive towards rewarding stimuli (Doremus-Fitzwater & Spear, 2016; Spear, 2000). The reward-sensitive phenotype promotes goal-direct behaviour and evolutionarily would assist species to focus on obtaining food, water and a mate (Spear, 2011; Romer, 2010; Steinberg, 2010). Beyond its evolutionary role, this phenotype causes adolescent's to engage in positive behaviours including establishing meaningful friendships and pursuing education (Telzer, 2016). However, this phenotype can also cause misaligned, detrimental goal-seeking behaviour such as unprotected sex, reckless driving and alcohol abuse (Johnston et al., 2015; Hingson et al., 2009; 2003). This phenotype is mirrored in rodents (Alaux-Cantin et al., 2013; Vetter et al., 2007) and is exacerbated as adolescent rodent's sensitivity to aversive stimuli is attenuated (Vetter-O'Hagen et al., 2009; Andersen et al., 1997). Collectively, the increased reward and decreased aversion promotes high levels of alcohol intake with very little immediate consequences. This pattern of drinking (binge drinking) is particularly problematic to neurodevelopment as alcohol reinforces an immature brain state which is primed/sensitised towards alcohol (Spear & Swartzwelder, 2014).

4.2 Reward development

The increased reward sensitivity and attenuated aversion is attributable to developmental changes occurring in the limbic system (Crews et al., 2006). For example, the prefrontal cortex (PFC), amygdala, nucleus accumbens and ventral tegmental area undergo substantial reorganisation inferred by volumetric changes in

MRI scans (Uematsu et al., 2012; Ernst & Mueller, 2008; Giedd et al., 1999). The volume of these regions generally follows an inverted U shape across adolescence (Gogtay et al., 2004; Sowell et al., 1999). The volume initially increases until mid-adolescence owing to the overproduction of axons and synapses. These are subsequently pruned and refined, reducing brain volume from mid to late adolescence. In terms of reward sensitivity, emphasis has been placed on neurodevelopmental alterations to the dopamine pathway. During adolescence, the number of dopaminergic fibres linking the prefrontal cortex to the nucleus accumbens increases (Naneix et al., 2012; Wahlstrom et al., 2010; Benes et al., 2000; Rosenberg & Lewis, 1995) and inhibitory control of PFC activity by dopaminergic projections from the VTA increases (Tseng & O'Donnell, 2007). This indicates that as adolescence progresses there is greater top-down feedback from regions involved in attention, memory and reward processing (PFC) thereby reducing impulsivity (Gogtay et al., 2004).

On a molecular level, there is conflicting evidence regarding the expression of dopamine's receptors, enzymes and transporters and the activity of dopaminergic neurons across adolescence. For example, studies found that while the levels of dopamine peak during adolescence (Andersen et al., 1997; Teicher et al., 1993), there was an overall reduction in dopamine synthesis and turnover rates in the nucleus accumbens compared to adult mice (Trantham-Davidson et al., 2017; Stamford, 1989). Further, adolescents exhibited reductions in basal dopamine release but had a greater dopamine reserve compared to adults (Andersen & Gazzara, 2006; Stamford, 1989). It was therefore hypothesized that adolescents exhibit greater dopamine release when stimulated (Laviola et al., 2001). However, other groups have reported contrary findings with adolescent rodents exhibiting increased tyrosine hydroxylase and dopamine receptors in the nucleus accumbens (Naneix et al., 2012; Mathews et al., 2009; Andersen, 2002; Tarazi & Baldessarini, 2000) and greater dopaminergic tone

(Andersen, 2002; Andersen et al., 1997) and dopamine turnover rates (Spear, 2000). The discrepancies in results are likely due to the ontological period (age), sex, the behaviour assessed and the molecular biology techniques used to assess expression levels. Despite differing findings, the conclusions drawn by these studies are the same: the fluctuation in dopamine may underlie the heightened sensitivity towards rewarding stimuli and confer greater motivational importance upon obtaining the stimuli. This in turn predisposes adolescent's to engage in reward-seeking behaviour (Doremus-Fitzwater & Spear, 2016).

There is little consensus regarding the developmental progression of the endogenous opioid pathway in reward regions (a key mediator of the "liking" component of reward). The variability in results is again likely attributable to the differing brain regions, sex and method of assessment (Carretero et al., 2004; McDowell & Kitchen, 1987). For example, receptor binding studies (either autoradiography or displacement assays) demonstrated that the expression of μ, δ and κ opioid receptors in the amygdala, hippocampus and nucleus accumbens peak during early adolescence (around P25) or in adulthood (P56) (Kapcala, 1986; Seizinger et al., 1982; Tsang et al., 1982; Ng et al., 1984; Bloom et al., 1980; Patey et al., 1980). By contrast, immunohistochemical studies suggest the expression of μ opioid receptors declines from one week post-natal onwards (Carretero et al., 2004). Importantly, the pharmacological properties of the receptor such as binding affinity and dissociation constant remain unchanged (Kornblum et al., 1987; Petrillo et al., 1987; Tavani et al., 1985). However, the recruitment of μ opioid receptor's intracellular signalling pathways, as inferred by [^{35}S]GTPγS binding is decreased in adolescents relative to adults, and the ratio μ opioid receptors binding to [^{35}S]GTPγS is decreased in reward-related brain regions (Talbot et al., 2005). The increased (or similar) receptor expression but reduced receptor coupling suggests adolescent brains are somewhat resilient to the effects of

the endogenous opioids. This conclusion contrasts the wealth of behavioural studies demonstrating adolescent mice typically consume higher levels of saccharin compared to adult mice suggested an augmented “liking” component of reward (for example, Friemel et al., 2010; Wilmouth & Spear, 2009). It is important to note that opioids are not the only mechanism underlying the reward sensitive phenotype. For example, cannabinoid receptors exhibit peak binding activity during adolescence, indicating an increased sensitivity of this system (Belue et al., 1995) and decreased receptor-mediated tonic current of $GABA_A$ receptors during adolescence may also mediate reward sensitivity (Yan et al., 2010; Li et al., 2006; 2003). These processes may contribute to the increased saccharin intake observed during adolescence. However, an in-depth discussion of the neuronal processes underlying these behaviours are beyond the scope of this thesis. Please refer to Crews et al., (2016) and Spear, (2000) for reviews.

Overall, the developmental effects occurring within the limbic pathway confer enhanced sensitivity to reward and attenuated sensitivity to aversion in adolescent’s. When these findings are applied to the context of alcohol use, adolescent’s will exhibit increased “liking” and “wanting” of alcohol with the experience of aversive taste or dysphoria lessened. This enables adolescents to consume high levels of alcohol (binge drinking) with little immediate consequences. As such binge drinking is increasingly prevalent among adolescents. For example, approximately 20 per cent of teenagers report engaging in binge drinking within the past week (Johnston et al., 2015). Like humans, adolescent rodents typically display greater intake and preference for alcohol and exhibit greater conditioned place preference and IV self-administration of alcohol indicating a greater “liking” and “wanting” component of reward compared to adults (Carrara-Nascimento et al., 2012; Maldonado et al., 2008; Truxell et al., 2007; Brunell & Spear, 2006; Doremus et al., 2005; Philpot et al., 2003). Further, adolescent rodents

are insensitive to the detrimental effects of alcohol and are protected against motor impairment, social impairment, anxiety, and require higher doses of alcohol to exhibit conditioned taste aversion and resist conditioned odour aversion towards alcohol (see Spear & Swartzwelder, 2014 for review).

4.3 Reward priming and later life effects

The consumption of alcohol during adolescence can have enduring consequences (McCambridge et al., 2011). For example, human youths that engage in binge drinking exhibit alterations in brain volumes, integrity of white matter tracts and distinctive patterns of brain engagement during cognitive tasks (Risher et al., 2015; Squeglia et al., 2015; Ozsoy et al., 2013; McQueeny et al., 2009; De Bellis et al., 2005). Further, epidemiological studies demonstrate correlations between the age of first alcohol use and alcohol dependence later in life suggesting that the brain is sensitised to the effects of alcohol (for example, Green et al., 2016; Irons et al., 2015; McCambridge et al., 2011). These effects are mirrored *in vivo* (Vetreno et al., 2017; Montesinos et al., 2016; Swartzwelder et al., 2014; Alaux-Cantin et al., 2013) However, establishing causation in humans is difficult as discerning the effects of adolescent alcohol from individual/genotype differences, personality traits and environmental factors such as socio-economic status, peer pressure and abusive/alcohol-dependent parents confounds findings (Stone et al., 2012; Donovan, 2004).

In rodents, adolescents exposed to alcohol exhibit attenuated sensitivity towards the aversive components of alcohol in adulthood (Crews et al., 2016; Spear & Swartzwelder, 2014). For example, adolescent alcohol exposure decreases alcohol-induced motor impairment, acute withdrawal, conditioned taste aversion, social inhibition, sedation time and anxiety behaviour in adulthood (for example, Mejia-Toiber et al., 2014; Varlinskaya et al., 2014; Matthews et al., 2008; Diaz-Granados & Graham,

2007). Furthermore, the increased sensitivity towards the rewarding components are persistently increased (Alaux-Cantin et al., 2013). Similar to humans, adolescent rodents exposed to alcohol increases 2 and 24 h alcohol intake and conditioned place preference in adulthood – indicating increased “liking” and “wanting” of alcohol (Alaux-Cantin et al., 2013; Broadwater & Spear, 2013; Gilpin et al., 2012; Pascual et al., 2009). Further, these mice exhibit higher breakpoints across progressive ratios when using self-administration furthering the potentiated “wanting” of alcohol (Amodeo et al., 2017). However, these findings are far from uniform with studies demonstrating the opposing effects (Slawecki & Betancourt, 2002). The different results are likely attributable to early vs late adolescence, route of alcohol exposure, and the behaviour examined later in life. In addition to alterations in reward and aversion, mice exposed to alcohol during adolescence exhibit greater levels of impulsivity and reduced behavioural flexibility as inferred by altered performance in the Barnes maze (Vetreno & Crews, 2015). This state further perpetuates the enhanced reward sensitivity as these mice become solely focused on obtaining alcohol. Collectively, these changes underlie the “locked-in” hypothesis of adolescent alcohol exposure. It suggests adolescent sensitivities towards alcohol are retained into adulthood. Consequently, the phenotype of an adult closely resemble that of an adolescent (increased novelty seeking/risk taking) (Crews et al., 2016; Spear & Swartzwelder, 2014).

There are shared mechanisms between adolescent and adult priming such as alterations to dopamine signalling (Karkhanis et al., 2015; Franklin et al., 2009; Pascual et al., 2009). However, there are also mechanisms unique to adolescence. Adolescent alcohol exposure alters the epigenetic profile of plasticity and reward related genes such BDNF, ARC and CREB (Sakharkar et al., 2016; Pandey et al., 2015). This process is likely mediated by increases in HDAC activity leading to decreased acetylation in the promoter regions of ARC and BDNF (Pandey et al., 2015). Altered

expression of BDNF and ARC is thought to alter dendritic spine density in the amygdala which in turn influences anxiety and drinking behaviour in adulthood (Trantham-Davidson et al., 2017; Pandey et al., 2015). In addition, perturbations to dendritic spine density is associated with altered dopaminergic signalling (Lin et al., 2015; Goldwater et al., 2009) and may therefore potentially alter reward as well. Furthermore, adolescent alcohol intake increases the number of immature dendritic spine formation, causing long-term potentiation at lower intensities (Risher et al., 2015). This finding mirrors those observed during neurodevelopmental disorders that lead to long-term memory deficits and other cognitive impairments (Penzes et al., 2011; Chen et al., 2010). Adolescent alcohol exposure induces many other epigenetic processes including the expression of regulatory microRNAs and histone methylation (Prins et al., 2014; You et al., 2014; Pietrzykowski et al., 2008) which have the potential to alter reward-related processes.

Further, the increased reward-sensitivity later in life may be due to alterations in the dopamine pathway. Following adolescent alcohol exposure, rodents exhibit increased baseline dopamine release in the nucleus accumbens (Philpot et al., 2009) and a reduction in dopamine D2 auto-receptor expression (negative regulator of dopamine) (Pascual et al., 2009). Upon re-exposure to alcohol in adulthood, these rodents exhibit decreased dopamine release despite increased activity in the nucleus accumbens (Liu & Crews, 2015) suggesting a state of reward deficiency and further potentiating the “wanting” of alcohol (Zandy et al., 2015). This effect is potentially attributable to the decreased feedback between the nucleus accumbens and the PFC indicating reduced top-down control of the reward pathway (Liu & Crews, 2015). This further suggests alcohol has altered the developmental pattern of the reward pathway via synaptic remodelling predisposing an individual to increased alcohol “wanting”.

In addition, the GABAergic system, the main inhibitory pathway is also altered. For example, adolescent alcohol exposure decreases $GABA_A$ α4 and the extrasynaptic $GABA_A$ δ receptor mRNA in adulthood (McClintick et al., 2016; Risher et al., 2015; Centanni et al., 2014). This decreases basal GABAergic currents however, upon re-exposure to alcohol, this current is potentiated. This suggest adults under basal circumstances exhibit less inhibition, however, upon re-exposure there is an increase in inhibition potentially leading to tolerance (Risher et al., 2015; Fleming et al., 2011).

The examples provided above are a select handful. Alcohol affects almost all brain regions and neurotransmitter systems. Consequently, the effects of adolescent alcohol are likely to be widespread and involve almost all neurotransmitter systems within the brain (McClintick et al., 2016). Recent research however, has highlighted the importance of the neuroimmune system, specifically the innate pattern recognition receptor TLR4, in mediating the molecular actions of alcohol, reward and development.

4.4 TLR4 and neurodevelopment

Toll-like receptor 4 and the development of the CNS are inextricably linked. This is perhaps unsurprising as Toll-like receptors were first identified as a crucial regulators of embryogenesis in the fly (Belvin and Anderson, 1996; Nüsslein-Volhard & Wieschaus, 1980). Recent research has extended these findings demonstrating TLR4 is involved in key aspects of neurodevelopment such as axonal growth and neural progenitor cell proliferation and development (Barak et al., 2014; Okun et al., 2011). Further, genetic knockout of TLR4 can severely alter the phenotype of the central nervous system (Okun et al., 2012). For example, $TLR4^{-/-}$ mice contain relatively fewer glia and more neurons compared to wildtype mice (Rolls et al., 2007). TLR4's downstream signalling molecules, such as NFκB, are additionally crucial to neuronal development. For example, NFκB is involved in synaptic scaling, synaptic positioning

and ensures the correct expression of neurotransmitter receptors within neurons (Zhang & Hu, 2012).

Given TLR4 is pivotally involved in development, activation of this receptor during periods of neurodevelopment can be detrimental. For example, high doses of LPS impair spatial learning and increase anxiety behaviour in adulthood an effect attributable to neuronal cell death and synaptic loss- particularly in cholinergic and serotonergic neurons (Vetreno et al., 2017; 2014). Lower doses of LPS transiently alter social and anxiety-like behaviour and lead to long-lasting modifications in electrophysiological properties of neurons (Ming et al., 2015a; 2015b) further highlighting the detrimental effects of immune activation during adolescence.

4.5 Alcohol-induced TLR4 signalling during adolescence

Similar to adults, alcohol exposure during adolescence initiates an immune response within the CNS. For example, adolescent alcohol exposure activates microglia and astrocytes; increases expression of innate immune receptors RAGE and TLR2, 3 and 4, TLR agonist HMGB1, inflammatory cytokines and reactive oxygen species in the brain of rodents (Montesinos et al., 2016; Pascual et al. 2016; Alfonso-Loeches et al., 2013; Vetreno & Crews, 2012; Pascual et al., 2007). It is unclear however, whether alcohol-induced TLR4 activation results in the activation of the MyD88 and TRIF pathways – an area requiring future research.

It is important to note, that in general the elevations in immune responses of an adolescent are blunted compared to those of adults. For example, alcohol increased IL-6 and IκBα in the amygdala, hippocampus and hypothalamus with adults exhibiting a more robust response compared to adolescence – an effect which coincided with reduced BACs (Doremus-Fitzwater et al., 2015). Further, adolescent mice exhibited

blunted alcohol-induced cytokine expression compared to adults in the cortex and hippocampus (Doremus-Fitzwater et al., 2015; Kane et al., 2013). This finding mirrors the age differences in the neuroimmune response towards LPS. Adult mice exhibit greater expression of inflammatory mediators following LPS compared to adolescent mice. This effect was attributable to circulating gonadal hormones as gondectomy mitigated the ontological differences (Cai et al., 2016). Additional studies suggest CREB may additionally contribute to the ontological differences in the neuroimmune response. Compared to adults, CREB is upregulated in the adolescent brain (Pennypacker et al., 1995). Given that CREB competitively competes for CBP, a co-factor required for NFκB signalling within the brain (Wen et al., 2010) it is hypothesised the NFκB activity would be decreased, dampening a potential immune response towards LPS and alcohol. However, the mechanisms underlying the attenuated neuroimmune response is an area which requires further research. Regardless, the limited inflammatory response is presumably designed to stop the detrimental effects of an over-active immune system on neurodevelopment.

4.6 Alcohol-induced TLR4 priming/sensitisation

Neuroimmune cells can enter a “primed” state following alcohol-induced immune activation. It is important to note, “primed” neuroimmune cells appear morphologically active however, they do not over produce immune mediators basally (Bilbo, 2009). Neuroimmune priming has been observed in the context adolescent alcohol exposure. For example, alcohol exposure during adolescence increased the number of amoeboid microglia and CD68 expression (a marker of “activated microglia”) in adulthood (McClain et al., 2011). Interestingly however, immune receptors such as MHCII, TLR2 – 4, reactive oxygen-inducing enzymes, NFκB, cytokines and danger signals are additionally elevated in the brains of adult rodents that received alcohol during adolescence (Cruz et al., 2017; Montesinos et al., 2016; Vetreno & Crews, 2012;

McClain et al., 2011). It is presently unclear whether the MyD88 and TRIF pathways remain elevated or what cell type is responsible for these elevations following adolescent alcohol use. The results therefore suggest rather than remaining in a "primed" state, the neuroimmune system is continually sensitised exhibiting basal increases in the expression of immune mediators.

The ongoing elevation in TLR4-related mediators is likely to perturb the development of neurons. As alluded to in chapter 1.8, TLR4 and its downstream mediators alter the functional, epigenetic and structural properties of neurons. These effects are also observed in the adult brains of rodents who received alcohol during adolescence (Montesinos et al., 2016; 2015). For example, adolescent alcohol exposure reduces markers of plasticity (ARC and BDNF), epigenetic processes (HDAC and histone proteins) and myelin as well as increases in markers of the TLR4 pathway and apoptosis in adulthood (Montesinos et al., 2016; Sakharkar et al., 2016). The reduction in plasticity and potentiated neuroimmune responses, reinforces an immature brain which is sensitive to the effects of alcohol later in life (Montesinos et al., 2016). In addition to the developmental effects, it has been hypothesised that subsequent activation of the immune system by the original or a new immunogen, will result in an exaggerated inflammatory response. This exaggerated response will act on neighbouring cells influencing their function and potentially increasing the hedonic and anhedonic aspects of drugs of abuse later in life.

Crucially however, attenuating TLR4 protects against the enduring effects of adolescent alcohol exposure. Inhibiting alcohol-induced TLR4 signalling prevents the reduction in plasticity and epigenetic processes, decreases markers of inflammation and prevents adolescent alcohol-induced potentiation of reward ("wanting" and "liking") and anxiety behaviour later in life (Montesinos et al., 2016; 2015). Collectively, the

results suggest that adolescent mice are hypo-sensitive to the immediate effects of an alcohol-induced immune response. However, the neuroimmune system, in particular the TLR4 pathway, readily undergoes sensitisation exhibiting long-term increases in basal expression. Upon re-exposure to alcohol, the response of this pathway will potentially be exaggerated. This in turn contributes to molecular and behavioural alterations which underlie the enduring consequences of adolescent alcohol exposure including reward sensitisation.

4.7 Study 2 aims and hypothesis

The previous sections aimed to highlight the importance of TLR4 in mediating the effects of alcohol, development and reward. These sections further illustrated that alcohol-induced activation of TLR4 during adolescence can sensitise this pathway which may contribute to developing behavioural deficits associated with adolescent alcohol use later in life; specifically, increased reward-seeking behaviour. However, definitive evidence demonstrating TLR4's involvement in this process is lacking. Thus far, only one study has sought to determine the role TLR4 in mediating the consequences of adolescent alcohol use on reward and anti-reward behaviour later in life. Montesinos et al., (2016) demonstrated adolescent alcohol exposure increased cocaine-induced conditioned place preference, 48 h alcohol two bottle choice, saccharin intake and time spent in the enclosed arms of the elevated plus maze. Collectively the results suggest adolescent alcohol exposure potentiated the "liking" and "wanting" components of reward and increased "anti-reward" later in life (Montesinos et al., 2016). These behaviours coincided with increased inflammatory mediators, HDAC expression and acetylation of histones. The behavioural and molecular alterations induced by adolescent alcohol exposure were all absent in TLR4$^{-/-}$ mice. This suggests TLR4 is crucial in mediating alcohol-induced epigenetic remodelling and sensitisation which leads to potentiated "wanting", "liking" and anti-

reward later in life. However, given TLR regulates neurodevelopmental processes, the results above are inherently confounded. For example, TLR4$^{-/-}$ mice contain higher levels of neurons and comparatively fewer glia compared to wildtype mice (Okun et al., 2012; Rolls et al., 2007). Consequently, the reduced immune response observed by Montesinos et al., (2016) may simply be due to a reduction in the number of primary immune cell in the CNS. Further, TLR4 is involved in the regulation of neurogenesis, neurite/axonal growth and neuronal differentiation and survival suggesting that TLRs may influence cognition (Okun et al., 2011). Therefore, the precise role of TLR4 in the effects of alcohol, reward sensitisation and adolescence can only be studied using conditional knockouts or a pharmacological antagonist. Further, these studies failed to differentiate or discuss differences between the MyD88 and TRIF pathway in mediating these effects and they used chronic, high doses of alcohol which do not accurately follow human drinking behaviour. Therefore, the aims of this study were to determine:

1. whether a more relevant model of adolescent alcohol exposure;
    a. modifies the “liking” and “wanting” components of alcohol reward and alcohol-induced anti-reward later in life;
    b. modifies the expression of TLR4- and reward-related pathways later in life;
        i. and whether it preferentially modifies the TRIF or MyD88 pathway;
2. whether attenuating the TLR4-TRIF either before or after adolescent alcohol exposure;
    a. modifies alcohol-induced alterations to the “liking” and “wanting” components of reward and anti-reward later in life; and
    b. modifies alcohol-induced alterations to the TLR4- and reward pathway later in life.

Given that adolescent alcohol exposure modifies the reward pathway and the neuroimmune system, we hypothesised that inhibiting the TLR4-signalling pathway before or after adolescent alcohol exposure would reduce, but not completely reverse increases in reward and anti-reward behaviour in adulthood.

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Chapter 5: Administering (+)-Naltrexone before or after adolescent alcohol exposure prevents alcohol drinking but not seeking behaviour in adulthood.

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5.1 Abstract

Adolescents frequently engage in risky behaviours such as binge drinking. Binge drinking, in turn, perturbs neurodevelopment reinforcing reward seeking behaviour in adulthood. Current animal models are limited in their portrayal of this behaviour and in their assessment of the neuroimmune systems involvement, specifically the role of Toll-like receptor 4 (TLR4). Therefore, the aims of this project were to develop a more relevant animal model of adolescent alcohol exposure and to characterise its effects on TLR4 signalling and alcohol-related behaviours later life. Balb/c mice received a short (P22 – P25) alcohol binge during in early adolescence, and underwent tests to investigate anxiety (elevated plus maze), alcohol seeking (conditioned place preference) and drinking behaviour (drinking in the dark) in adulthood. Four doses of alcohol during adolescence increased alcohol-induced conditioned place preference and alcohol intake in adulthood. However, this model did not affect basal elevated plus maze performance. Subsequent analysis of nucleus accumbal mRNA, revealed increased expression of TLR4-related mRNAs in mice who received alcohol during adolescence. To further elucidate the role of TLR4, (+)-Naltrexone, a biased TLR4 antagonist was administered 30 mins before or after the adolescent binge paradigm. When tested in adulthood, (+)-Naltrexone treated mice exhibited reduced alcohol intake however, alcohol seeking and anxiety behaviour was unaltered. This study highlights that even a small amount of alcohol, when given during a critical neurodevelopmental period, can potentiate alcohol-related behaviours and TLR4 activation later in life. Interestingly, attenuation of TLR4 before or after adolescent alcohol exposure reduced alcohol intake but not seeking behaviour in adulthood.

Keywords: Toll-like receptor 4, TRIF, alcohol, development, neurodevelopment, adolescent, GABA

5.2 Introduction

Adolescence is a unique neurodevelopmental period characterized by an increased sensitivity towards rewarding stimuli and an attenuated sensitivity to aversive stimuli (Spear, 2011). This phenotype causes adolescents to engage in risk-taking behaviors such as unprotected sex, reckless driving and binge drinking (Johnston et al., 2015; Hingson et al., 2009; 2003). Binge drinking in turn profoundly perturbs neurodevelopment causing a retention of adolescent-like phenotypes such as reward-sensitivity in adulthood (the “locked-in” hypothesis) (Crews et al., 2016; Doremus-Fitzwater & Spear, 2016). Consequently, individuals that consume alcohol during adolescence are more likely to develop problems associated with alcohol use in adulthood (see Spear, 2011 for review). This finding is reinforced by the link between age of first use and alcohol dependence later in life (DeWit et al., 2000). Crucially, these phenomen are readily translatable to rodents (Spear, 2011). Adolescent rodents exposed to alcohol exhibit potentiated alcohol-reward behaviors in adulthood as inferred by increased conditioned place preference, self-administration and two bottle choice drinking (Pandey et al., 2015; Alaux-Cantin et al., 2013; Maldonado et al., 2008; Rodd-Henricks et al., 2002). However, the magnitude of this potentiation is variable owing to differences in sex, genetic background, age and the model of adolescent alcohol exposure (Strong et al., 2010; Walker & Ehlers, 2009; Blizard et al., 2004; Siciliano & Smith, 2001). The model of alcohol exposure is a particularly important variable. To reach high blood alcohol concentrations (BACs) researchers often use methods that bypass the natural route of administration (for example Gass et al., 2014; Gilpin et al., 2012). This in turn, influences the molecular and behavioral responses towards alcohol (Osterndorff-Kahanek et al., 2015; 2013; Gilpin et al., 2012) and consequently, it is unclear how much these models reflect the human condition (Ward et al., 2014).

Despite different exposure methodologies rodent studies have identified multiple mechanisms underlying adolescent alcohol-induced reward sensitivities in adulthood with particular emphasis placed upon the molecular and cellular alterations within the nucleus accumbens and amygdala (Spear & Swartzwelder, 2014). For example, adolescent alcohol exposure reduces the expression of plasticity-related genes (BDNF, ARC and CREB), negative regulators of dopaminergic function (dopamine D2 receptor and GABA receptors) and alters dopaminergic firing and tone in adulthood (Sakharkar et al., 2016; Philpot et al., 2009; Pascual et al., 2009; Pietrzykowski et al., 2008). These alterations enhance an individual's sensitivity towards dopamine-inducing experiences such as alcohol use, and reduced the ability to alter learnt behavior (Vetreno et al., 2015; Alaux-Cantin et al., 2013; Maldonado-Devincci et al., 2010).

Recent research has additionally highlighted the importance of the neuroimmune system in contributing to the adverse neurodevelopmental consequences of adolescent alcohol exposure (Crews et al., 2016; Montesinos et al., 2016). Particular emphasis has been placed on Toll-like receptor 4 (TLR4), a pattern recognition receptor broadly expressed throughout the central nervous system (Akira & Takeda, 2004; Bsibsi et al., 2002). Following activation, TLR4 signals via the MyD88 or TRIF pathways culminating in the expression of classical pro-inflammatory cytokines and type 1 interferon's respectively (see Akira & Takeda, 2004 for review). Alcohol indirectly activates TLR4 recruiting MyD88 and TRIF *in vitro* (Crews et al., 2013; Fernandez-Lizarbe et al., 2009). However, whether both pathways are activated *in vivo* remains to be determined. Alcohol-induced recruitment of these adapters causes a signaling cascade resulting in the translocation of immune-related transcription to the nucleus. This in turn increases the expression of inflammatory proteins from both microglia and astrocytes (Fernandez-Lizarbe et al., 2009; Blanco et al., 2005).

Importantly, TLR4$^{-/-}$ mice display reduced levels of cytokines, chemokines and inflammatory transcription factors immediately following adolescent alcohol exposure and later in adulthood compared to wildtype mice (Montesinos et al., 2016; Pascual et al., 2016; Kane et al., 2013). This coincides with reduced synaptic and myelin derangements, long-term aberrant synaptic remodelling, decreased histone acetylation at BDNF and FosB promoter regions (Montesinos et al., 2016). Behaviourally, TLR4$^{-/-}$ mice do not exhibit long-term cognitive impairments (Montesinos et al., 2015), display less anxiety-like and drug seeking behaviour in adulthood compared to wildtype following adolescent exposure (Montesinos et al., 2016). While the precise neuroanatomical area underlying the long-term actions of adolescent alcohol-induced TLR4 activation remains to be determined, studies using morphine (another TLR4 agonist) have identified the nucleus accumbens as a key substrate (Schwarz et al., 2013).

TLR4 is additionally pivotal to normal neurodevelopmental processes (see Okun et al., 2011 for review), therefore, studies using TLR4$^{-/-}$ animals are inherently confounded. For example, TLR4$^{-/-}$ mice have higher levels of neurons and relatively fewer glia compared to wildtype mice (Rolls et al., 2007). Further, the use of TLR4$^{-/-}$ mice does not enable researchers to investigate the relative contribution of the MyD88 or TRIF pathways in the behavioral and molecular response to alcohol. Lastly, studies investigating the TLR4 often use excessive doses/treatments of alcohol exposure which may exaggerate endpoints. Therefore, the aims of this study were to determine whether a more relevant model of adolescent alcohol exposure alters reward-related behavior and mRNA and the TLR4 pathway later in life and secondly, to determine whether pharmacologically attenuating TLR4 prevents any alcohol-induced reward alterations later in life. These alterations were assessed using conditioned place preference, drinking in the dark and the elevated plus maze with the transcription of a

selection of gene targets relating to reward (dopaminergic, opioidergic, GABAergic and glutamatergic processes) and plasticity (BDNF and CREB) within the nucleus accumbens additionally assessed.

5.3 Methods

5.3.1 Animals

Pregnant female Balb/c mice (10 – 15 days into their gestation cycle) were obtained from the University of Adelaide Laboratory Animal Services, Adelaide, SA, Australia. Following their arrival to the animal facility, mice were housed in light/dark (12/12h, lights on/off at 7am/7pm respectively) and temperature (23 ± 3°C) controlled rooms. Food and water was available *ad libitum*.

After the dams had given birth, their offspring developed undisturbed until postnatal (P) day 22 at which point they began the adolescent alcohol exposure paradigm (figure 1a – b). The young age selected for this study was designed to reflect the age at which individuals are particularly sensitive to the effects of alcohol (DeWit et al., 2000). After the completion of the paradigm, mice were weaned and separated into single sex housing (P25) and were left undisturbed until P51. At beginning of adulthood (P56) mice began behavioural testing. Mice undergoing conditioned place preference or elevated plus maze remained group housed. Mice undergoing drinking in the dark were separated into individual cages.

Adult mice were handled by the experimenter for five days prior to testing. Conditioned place preference and elevated plus maze occurred during the light phase of the mouse's light/dark cycle. Drinking in the dark (2 – 4 h access alcohol drinking) began 2 h into the mouse's dark cycle. Both male and female mice were used for behavioural experiments. Statistical analysis determined sex was not a significant variable for

behavioural experiments and consequently, data from both male and female animals were pooled together for data analysis.

All animal care and experiments complied with the principles of the Australian Code of Practice for the care and use of animals for scientific purposes and was approved by the University's Animal Ethics Committee.

5.3.2 Drugs

Ethanol (99.5%) (herein referred to as alcohol) was purchased from Chemsupply (Gliman, SA, Australia). Alcohol was administered as an oral gavage (10 – 30 per cent v/v). The dose of alcohol ranged from 0.5g/kg to 3.5g/kg for adolescent alcohol exposure paradigm and 1.5g/kg for conditioned place preference. Saline oral gavages were volume-matched.

(+)-Naltrexone, a pharmacological TLR4 antagonist was synthesised and supplied by Dr Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute of Alcohol Abuse and Alcoholism, Bethesda, MD, USA). (+)-Naltrexone was administered via intraperitoneal injections at a dose of 60mg/kg (dose volume 10 ml/kg). Saline intraperitoneal injections werer volume-matched.

5.3.3 Adolescent alcohol exposure

5.3.3.1 Rationale

Consuming alcohol during adolescence can impair neurodevelopment, reinforcing an underdeveloped, immature brain. In adulthood, these individuals are at risk for developing anxiety and alcohol-drinking disorders indicating alcohol specifically alters the development of brain regions governing hedonia, reward, motivation and emotion (Doremus-Fitzwater & Spear, 2016). This phenomenon is translatable to animal

models with adolescent mice and rats exposed to alcohol exhibiting potentiated alcohol preference and anxiety later in life (for example, Sakharkar et al., 2016). However, generalising the magnitude of effects is difficult owing to differences in experimental design. For example, current rodent models by-pass the natural oral route of administration (Gass et al., 2014; Gilpin et al., 2012) to produce greater blood alcohol concentrations and are prolonged/chronic in nature (Vetreno et al., 2015). Consequently, the behavioural and molecular responses attributable to alcohol are either exaggerated, minimised or clouded (Ward et al., 2014). To circumvent these confounding variables, a shorter model was utilised.

5.3.3.1 Adolescent exposure model

Mice received an oral gavage of alcohol (0.5, 1.5, 2.5, 3.5g/kg) or saline (volume matched) for four consecutive days (P22 – 25). An hour after the last oral gavage, tail blood was collected and blood alcohol concentration (BAC) was quantified. Mice were then weaned (P25), separated into single sex cages and allowed to mature undisturbed until P51(Figure 1a).

For studies assessing the role of TLR4 on the neurodevelopmental outcomes following adolescent alcohol exposure, a similar protocol was used. However, thirty minutes pre- or post adolescent alcohol exposure, mice received an intraperitoneal injection of (+)-Naltrexone or saline (Figure 1b). The objective of using both a pre- and post-treatment paradigm was to ascertain the mechanism by which (+)-Naltrexone works (pretreatment) and to determine its efficacy once the pathology has commenced (post-treatment). Mice in this experiment received 2.2 g/kg of alcohol rather than a range of doses to minimise the number of rodents used in this study. The dose of alcohol was calculated by determining the effective dose 50 (ED_{50}) from conditioned place

preference later in life (figure 3a). An hour after the last gavage of alcohol, blood was harvested from the tail to quantify BAC.

5.3.4 Adult behavioural tests

At the beginning of adulthood (P56) mice underwent elevated plus maze, conditioned place preference or drinking in the dark (P63).

5.3.4.1 Conditioned place preference

Conditioned place preference was used to infer alcohol-seeking and -rewarding behaviour and the ability to form an alcohol-associated memory (Bardo & Bevins, 2000).

Apparatus

The conditioning apparatus consisted of two conditioning chambers (10.9 (length) x 9.3 (width) x 35 (height) cm) separated by a neutral chamber (16.6 x 4.8 x 35 cm). The neutral chamber contained black walls with grey flooring. The conditioning chambers differed in tactile and visual cues. The flooring of the conditioning chambers were either black plexiglass perforated holes (5mm apart) or black plexiglass grids (5mm apart). The walls of each chamber were white or black. The combination of floor texture and wall colour were altered for each cohort to prevent any inherent bias the rodents have for a specific texture/colour combination.

During conditioning, a sliding partition restricted access to only one chamber. Movement and time spent in each chamber was recorded using Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA).

Procedure

Pre-test (day 1): Mice were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

Conditioning (day 2 – 9): Mice received an oral gavage of alcohol (1.5 g/kg) and placed within their conditioning chamber for 30 min on days 1, 3, 5, 7. On days 2, 4, 6 and 8, mice received an oral gavage of saline and placed within the unconditioned chamber for 30 min. Mice received a total of four conditioning sessions with each drug (alcohol or saline).

Test (Day 10): Mice received an oral gavage of saline and were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

To infer whether the conditioning was successful, the time spent in the conditioned chamber during the post-test was subtracted from the time spent in the conditioned chamber during the pre-test.

5.3.4.2 Drinking in the dark

Binge-like consumption of alcohol was assessed using the drinking in the dark procedure (Thiele & Navarro, 2014). At P56 mice were individually housed and acclimatised to their new environment for one week prior to experimentation. 2 h into the mouse's dark cycle, the bottle of water was removed and replaced with a bottle of 20 per cent (v/v) alcohol for 2 h (P63 – 65). After 2 h, the alcohol bottle was removed, weighed and replaced with a bottle of water. On the fourth and final day of testing (P66), mice received alcohol for 4 h.

5.3.4.3 Elevated plus maze

To infer basal anxiety-like behaviour mice underwent the elevated plus maze (Carola et al., 2002). The elevated plus maze consisted of two areas characterised by high walls and a relatively dark environment and an open area.

Apparatus

The maze is made of black PVC and consists of four arms: two open and two closed. All arms were 30 cm long and 5 cm wide. The two enclosed arms had walls 25 cm high. The maze was elevated 1.2 m off the ground.

Procedure

Mice were moved into the behavioural testing room 30 minutes prior to testing to acclimatise them to a new environment. Mice were subsequently placed into the centre of the elevated plus maze with their head facing towards the open arm and allowed to explore the apparatus for five minutes. The time spent, number of exits, distance travelled and the number of immobile episodes was recorded using a Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA).

5.3.5 Blood alcohol concentration assay

Serum alcohol concentration was measured using a commercial kit (ADH-NAD Reagent Multiple Test Vial; Sigma-Aldrich) and performed as per the manufacturer instructions. In brief, it estimates alcohol induced reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH in the presence of alcohol dehydrogenase. The reaction is observed by recording the absorbance of 340 nM by the solution. Serum alcohol was acquired immediately after behavioural testing or adolescent alcohol exposure.

5.3.6 RNA isolation, reverse transcription and quantitative PCR (qPCR)

The nucleus accumbens region was isolated using micropunches (Kai Medical, Seki City, Japan) from whole brains and submerged in RNAlater® ICE (ThermoFisher Scientific, Waltham, MA, USA) prior to performing RNA isolation. RNA was isolated using Maxwell® 16 LEC simply RNA Tissue Kit (Promega, Madison, WI, USA) as per manufacturer instructions. RNA was quantified using spectrophotometric analysis, with the quality of RNA verified by the OD260/280 ratio. Isolated RNA (900ng) was reversed transcribed into cDNA using iScript™ cDNA reverse transcription kit (BioRad, Hercules, CA, USA) as per manufacturer instructions.

Gene expression was assessed using iTaq™ Universal SYBR® Green Supermix as per manufacturer instructions. Real time PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Mouse *Bdnf*, *Ccl2*, *Cd14*, *Creb1*, *Drd1*, *Drd2*, *Gabra1*, *Gabra2*, *Gapdh*, *Gria1*, *Grin1*, *Hmgb1*, *Ifnb*, *Il1b*, *Il10*, *Md2*, *Myd88*, *Ntrk2*, *Oprm1*, *Th*, *Tlr4* and *Trif* forward and reverse primers were synthesised by Integrated DNA Technologies Pty. Ltd. (Baulkham Hills, NSW, Australia). For primer sequences refer to supplementary materials. The genes assessed were based upon previous studies demonstrating differences in dopaminergic, opioidergic, GABAergic and glutamatergic processes following adolescent alcohol exposure (Alaux-Cantin et al., 2013; Pascual et al., 2009).

The relative difference in expression level of each of the genes of interest were normalised to the CT of GAPDH for both the test and control sample. The ΔCT of the test sample was normalised to the ΔCT of a control sample (a equal amount of cDNA from all the different groups), and then expressed as a ratio (2^-ΔΔCT).

5.3.7 Statistical analysis

Experiment 1: Conditioned place preference (chamber x dose), elevated plus maze (arm x dose) and drinking in the dark (day x dose) were analysed using a two-way ANOVA with repeated measures and Tukey post hoc (figures 2 – 3).

Experiment 2: qPCR was analysed using a one-way ANOVA with Bonferonni post hoc (figures 4 – 5).

Experiment 3: qPCR analysed using a two-way ANOVA with Bonferonni post hoc (intervention x gavage) (figures 6 – 7).

Experiment 4: Conditioned place preference (chamber x intervention x gavage x order), elevated plus maze (arm x intervention x gavage x order) and drinking in the dark (day x intervention x gavage x order) was assessed using a four-way ANOVA with Tukey post hoc (figure 8 – 10).

All summary values presented as mean ± standard error of mean (SEM). p-values ≤ 0.05 were considered statistically significant.

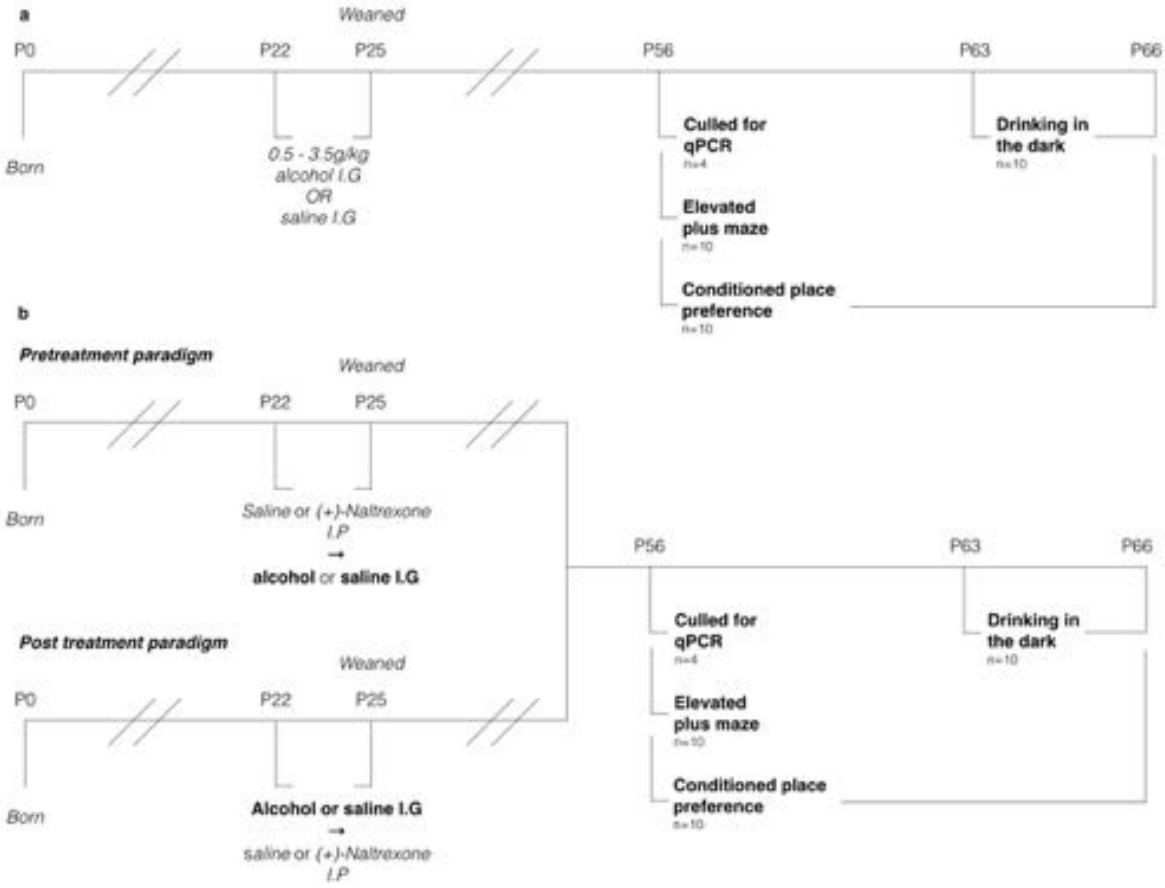

Figure 1 Timelines for behavioural experiments. (a) Between postnatal days (P) 22 and 25 adolescent mice received a gavage of alcohol (0.5 g/kg – 3.5 g/kg) or saline daily. On P25, mice were weaned and separated into single sex cages and left to develop undisturbed until adulthood. Mice were subsequently tested for anxiety-like, alcohol-seeking or alcohol drinking in adulthood using the elevated plus maze (on P56), conditioned place preference (P56 – 66) and drinking in the dark (P63 – P66) respectively. (b) Adolescent mice received either (+)-Naltrexone or saline 30 minutes before or after an oral gavage of saline or alcohol (2.2 g/kg) for four consecutive days (P22 – 25). Mice were left to develop undisturbed until adulthood upon which they were tested using the elevated plus maze, conditioned place preference or drinking in the dark or culled for qPCR.

5.4 Results

5.4.1 Experiment 1: Does a short adolescent alcohol exposure model potentiate anxiety and alcohol-reward behaviour in adulthood?

An important consideration when examining the effects of adolescent alcohol exposure on later life behaviour is the relative rise in blood alcohol following the initial alcohol experience. One hour after the last gavage tail blood was isolated and BAC was quantified. The gavage model produced a dose dependent increase in blood alcohol ranging from 57 to 431mg/100mL at the lowest (0.5 g/kg) and highest (3.5 g/kg) doses respectively (effect of dose, $F_{(3.\ 32)} = 319.8$, $p < 0.0001$). The precise statistical information and figures can be viewed in the supplementary material (figure s1).

Basal anxiety-behaviour in adulthood (P56) was assessed using the elevated plus maze. A two-way ANOVA determined alcohol exposure during adolescence did not influence the time spent, number of exits, distance travelled or immobile episodes in the elevated plus maze in adulthood (effect of dose; time, $F_{(4, 36)} = 1.1$, $p = 0.37$; exits, $F_{(4, 36)} = 1.0$, $p = 0.42$; distance, $F_{(4, 36)} = 1.18$, $p = 0.34$; and immobile episodes, $F_{(4, 36)} = 0.57$, $p = 0.68$, respectively) (figure 2a – d). Post hoc analysis did not reveal any significant differences between the treatment groups with respect to the dose of alcohol. However, there was a significant effect of maze arm (open or closed) with respect to time, number of exits, distance travelled and immobile episodes (effect of maze arm; time, $F_{(1, 9)} = 126.1$, $p < 0.0001$; exits, $F_{(1, 9)} = 403.8$, $p < 0.0001$; distance, $F_{(1,\ 9)} = 4.952$, $p = 0.05$; and immobile episodes, $F_{(1,\ 9)} = 135.7$, $p < 0.0001$, respectively). No interactive effects (effect of dose x maze arm) or post hoc differences were present for any of the variables ($p > 0.05$, see supplementary material for full statistical description). These findings suggest that four consecutive doses of alcohol during adolescence are insufficient to alter baseline anxiety-like behaviour in adulthood using this model in Balb/c mice.

To determine whether adolescent alcohol exposure modifies alcohol-reward behaviour in adulthood, adult mice underwent conditioned place preference (figure 3a). Irrespective of the adolescent treatment, all mice exhibited conditioned place preference towards alcohol (effect of conditioning chamber, $F_{(1, 11)} = 47.12$, $p < 0.001$) (figure 3a). Further, there was an effect of the adolescent alcohol dose on the change in time alcohol-conditioned chamber time in adulthood (effect of dose, $F_{(4, 44)} = 4.36$, $p = 0.0047$). The Tukey post hoc revealed significant differences between vehicle and 0.5g/kg, 1.5g/kg, 2.5g/kg and 3.5g/kg of alcohol, with the greatest difference observed at 2.5g/kg. Interestingly, however was no interactive effect between the dose of alcohol and the conditioning chamber suggesting the differences between doses is small (interaction, $F_{(4, 44)} = 1.69$, $p = 0.17$). These results highlight that a comparatively minor dose of alcohol during adolescence is sufficient to potentiate alcohol-seeking behaviour in adulthood.

To verify that the adolescent alcohol model potentiates alcohol reward-behaviour in adulthood, mice underwent drinking in the dark, a limited access-drinking paradigm (figure 3b). One concentration of alcohol (2.2 g/kg) was selected for this experiment based from the ED_{50} of the conditioned place preference results in figure 3a. A two-way ANOVA determined adolescent alcohol exposure significantly influenced alcohol intake in adulthood (effect of adolescent drug, $F_{(1, 9)} = 8.18$, $p = 0.019$) (figure 3b). There was an additional effect of testing day (effect of day, $F_{(3, 27)} = 109.9$, $p < 0.001$ respectively) with post hoc analysis demonstrating significant differences between saline and alcohol groups on day 2 and 4. Collectively, the results indicate that four consecutive doses of alcohol during adolescence does not influence baseline anxiety-like behaviour but increases the alcohol-seeking behaviour and intake in adulthood. Importantly, a dose-dependent effect on alcohol seeking was shown.

5.4.2 Experiment 2: Does adolescent alcohol exposure "sensitise" molecular mediators of reward and the TLR4-signalling pathway in adulthood?

The increased alcohol seeking behaviour is potentially explained by alterations in reward-related genes in adulthood caused by adolescent alcohol exposure. Thus, the expression of genes relating to alcohol reward, seeking and synaptic plasticity in the nucleus accumbens were examined in adulthood prior to behavioural testing (P56) (figure 4). The genes assessed were based upon previous studies demonstrating differences in dopaminergic, opioidergic, GABAergic and glutamatergic processes following adolescent alcohol exposure (Alaux-Cantin et al., 2013; Pascual et al., 2009). A one-way ANOVA determined a significant effect of alcohol dose on the expression of *Drd1*, *Th*, *Oprm1*, *Gabra1*, *Gabra2* and *Creb1* mRNA in adulthood (effect of dose; *Drd1,* $F_{(4, 10)} = 3.74$, $p = 0.016$; *Th,* $F_{(4, 10)} = 3.4$, $p = 0.041$; *Oprm1*, $F_{(4, 10)} = 4.46$, $p = 0.0073$; *Gabra1*, $F_{(4, 10)} = 4.09$, $p = 0.011$; *Gabra2*, $F_{(4, 10)} = 2.89$, $p = 0.035$; and *Creb1*, $F_{(4, 10)} = 3.60$, $p = 0.014$). This effect was not consistent however, as no alcohol-dose effect was observed for *Drd2*, *Gria1*, *Grin1*, *Bdnf* or *Ntrk2* mRNA levels (effect of dose; *Drd2,* $F_{(4, 10)} = 2.04$, $p = 0.12$; *Gria1*, $F_{(4, 10)} = 2.11$, $p = 0.10$; *Grin1*, $F_{(4, 10)} = 0.52$, $p = 0.71$; *Bdnf*, $F_{(4, 10)} = 2.34$, $p = 0.080$; and *Ntrk2*, $F_{(4, 10)} = 1.01$, $p = 0.41$). Collectively, these data indicate that adolescent alcohol exposure significantly increased the expression of receptors previously associated with alcohol seeking behaviour and intake (*Drd1*, *Th*, *Oprm1*, *Gabra1 and 2* and *Creb1*), while having no effect on genes related to glutamate (*Gria1* and *Grin1*) or plasticity support (*Bdnf* and *Ntrk2*).

The role of the neuroimmune system in mediating the long-term consequences of adolescent alcohol exposure is of increasing interest (Montesinos et al. 2016). Therefore, the expression of the genes pertinent to the TLR4 pathway was assessed (figure 5). A one-way ANOVA determined a significant effect of alcohol dose on the expression of *Tlr4*, *Md2*, *Trif*, *Ccl2*, *Ifnb* and *Hmgb1* mRNA (effect of dose; *Tlr4*, $F_{(4, 10)}$

= 3.42, p = 0.016; *Md2*, $F_{(4, 10)}$ = 3.25, p = 0.023; *Trif*, $F_{(4, 10)}$ = 3.90, p = 0.0090; *Ccl2*, $F_{(4, 10)}$ = 3.70, p = 0.012; *Ifnb,* $F_{(4, 10)}$ = 2.68, p = 0.044; and *Hmgb,1* $F_{(4, 10)}$ = 3.63, p = 0.014). There was no effect of alcohol dose on the expression of *Cd14, Myd88*, *Il1b* or *Il10* mRNA (effect of dose; *Cd14,* $F_{(4, 10)}$ = 1.72, p = 0.16; *Myd88*, $F_{(4, 10)}$ = 1.026, p = 0.40; *Il1b,* $F_{(4, 10)}$ = 1.50, p = 0.22; and *Il10*, $F_{(4, 10)}$ = 2.53, p = 0.056). Interestingly, adolescent alcohol exposure increased the expression of genes associated with the TRIF and not the MyD88 pathway in the nucleus accumbens of adult mice. This suggests an inherent bias of the immune system in the brains of these animals induced by adolescent alcohol exposure.

5.4.3 Experiment 3: Does (+)-Naltrexone attenuate the long-term increases of the TLR4 pathway induced by adolescent-alcohol?

Given that adolescent alcohol exposure potentiated the expression of *Trif* and *Ifnb* mRNA within the nucleus accumbens, the question arose as to whether the TLR4-TRIF pathway was associative or causative in mediating alcohol seeking and intake behaviours observed later in life. Therefore, (+)-Naltrexone, a pharmacological biased antagonist of the TLR4-TRIF pathway (Wang et al., 2016) was administered either before or after exposure to adolescent alcohol exposure and later life behaviour and mRNA expression was assessed. The decision to include both pre- and post-treatment was to ascertain whether TLR4-TRIF pathways were involved in these behaviours and whether the isomer is of any benefit once the pathology has commenced. Importantly, (+)-Naltrexone did not influence BAC following adolescent alcohol exposure suggesting any alteration in behaviour was unlikely to be attributable to alterations in pharmacokinetics (figure s2).

The ability of (+)-Naltrexone to selectively attenuate adolescent alcohol induced TLR4 gene expression was investigated using qPCR. A two-way ANOVA determined a

significant effect of gavage (alcohol or saline) on *Tlr4, Ifnb* and *Hmgb1* mRNA in the nucleus accumbens of mice in the pretreatment paradigm (figure 6a) (effect of gavage; *Tlr4*, $F_{(1, 4)} = 40.51$, $p = 0.0007$; *Ifnb*, $F_{(1, 4)} = 2.59$, $p = 0.015$; and *Hmgb1*, $F_{(1, 4)} = 8.71$, $p = 0.025$). There was an additional effect of intervention (saline vs (+)-Naltrexone) for these genes (effect of intervention; *Tlr4*, $F_{(1, 4)} = 10.09$, $p = 0.019$; *Ifnb*, $F_{(1, 4)} = 44.68$, $p = 0.022$; and *Hmgb1*, $F_{(1, 4)} = 0.035$, $p = 0.85$). There were interactive effects for *Ifnb* and *Hmgb1* but not *Tlr4* mRNA (interaction; *Tlr4*, $F_{(1, 4)} = 0.17$, $p = 0.68$; *Ifnb*, $F_{(1, 4)} = 9.28$, $p = 0.02$; and *Hmgb1*, $F_{(1, 4)} = 0.073$, $p = 0.79$). The expression of *Trif* was unaffected by intervention ($F_{(1, 4)} = 0.83$, $p = 0.39$) or gavage ($F_{(1, 4)} = 2.25$, $p = 0.18$). However, an interactive effect was observed ($F_{(1, 4)} = 19.57$, $p = 0.0045$).

A two-way ANOVA determined the expression of *Trif* and *Ifnb* was influenced by gavage (effect of gavage; *Trif*, $F_{(1, 3)} = 0.45$, $p = 0.52$; and *Ifnb*, $F_{(1, 3)} = 3.04$, $p = 0.013$) and intervention (effect of intervention; *Trif*, $F_{(1, 3)} = 17.76$, $p = 0.0056$; and *Ifnb*, $F_{(1, 3)} = 12.90$, $p = 0.011$) in the post-treatment paradigm (figure 6b). There was no significant interactions between gavage and intervention for these two genes (interaction; *Trif*, $F_{(1, 3)} = 4.87$, $p = 0.069$; and *Ifnb*, $F_{(1, 3)} = 0.26$, $p = 0.62$). In contrast, to the pretreatment paradigm however, *Tlr4* mRNA was only significantly modified by intervention ($F_{(1, 3)} = 5.13$, $p = 0.040$) but not gavage ($F_{(1, 3)} = 2.4$, $p = 0.17$). There was no interaction between the two variables (interaction, $F_{(1, 3)} = 4.14$, $p = 0.08$). There was no effect of intervention ($F_{(1, 3)} = 1.17$, $p = 0.31$), gavage ($F_{(1, 4)} = 5.76$, $p = 0.05$) or an interactive effect ($F_{(1, 3)} = 2.35$, $p = 0.16$) on *Hmgb1* expression. All remaining genes did not exhibit a significant effect of intervention or gavage with statistical information available in the supplementary material (figure s3 – 4).

Interestingly, both pre- and post-treatment paradigms had a signficant effect of the intervention (saline vs (+)-Naltrexone) on the expression of *Gabra2* mRNA (effect of

intervention; pretreatment, $F_{(1, 3)}$ = 17.84, p = 0.05; and post-treatment, $F_{(1, 3)}$ = 15.79, p = 0.048) (figure 7a and b). There was no effect of gavage on the expression of *Gabra2* mRNA in either paradigms (effect of gavage; pretreatment, $F_{(1, 3)}$ = 1.63, p = 0.33; and post-treatment, $F_{(1, 3)}$ = 1.96, p = 0.30). However, a significant interactive effect between gavage and intervention was observed for both cohorts (interaction; pretreatment, $F_{(1, 3)}$ = 349.1, p = 0.0029; and post-treatment, $F_{(1, 3)}$ = 24.61, p = 0.038). Bonferonni post hoc determined (+)-Naltrexone significantly reduced the expression of *Gabra2* mRNA compared to saline. The expression of *Th* was significantly influenced by the intervention in the pre- but not post-treatment paradigm (effect of intervention; pretreatment, $F_{(1, 3)}$ = 117.1, p = 0.008; and post-treatment, $F_{(1, 3)}$ = 5.01, p =0.15). The expression of *Th* was not influenced by gavage (effect of gavage; pretreatment, $F_{(1, 3)}$ = 6.56, p =0.12; post-treatment, $F_{(1, 3)}$ = 3.78, p = 0.19) nor was there an interactive effect for the pre- and post-treatment paradigms (interaction; pretreatment, $F_{(1, 3)}$ = 4.97, p = 0.15; post-treatment, $F_{(1, 3)}$ = 3.54, p = 0.20, respectively). There was no effect of intervention for any other reward pathway-related mRNA (figure s2, see supplementary material for full list of statistical results).

5.4.4 Experiment 4: Does (+)-Naltrexone attenuate behavioural alterations in adulthood induced by adolescent alcohol exposure?

To verify that (+)-Naltrexone selectively attenuated the enhanced rewarding properties of alcohol and did not modify basal behaviour adult mice underwent the elevated plus maze (figure 8a and b). There was a significant effect of arm on performance in the elevated plus maze (effect of arm, $F_{(1, 144)}$ = 39.71, p < 0.0001), with post hoc analysis determining all cohorts of mice spent significantly longer in the closed arm relative to the open arm.

A 4-way ANOVA determined percent of time spent in each of the arms was not influenced by the gavage, intervention or the order in which that intervention was received (pre- or post-treatment) (effect of gavage, $F_{(1, 144)} = 0.12$, $p = 0.73$; effect of intervention, $F_{(1, 144)} = 0.12$, $p = 0.73$; and effect of order $F_{(1, 144)} = 0.80$, $p = 0.37$, respectively) – confirming the previous findings that this model exclusively augments reward behaviour. However, the multiple comparisons test determined that mice receiving an IP injection of saline followed by a gavage of saline (pretreatment paradigm) exhibited an increase in open arm time compared to all other cohorts (figure 8a). This effect was not observed in the post-treatment paradigm. This finding is furthered as an interactive effect between arm and order was found ($F_{(1, 144)} = 39.87$, $p < 0.0001$). Collectively, this suggests that under specific circumstances, alcohol and (+)-Naltrexone may modify performance in the elevated plus maze. A list of all interactive effects can be found in the supplementary material.

The remaining markers of elevated plus maze performance (distance travelled, number of exits and immobile episodes) all exhibited a similar trend in their main effects. There was a significant effect of arm ($p < 0.001$) but not gavage, intervention or the order of the intervention (effect of gavage, intervention and order $p > 0.05$). Significant interactions were observed for arm x adolescent exposure x order and arm x adolescent exposure x order x intervention ($p < 0.05$) (a complete list of statistical analyses can be found in the supplementary materials).

(+)-Naltrexone's ability to attenuate the rise in alcohol-reward behaviour in adulthood was assessed using conditioned place preference (figure 9a and b). The change in conditioning time was significantly modified by conditioning chamber but not gavage, intervention or order (effect of conditioning chamber, $F_{(1, 144)} = 56.09$, $p < 0.0001$; effect of gavage, $F_{(1, 144)} = 0.16$, $p = 0.69$; effect of intervention, $F_{(1, 144)} = 0.051$, $p = 0.82$; and

effect of order $F_{(1, 144)} = 0.018$, p = 0.89). Thus, while mice overall preferred the alcohol-conditioned chamber compared to the unconditioned chamber, there was no overall effect of alcohol or (+)-Naltrexone on modifying alcohol-induced conditioned place preference. Post hoc analysis demonstrated that both control cohorts (Saline IP -> Saline IG and (+)-Naltrexone IP -> Saline IG) exhibited a reduced change in alcohol-conditioned chamber time compared to Saline IP -> Alcohol IG group, supporting earlier findings that adolescent alcohol potentiates time spent in the alcohol-conditioned chamber in adulthood (figure 9a). Similarly, in the post-treatment the Saline IG -> Saline IP cohort exhibited a reduced change in chamber time compared to alcohol IG -> Saline IP. This suggests despite no main effect of gavage, there was still a post hoc effect of adolescent alcohol exposure on later life behaviour. This is further supported by the significant interactive conditioning chamber x gavage ($F_{(1, 144)}$ = 4.88, p = 0.037). This indicates that adolescent alcohol exposure still potentiated alcohol-induced conditioned place preference under specific circumstances. For the remaining interactive effects refer to supplementary material.

In contrast to conditioned place preference, drinking in the dark was significantly affected by gavage, intervention and testing day but not the order, (effect of gavage, $F_{(1, 256)} = 4.64$, $p = 0.032$; effect of intervention, $F_{(1, 256)} = 82.58$, $p < 0.0001$; effect of testing day, $F_{(3, 256)} = 8.81$, $p < 0.0001$; and effect of order $F_{(1, 256)} = 0.004$, $p = 0.95$) (figure 10a and b). Post hoc analysis determined: mice that received alcohol during adolescence exhibited potentiated alcohol intake in adulthood compared to mice that received saline. Furthermore, mice that received alcohol and (+)-Naltrexone in the pre- or post-treatment paradigms exhibited reduced intake compared to mice that received alcohol and saline. Interactions of intervention x gavage ($F_{(1, 256)} = 38.40$, $p < 0.0001$), testing day x intervention x adolescent gavage ($F_{(3, 256)} = 2.57$, $p = 0.054$) and order x intervention x gavage ($F_{(1, 256)} = 42.14$, $p < 0.0001$) were additionally observed.

5.4.5 Figures

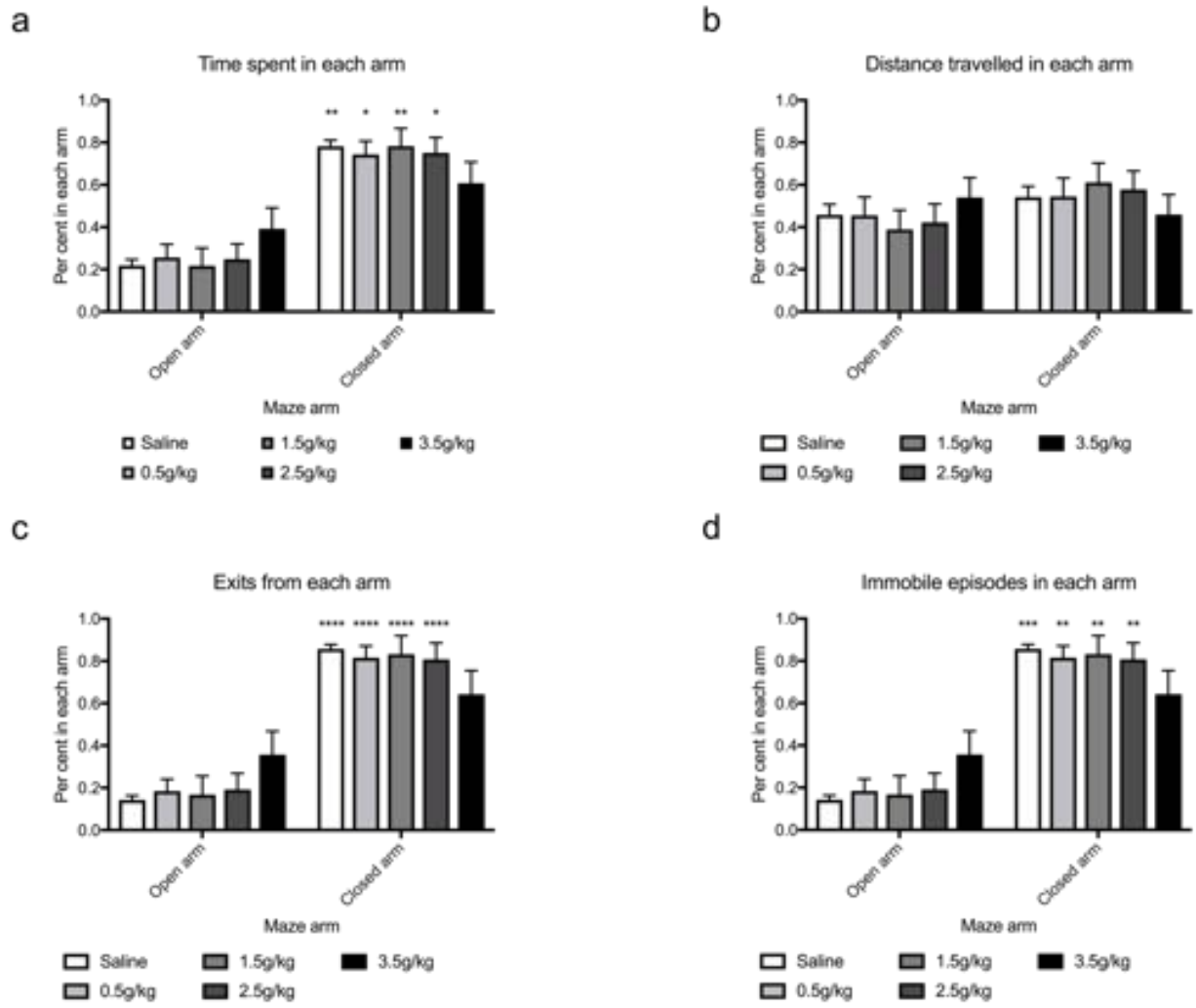

Figure 2 Adolescent alcohol exposure has no effect on performance in the elevated plus maze in adult mice. Increasing the dose of alcohol does not influence the time (a), distance (b), exits (c) or immobile episodes (d) in each arm during a five-minute test. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10; between arms (open vs closed) *p < 0.05; **p < 0.01, *** p < 0.001, ****p < 0.0001.

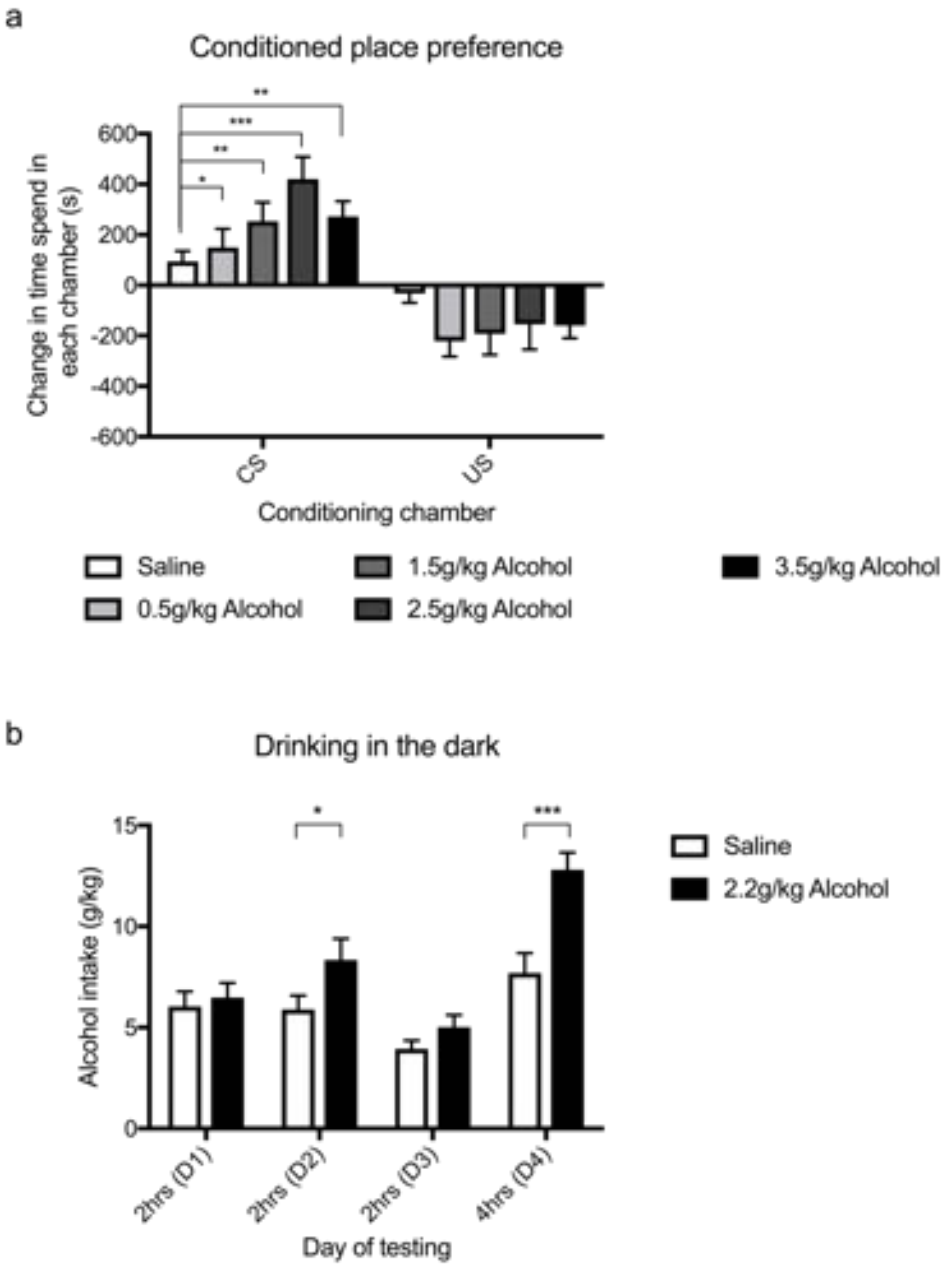

Figure 3 Adolescent alcohol exposure potentiates alcohol-induced reward behaviours in adulthood. Adolescent alcohol exposure dose-dependently increases the time spent in the alcohol-conditioned chamber relative to saline (a) and alcohol intake (b). CS, conditioning stimuli; US, unconditioned stimuli. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10; *$p < 0.05$; **$p < 0.01$, *** $p < 0.001$.

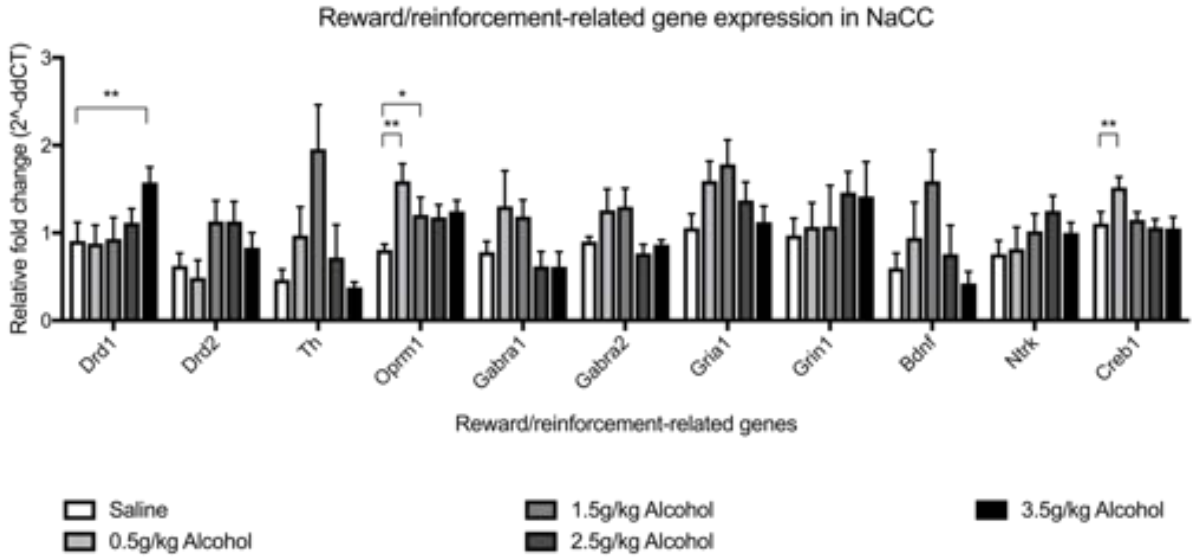

Figure 4 Adolescent alcohol exposure dysregulates the expression of genes associated with reward/reinforcement within the nucleus accumbens. Alcohol during adolescence increased the expression of *Drd1*, *Th*, *Oprm1*, *Gabra1*, *Gabra2* and *Creb1* but did not affect the expression of *Drd2*, *Gria1*, *Grin1*, *Bdnf* or *Ntrk2* mRNA in adulthood. All data was analysed using a one-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4; *p < 0.05; **p < 0.01, *** p < 0.001.

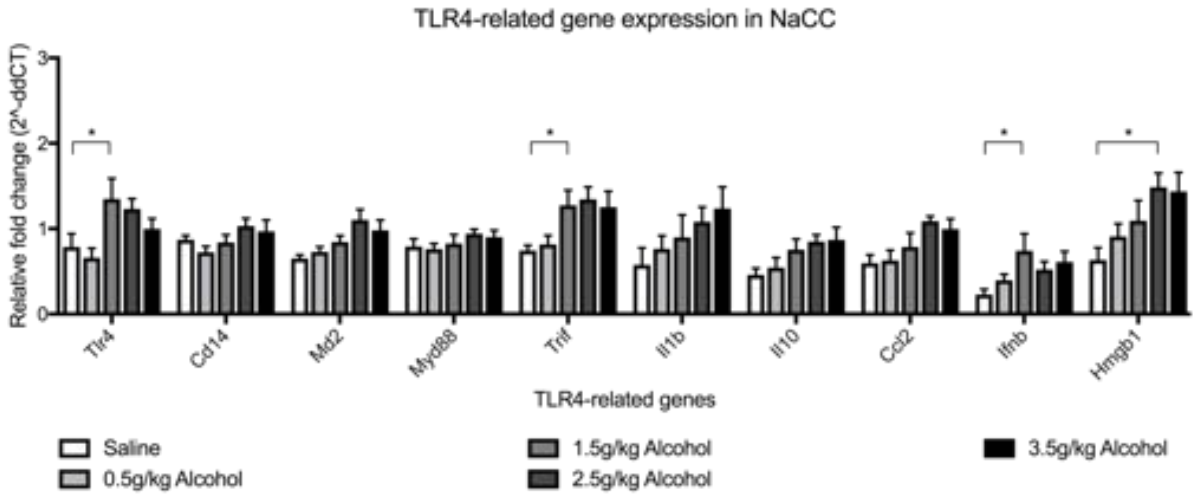

Figure 5 Adolescent alcohol exposure increases the expression of TLR4-related genes within the nucleus accumbens. Alcohol during adolescence increased the expression of *Tlr4*, *Md2*, *Trif, Ccl2*, *Ifnb* and *Hmgb1* but did not affect the expression of *Cd14*, *Myd88*, *Il1b* or *Il10* mRNA in adulthood. All data was analysed using a one-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4; *p < 0.05; **p < 0.01, *** p < 0.001.

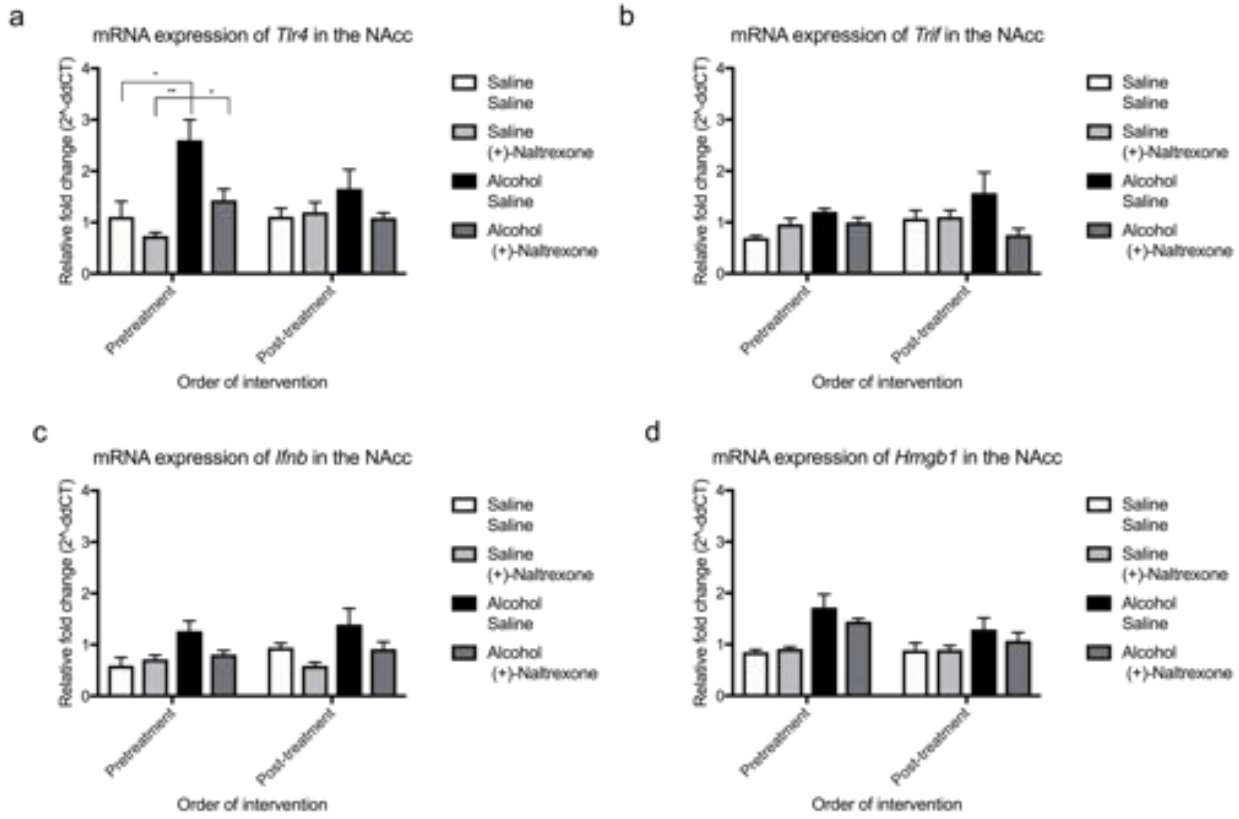

Figure 6 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure prevents long-term increases of *Tlr4* and *Ifnb* mRNA in the nucleus accumbens of adult mice. (+)-Naltrexone selectively reduces alcohol-induced sensitisation of *Tlr4* and *Ifnb* mRNA in adulthood but does not alter the expression of MyD88-related genes. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

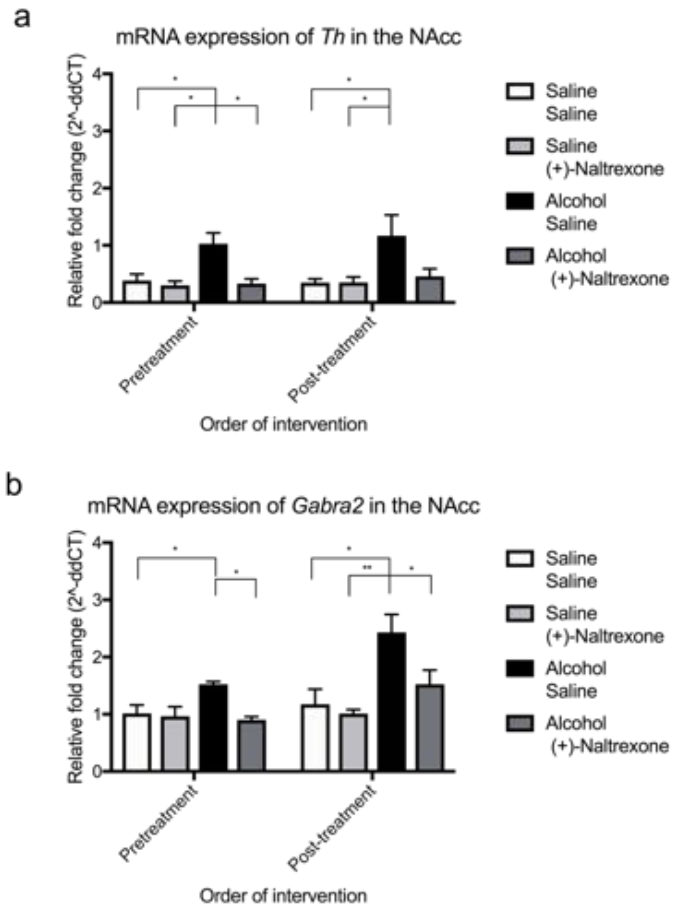

Figure 7 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure prevents long-term increases of *Th* and *Gabra2* mRNA in the nucleus accumbens of adult mice. (+)-Naltrexone selectively reduces alcohol-induced sensitisation of *Gabra2* mRNA in adulthood but does not alter the expression of other reward/reinforcement related genes. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

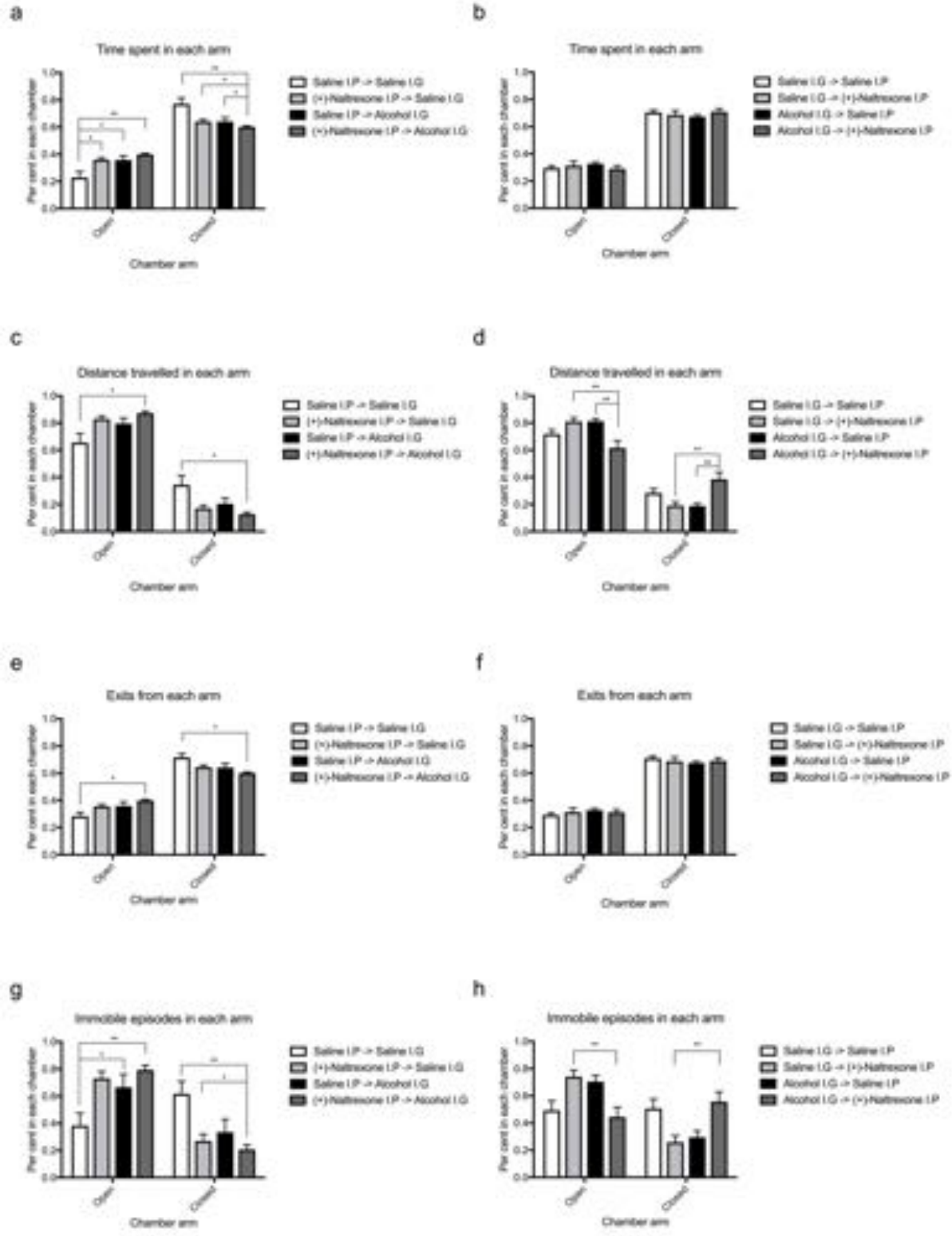

Figure 8 Antagonising TLR4 signalling either before (a, c, e, g) or after (b, d, f, h) adolescent alcohol exposure has no effect on time spent (a and b), distance travelled (c and d), number of exits (e and f) or immobile episodes (g and h) in the elevated plus maze in adult mice. Adolescent alcohol and (+)-Naltrexone does not influence the time, distance, number of exits or immobile episodes (d) in each arm.

All data was analysed using a four-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10, *$p < 0.05$; **$p < 0.01$.

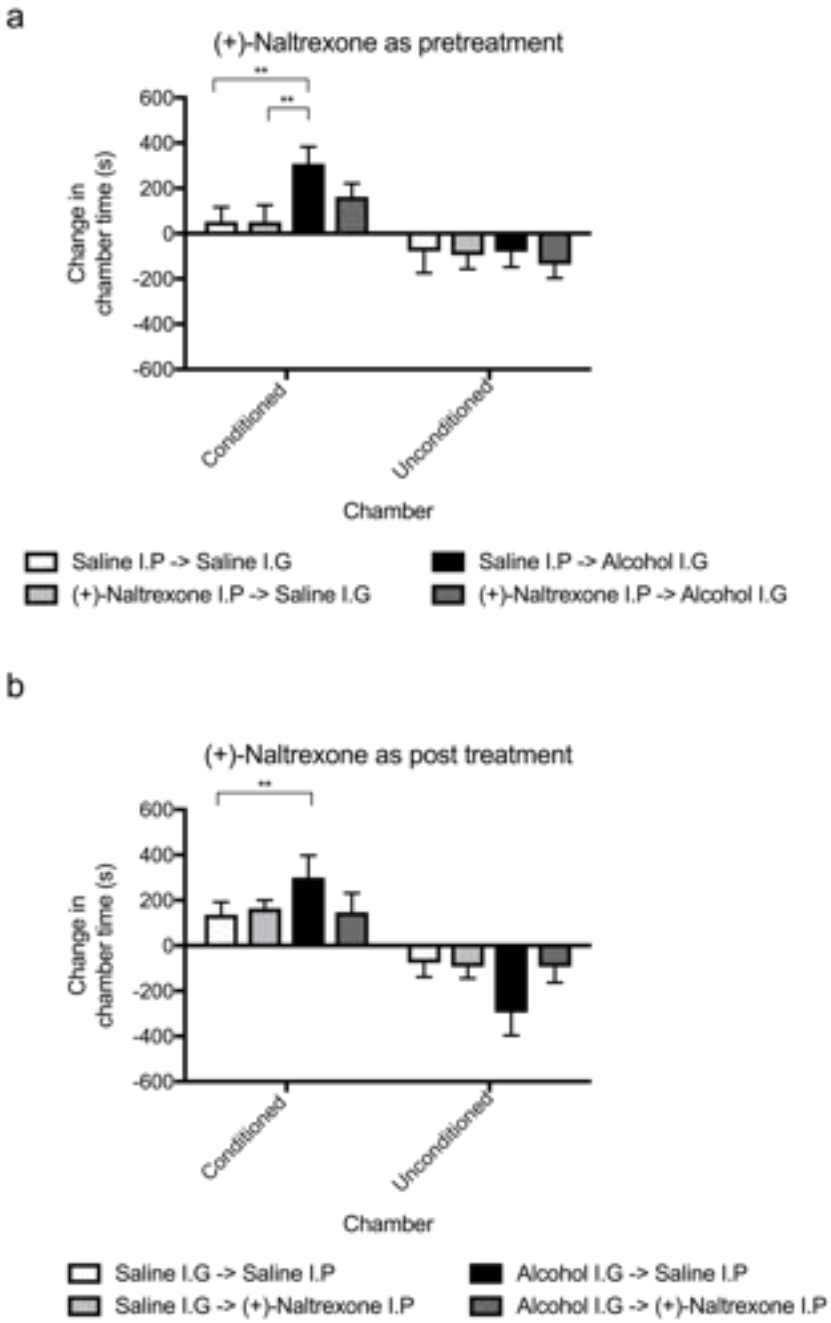

Figure 9 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure has no effect on preference for an alcohol-conditioned stimulus in adult mice. (+)-Naltrexone does not influence alcohol-induced conditioned place preference. All data was analysed using a four-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10, *p < 0.05; **p < 0.01.

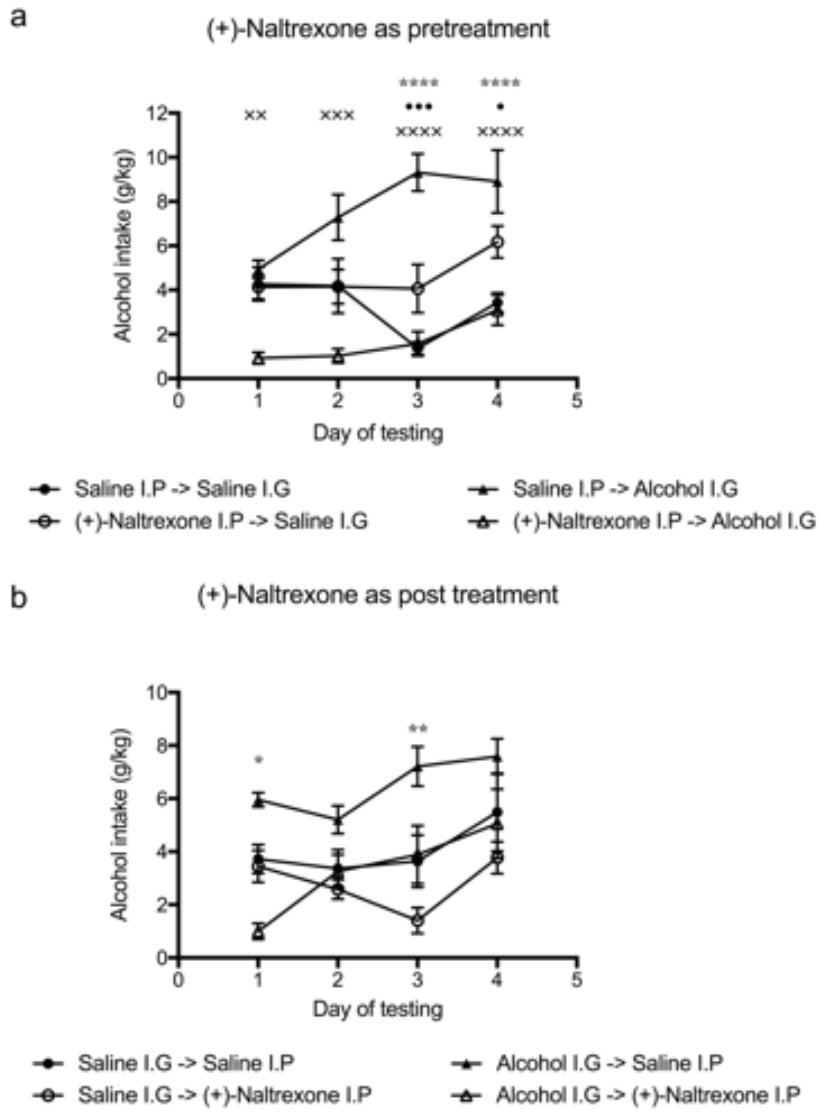

Figure 10 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure decreases alcohol intake in adult mice. (+)-Naltrexone reduces the intake of alcohol irrespective of whether the mice received alcohol or saline during their adolescence. All data was analysed using a four-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10. All post hoc differences presented in comparison to Saline I.P -> Alcohol I.G (a) and Alcohol I.G -> Saline I.P (b).

* Saline I.P -> Saline I.G; • Naltrexone I.P -> Saline I.G; ^{x} Naltrexone I.P -> Alcohol I.G

(a) ****p < 0.0001; •p < 0.05; •••p < 0.001; ^{xx}p < 0.01; ^{xxx}p < 0.001; ^{xxxx}p < 0.0001

• Saline I.G -> Naltrexone I.P (b) *p < 0.05; **p < 0.01

5.5 Discussion

Adolescence is a vulnerable stage of neurodevelopment, throughout which the brain undergoes substantial reorganisation and maturation. Exposure to drugs of abuse, in particular alcohol, can perturb normal brain development, reinforcing an immature brain state in both rodents and humans (Spear & Swartzwelder, 2014). As adults, these individuals are at risk of developing psychiatric disorders such as addiction and anxiety disorders (Spear & Swartzwelder, 2014). Results from our study demonstrated four oral gavages during early adolescence potentiated alcohol-induced conditioned place preference and alcohol drinking when tested in adulthood. However, performance in the elevated plus maze was not altered. These behavioural alterations coincided with elevations in the expression of genes relating to dopamine, opioid and GABA receptors but not other neurotransmitter or neurotropic systems in the nucleus accumbens of adult mice. Furthermore, the expression of genes relating to the TLR4 pathway (*Tlr4*, *Md2*, *Trif*, *Ccl2*, *Ifnb* and *Hmgb1*) were also increased. Administration of (+)-Naltrexone either before or after adolescent alcohol exposure, prevented the increase in *Tlr4*, *Ifnb* and *Gabra2* mRNA and decreased alcohol intake later in life. However, (+)-Naltrexone failed to modify adolescent alcohol potentiated conditioned place preference, elevated plus maze performance or the increased expression of other neurotransmitter and neurotrophic-related genes. Collectively, the results highlight the potential importance of the alcohol-TLR4-IFNβ axis in mediating adolescent-induced potentiation of later life drinking behaviour but not alcohol-seeking or anxiety behaviour.

Current models examining the effects of adolescent alcohol exposure are often limited in the generalisability of their effects as they use clinically irrelevant routes of administration (i.e. intraperitoneal, Gilpin et al., 2012); are prolonged in nature (Vetreno et al., 2015; Pascual, et al., 2009); or use very high doses of alcohol (Vetreno & Crews 2012). These limitations are particularly important, as the dose of alcohol and route of

administration influences the response to alcohol (for example, Ward et al., 2014; Osterndorff-Kahanek et al., 2013). Consequently, an aim of this study was to evaluate the effects of a shorter model of adolescent alcohol exposure and characterise its behavioural and molecular outcomes. Similar to study's using more chronic models (Montesinos et al., 2016; Alaux-Cantin et al., 2013; Maldonado-Devincci et al., 2010), our adolescent alcohol exposure model potentiated conditioned place preference and alcohol drinking behaviour later in life. Interestingly, peak conditioned place preference was not observed at the highest dose of alcohol - an effect potentially attributable to alcohol's memory impairing effects at higher doses (Land, 2004). However, unlike chronic studies (Montesinos et al., 2016) this shorter model did not alter anxiety-like behaviour suggesting higher or more chronic doses of alcohol are required to engage brain regions governing anxiety (He & Crews, 2008). Alternatively, the lack of difference in anxiety behaviour may be related to the mouse strain used in the study. Balb/c are an anxiety-sensitive strain of mice (Carola et al., 2002; Griebel et al., 2000; Makino et al., 1991) potentially masking an alcohol response.

To ascertain why these rodents exhibited potentiated reward-like behaviour, the nucleus accumbens of adolescent alcohol exposed mice was collected in adulthood and genes pertaining to reward were assessed. The nucleus accumbens was selected owing to its pivotal importance in the generation of reward. Similar to Alaux-Cantin et al., (2013) our study demonstrated genes pertaining to GABA and the endogenous opioid system were elevated in adulthood following adolescent alcohol exposure. The current study additionally demonstrated increases in genes relating to dopamine synthesis (*Th*) and receptors (*Drd1*). Tyrosine hydroxylase, dopamine and opioid receptors are associated with the hedonic and salient motivational properties of alcohol (Berridge & Robinson, 2016). Therefore, persistent elevation in these genes is likely to increase the sensitivity of these individuals to hedonic and motivational properties of

alcohol in adulthood. In contrast to Alaux-Cantin et al., (2013), mRNA from other neurotransmitter systems such as glutamate, were not significantly altered by adolescent alcohol exposure. Closer analysis demonstrates a unique expression pattern, which would not prove statistically significant using conventional data analysis that relies on a linear change (ANOVA). For example, the alcohol dose response effect on *Grin1* expression is bell-shaped, highlighting the importance of examining a broad range of doses when examining adolescent alcohol exposure. Lastly, despite this shorter exposure model demonstrating increased expression of genes relating to reward, and elevated alcohol seeking and drinking later in life, it remains to be determined whether this result is ontologically specific or can occur irrespective of developmental stage.

This study's primary focus was to investigate the effects of adolescent alcohol exposure on the neuroimmune system. Specifically, the role of the TLR4 pathway was examined based on the recent studies implicating this receptor and its signalling pathway in alcohol-related behaviours (for example, Blednov et al., 2017; Harris et al., 2017; Aurelian et al., 2016; Montesinos et al., 2016; Liu et al., 2011; Pascual et al., 2011). Despite its purported importance in mediating these behaviours, no study has examined how alcohol modifies the gene expression of TLR4's signalling pathways during crucial neurodevelopment periods such as adolescence. TLR4 has two main signalling pathways (the MyD88 and TRIF pathway) with their activation leading to increased production of classical proinflammatory cytokines (IL-1β) and type 1 interferons, respectively (Akira & Takeda, 2004). Results from our study demonstrate adolescent alcohol exposure resulted in the persistent elevation of *Tlr4*, *Md2*, *Trif*, *Ccl2*, *Ifnb* and *Hmgb1* mRNA in adulthood within the nucleus accumbens. Interestingly, alcohol exposure did not alter the expression of genes classically associated with the MyD88 pathway, suggesting that the long-term neuroimmune effects of alcohol may

have a more pronounced effect on the TRIF pathway. However, studies determining whether the mRNA increases translate to protein-level differences are required to verify these conclusions.

While this study did not address the immediate effects of alcohol exposure during adolescence, published literature from *in vitro* experiments suggests acute alcohol activates both the MyD88 and TRIF pathways (Fernandez-Lizarbe et al., 2009). However, the degree of immune activation appears to be dampened compared to adults (Doremus-Fitzwater et al., 2015; Kane et al., 2013). While the mechanism underlying the limited immune response is unknown, it is hypothesised that this phenomenon is designed to limit neuroinflammatory responses which can perturb neurodevelopment (Ismail & Blaustein, 2013; Ismail et al., 2013).

The rise in immune mediators has both short and long-term consequences. In the acute setting, the immune mediators act upon neighbouring neurons altering their function and behaviour (for example, Marshall et al., 2016). This in turn is hypothesised to potentiate hedonic and anhedonic aspects of drugs of abuse (see Lacagnina et al., 2016 for review). For example, both TLR4 and CCL2 modify dopaminergic neurotransmission in the striatum (Northcutt et al., 2015; Hutchinson et al., 2012; Guyon et al., 2009). In addition, activation of TLR4 during adolescence has long-term effects on neurodevelopment (see Bilbo & Schwarz 2012, for review). For example alcohol-induced TLR4 signalling reduces myelination, synaptic pruning, and increases neuronal and astrocyte cell death and alters epigenetic processes which reinforce an immature adolescent brain (Montesinos et al., 2016; Montesinos et al., 2015; Pascual et al., 2014; Pascual et al., 2009). These events may assist in producing an underdeveloped, immature brain that is uniquely sensitive to the hedonic aspects of alcohol exposure and is more susceptible to develop addiction with chronic use.

In addition to the immediate and neurodevelopmental effects, this study highlighted that adolescent alcohol exposure can lead to persistent increases in the TLR4 related mRNAs. The study demonstrated mice exposed to alcohol during adolescence exhibited an increase in the expression of multiple inflammatory genes in adulthood prior to re-exposure. This is in accordance with other studies demonstrating increased expression of microglial activation markers ED1 and MHCII (McClain et al., 2011), inflammatory cytokines, chemokines and proteins (Pascual et al., 2016) and immune receptors (TLR4, TLR3 and RAGE) (Vetreno & Crews, 2012) in adult mice that were exposed to alcohol during adolescence. The effects of this persistent elevation in immune-related genes are yet to be fully elucidated. However, it has been hypothesised that subsequent activation of the immune system by the original or a new immunogen, will result in an exaggerated inflammatory response. This exaggerated response will act on neighbouring cells influencing their function; potentially increasing the hedonic and anhedonic aspects of drugs of abuse. Crucially, TLR4 appears to assist in mediating the enduring upregulation of neuroimmune-related genes. However, studies examining the role of TLR4 in adolescent alcohol priming often use knock out animals and thus the model is confounded given the pivotal role of TLR4 in neurodevelopment. For example, $TLR4^{-/-}$ mice display increased neuronal differentiation, higher total neuron cell counts and fewer glia compared to wildtype mice (Rolls et al., 2007). Given the pivotal role of glial TLR4 in mediating the molecular and behavioural adaptations induced by alcohol (Fernandez-Lizarbe et al., 2009), it is interesting to speculate whether the reduced inflammatory effects observed in these studies (Montesinos et al. 2016; Alfonso-Loeches et al., 2010) is simply due to the reduced number of glial cells or whether it is a TLR4 specific event.

The current study is the first to consider the relative contribution of TLR4s signalling pathways on the effects of adolescent alcohol exposure. To separate out the potential

MyD88 and TRIF-dependent effects, (+)-Naltrexone was used. (+)-Naltrexone is a stereoisomer of the clinically approved (-)-Naltrexone used to treat alcohol dependence. Both isomers are thought to bind to the LPS-binding pocket of TLR4's co-receptor MD2, however the precise binding site and mechanism remain to be fully elucidated (Hutchinson et al., 2010). Unlike the (-)-isomer, the (+)-isomer is devoid of mu opioid receptor activity. This compound has been further screened against 70 neurotransmitter, peptide, growth factor receptors, ion channels, second messengers and enzymes without any additional interactive effects (Hutchinson et al., 2010). *In vitro* experiments demonstrate (+)-Naltrexone blocks LPS-induced IRF3 phosphorylation and the production of nitric oxide, TNFα and IFNβ production in BV2 cells. It had no effect on the phosphorylation of p65, p38, JNK or ERK1/2 or the expression of IL-1β in these cells (Wang et al., 2016). Collectively, these results suggest (+)-Naltrexone is a biased TLR4-TRIF antagonist, as it failed to attenuate markers classically associated with the TLR4-MyD88 pathway. *In vivo* studies report contradictory findings as (+)-Naltrexone attenuated cocaine-induced IL-1β production (Northcutt et al. 2015). Results from our study further reinforce the concept that (+)-Naltrexone is a biased TLR4-TRIF antagonist as the drug decreased the expression of *Ifnb* but not *Il1b* or *Tnfa* mRNA in adult mice who received alcohol as adolescence.

Attenuating the rise in interferon mRNA may assist in reducing reward-like behavior in adulthood. Recent research has demonstrated that interferons share structural and functional similarities to endorphin, an endogenous opioid (Blalock & Smith, 1981; Blalock & Smith, 1980). Critically, interferons can bind to μ opioid receptor causing endorphin-like effects (Jiang et al., 2000). Given that activation of the μ opioid receptor contributes to generating the hedonic sensations (or "liking" of alcohol), it is hypothesized that attenuating the rise in interferons may reduce the potentiated hedonic sensation induced by alcohol later in life. While both drinking in the dark and

conditioned place preference require opioidergic activity (Kamdar et al., 2007; Middaugh & Bandy, 2000), conditioned place preference additionally requires the dopaminergic system (Kamdar et al., 2007; Buccafusco, 2009). This may explain why a difference was observed for drinking in the dark and not conditioned place preference. Alternatively, given alcohol seeking and drinking behaviour engages different brain regions, the discrepancy in behavioural outcomes may be due to neuroanatomical restrictions in the expression of TLR4 or its required signalling components. For example, if TLR4 or related genes are not expressed to high levels in brain regions governing conditioned place preference, it is unlikely to have a substantial effect in mediating this behaviour. This may assist in explaining why siRNA knock down of TLR4 in the CeA but not ventral pallidum attenuates alcohol-binge drinking behaviour (Liu et al., 2011).

Interestingly, (+)-Naltrexone attenuated the expression of alcohol-induced *Gabra2* and *Th* mRNA. GABA A2 and tyrosine hydroxylase are associated with the molecular and behavioral effects of alcohol and are particularly important to the generation of reward behavior (Harris et al., 2008). Importantly, previous studies have highlighted a link between TLR4 and both GABA A2 and tyrosine hydroxylase potentially providing an explanation behind the effects of (+)-Naltrexone on alterations in reward behavior later in life (Harris et al., 2017; Aurelian et al., 2016; June et al., 2015; Yan, 2015; Bajo et al., 2014; Liu et al., 2011). However, future experiments are required to fully elucidate these links.

A limitation of this study is that the cell-type(s) responsible for the persistent rise in immune-related genes was not explored. Substantial evidence has established the role of neurons in mediating the actions of TLR4 and alcohol in adult rodents (Aurelian et al., 2016; June et al., 2015; Liu et al., 2011). However, these cells may lack

components of the TLR4 pathway that were elevated following our model of exposure (for example, *Trif* and *Ifnb* mRNA). For example, it is unclear whether neurons can transcribe IFNβ with the differing results likely attributable to the different mechanism of IFNβ activation. For example, LPS does not initiate the transcription of *Ifnb* or activate JNK or NFκB in neurons; raising doubts whether these cells can signal through the MyD88 or TRIF pathway (Okun et al., 2011). However, other studies have found neurons produce IFNβ in response to rabies virus infection (Prehaud et al., 2005). Given the conjecture, it is likely, that the primary immunocompotent cells (microglia and astrocytes) of the CNS are primarily responsible for mediating this effect as *in vitro* and *in vivo* experiments have demonstrated that alcohol indirectly activates TLR4 culminating in the increase expression of inflammatory cytokines and proteins (Fernandez-Lizarbe et al., 2009; Blanco et al., 2005).

It is becoming increasingly apparent that the neuroimmune system plays a profound role in neurodevelopment, behaviour and the molecular responses towards alcohol and other drugs of abuse. This study demonstrated that short exposure to alcohol during adolescence perturbs reward-related neurodevelopment increasing the preference for alcohol seeking and drinking later in life. In addition, this model demonstrated that alcohol exposure during adolescence increased the transcription of genes relating to the TLR4 pathway, an effect that persisted during adulthood. Attenuation of the TLR4-TRIF pathway, using (+)-Naltrexone, decreased adverse later life outcomes such as alcohol drinking (an effect potentially attributable to a TLR4-GABA A2 interaction), but had no effect on alcohol-seeking behaviour or basal anxiety behaviour.

5.6 Acknowledgements

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The authors declare no competing financial interests.

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5.8 Supplementary material

Table 1 Nucleotide sequence of primers.

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Bdnf – Brain derived neurotrophic factor transcript variant 1	CTCATCTTTGCCAGAGCCCC	GCTTTCTCAACGCCTGTCAC
Ccl2 – Chemokine (C-C motif) ligand 2	ACACTGGTTCCTGACTCCTCT	ACCTGAGGACTGATGGTGGT
Cd14 – cluster of differentiation 14 antigen	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT
Creb1 – cAMP responsive element binding protein 1 transcript variant 1, 2, 3, 4, 6, 7, 8	ATCTGGAGCAGACAACCAGC	TGAGCTGCTGGCATGGATAC
Drd1 – Dopamine receptor D1 transcript variant 1	GTTGAGTCCAGGGGTTTTGGG	ACTTTTCGGGGATGCTGCC
Drd2 – Dopamine receptor D2	GTGAACAGGCGGAGAATGGA	TGGGAGGGATGGGGCTATAC
Gabra1 – Gamma aminobutyric acid A receptor, subunit alpha 1	CCTGCTTCCTAGCTTGCGTT	AACCGATCCTTGTAACTCTGCT
Gabra2 – Gamma aminobutyric acid A receptor, subunit alpha 2 transcript variant X1	GCAGCAGAGACCATACATTGC	GCAGCAGAGACCATACATTGC

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Gapdh – Glyceraldehyde-3-phosphate dehydrogenase transcript variant 1	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Gria1 – Glutamate receptor, ionotropic, AMPA1 (alpha 1), transcript variant 1	AGGGGAATGTGGAAGCAAGG	CCAGCCTCCAATCAGGATG
Grin1 – Glutamate receptor, ionotropic, NMDA1 (zeta 1), transcript variant 1, 2, 3	CCTATGACAAGCGCGGA	AGCAGAGCCGTCACATTCTT
Hmgb1 – High mobility group box 1, transcript variant 1	CCATTGGTGATGTTGCAAAG	CTTTTTCGCTGCATCAGGTT
Ifnb – Interferon beta 1, fibroblast	TGGGAGATGTCCTCAACTGC	CCAGGCGTAGCTGTTGTACT
Il1b – Interleukin 1 beta	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Il10 – Interleukin 10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Md2 – Lymphocyte antigen 96 transcript variant 1, 2	CGCTGCTTTCTCCCATATTGA	CCTCAGTCTTATGCAGGGTTCA
Myd88 – Myeloid differentiation primary response gene 88	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCA
Ntrk2 – Neurotrophic tyrosine kinase, receptor, type 2, transcript variant X1, 3	GTCTGGAGGGTGCTATGCTAT	CAGAGCAGGGGCAGAAACTC

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Oprm1 – Opioid receptor, mu 1, transcript variant 1C, 1M, 1U	TCCGACTCATGTTGAAAAACCC	CCTTCCCCGGATTCCTGTCT
Th – Tyrosine hydroxylase	CCTTCCGTGTGTTTCAGTGC	TCAGCCAACATGGGTACGTG
Tlr4 – Toll-like receptor 4	GCCTTTCAGGGAATTAAGCTCC	GATCAACCGATGGACGTGTAAA
Trif – Toll-like receptor adaptor molecule 1	AACCTCCACATCCCCTGTTTT	GCCCTGGCATGGATAACCA

5.8.1 Statistics for in-text figures

Figure 8 Antagonising TLR4 signalling either before (a, c, e, g) or after (b, d, f, h) adolescent alcohol exposure has no effect on time spent (a and b), distance travelled (c and d), number of exits (e and f) or immobile episodes (g and h) in the elevated plus maze in adult mice. All data was analysed using a four-way ANOVA with Tukey post hoc.

Time (a – b)

Arm, $F_{(1, 144)} = 39.71$, $p < 0.0001$

Order, $F_{(1, 144)} = 0.799$, $p = 0.37$

Gavage, $F_{(1, 144)} = 0.12$, $p = 0.73$

Intervention, $F_{(1, 144)} = 0.082$, $p = 0.77$

Interaction (arm x order), $F_{(1, 144)} = 39.87$, $p < 0.0001$

Interaction (arm x drug), $F_{(1, 144)} = 3.11$, $p = 0.080$

Interaction (order x drug), $F_{(1, 144)} = 0.12$, $p = 0.73$

Interaction (arm x intervention), $F_{(1, 144)} = 2.28$, $p = 0.14$

Interaction (order x intervention), $F_{(1, 144)} = 0.082$, $p = 0.77$

Interaction (drug x intervention), $F_{(1, 144)} = 0.034$, $p = 0.85$

Interaction (arm x order x drug), $F_{(1, 144)} = 3.10$, $p = 0.0805$

Interaction (arm x order x intervention), $F_{(1, 144)} = 2.24$, $p = 0.14$

Interaction (arm x drug x intervention), $F_{(1, 144)} = 3.73$, $p = 0.06$

Interaction (order x drug x intervention), $F_{(1, 144)} = 0.034$, $p = 0.85$

Interaction (arm x order x drug x intervention), $F_{(1, 144)} = 3.72$, $p = 0.06$

Distance travelled (c – d)
Arm, $F_{(1, 144)} = 730.88$, $p < 0.0001$

Order, $F_{(1, 144)} = 0$, $p = 1$

Gavage, $F_{(1, 144)} = 0$, $p = 1$

Intervention, $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order), $F_{(1, 144)} = 6.40$, $p = 0.012$

Interaction (arm x drug), $F_{(1, 144)} = 1.18$, $p = 0.28$

Interaction (order x drug), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x intervention), $F_{(1, 144)} = 3.60$, $p = 0.060$

Interaction (order x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (drug x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order x drug), $F_{(1, 144)} = 13.13$, $p = 0.0004$

Interaction (arm x order x intervention), $F_{(1, 144)} = 20.63$, $p < 0.0001$

Interaction (arm x drug x intervention), $F_{(1, 144)} = 24.48$, $p < 0.0001$

Interaction (order x drug x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order x drug x intervention), $F_{(1, 144)} = 6.16$, $p = 0.014$

Exits (e – f)

Arm, $F_{(1, 144)} = 50.41$, $p < 0.0001$

Order, $F_{(1, 144)} = 0$, $p = 1$

Gavage, $F_{(1, 144)} = 0$, $p = 1$

Intervention, $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order), $F_{(1, 144)} = 1.98$, $p = 0.16$

Interaction (arm x drug), $F_{(1, 144)} = 3.71$, $p = 0.056$

Interaction (order x drug), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x intervention), $F_{(1, 144)} = 11.79$, $p = 0.00078$

Interaction (order x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (drug x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order x drug), $F_{(1, 144)} = 10.26$, $p = 0.0017$

Interaction (arm x order x intervention), $F_{(1, 144)} = 13.17$, $p = 0.00039$

Interaction (arm x drug x intervention), $F_{(1, 144)} = 29.40$, $p < 0.0001$

Interaction (order x drug x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order x drug x intervention), $F_{(1, 144)} = 4.58$, $p = 0.034$

Immobile episodes (g – h)

Arm, $F_{(1, 144)} = 50.305$, $p < 0.0001$

Order, $F_{(1, 144)} = 0$, $p = 1$

Gavage, $F_{(1, 144)} = 0$, $p = 1$

Intervention, $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order), $F_{(1, 144)} = 1.98$, $p = 0.16$

Interaction (arm x drug), $F_{(1, 144)} = 3.71$, $p = 0.056$

Interaction (order x drug), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x intervention), $F_{(1, 144)} = 11.79$, $p < 0.0001$

Interaction (order x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (drug x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order x drug), $F_{(1, 144)} = 10.26$, $p = 0.0017$

Interaction (arm x order x intervention), $F_{(1, 144)} = 13.18$, $p < 0.0001$

Interaction (arm x drug x intervention), $F_{(1, 144)} = 29.40$, $p < 0.0001$

Interaction (order x drug x intervention), $F_{(1, 144)} = 0$, $p = 1$

Interaction (arm x order x drug x intervention), $F_{(1, 144)} = 4.58$, $p = 0.034$

Figure 9 Attenuating TLR4 either before (c) or after (d) adolescent alcohol exposure has no effect on preference for an alcohol-conditioned stimulus in adult mice. All data was analysed using a four-way ANOVA with Tukey post hoc.

Conditioning chamber, $F_{(1, 144)} = 56.09$, $p < 0.0001$

Order, $F_{(1, 144)} = 0.018$, $p = 0.89$

Intervention, $F_{(1, 144)} = 0.051$, $p = 0.82$

Gavage, $F_{(1, 144)} = 0.164$, $p = 0.69$

Interaction (adolescent gavage x intervention), $F_{(1, 144)} = 0.012$, $p = 0.91$

Interaction (adolescent gavage x order), $F_{(1, 144)} = 0.011$, $p = 0.92$

Interaction (intervention x order) , $F_{(1, 144)} = 0.038$, $p = 0.85$

Interaction (adolescent gavage x conditioning chamber), $F_{(1, 144)} = 3.63$, $p = 0.059$

Interaction (intervention x conditioning chamber), $F_{(1, 144)} = 0.011$, $p = 0.92$

Interaction (order x conditioning chamber), $F_{(1, 144)} = 3.19$, $p = 0.076$

Interaction (adolescent gavage x intervention x order), $F_{(1, 144)} = 0.026$, $p = 0.87$

Interaction (adolescent gavage x intervention x conditioning chamber), $F_{(1, 144)} = 2.63$, $p = 0.11$

Interaction (adolescent gavage x order x conditioning chamber), $F_{(1, 144)} = 0.47$, $p = 0.50$

Interaction (intervention x order x conditioning chamber), $F_{(1, 144)} = 4.38$, $p = 0.038$

Interaction (adolescent gavage x intervention x order x conditioning chamber), $F_{(1, 144)} = 1.48$, $p = 0.23$

Figure 10 Attenuating TLR4 either before (a) or after (b) adolescent alcohol exposure decreases alcohol intake in adult mice. All data was analysed using a four-way ANOVA with Tukey post hoc.

Testing day, $F_{(3, 256)} = 8.81$, $p < 0.0001$

Order, $F_{(1, 256)} = 0.004$, $p = 0.95$

Intervention, $F_{(1, 256)} = 82.58$, $p < 0.0001$

Gavage, $F_{(1, 256)} = 4.64$, $p = 0.032$

Interaction (testing day x order), $F_{(3, 256)} = 0.26$, $p = 0.85$

Interaction (testing day x intervention), $F_{(3, 256)} = 0.036$, $p = 0.99$

Interaction (order x intervention), $F_{(1, 256)} = 0.269$, $p = 0.60$

Interaction (testing day x adolescent gavage), $F_{(3, 256)} = 12.096$, $p < 0.0001$

Interaction (order x adolescent gavage), $F_{(1, 256)} = 1.484$, $p = 0.2242$

Interaction (intervention x adolescent gavage), $F_{(1, 256)} = 38.40$, $p < 0.0001$

Interaction (testing day x order x intervention), $F_{(3, 256)} = 0.81$, $p = 0.49$

Interaction (testing day x order x adolescent gavage), $F_{(3, 256)} = 0.424$, $p = 0.73$

Interaction (testing day x intervention x adolescent gavage), $F_{(3, 256)} = 2.57$, $p = 0.054$

Interaction (order x intervention x Adolescent gavage), $F_{(1, 256)} = 42.14$, $p < 0.0001$

Interaction (testing day x order x intervention x Adolescent gavage), $F_{(3,256)} = 1.85$, $p = 0.14$

5.8.2 Supplementary figures

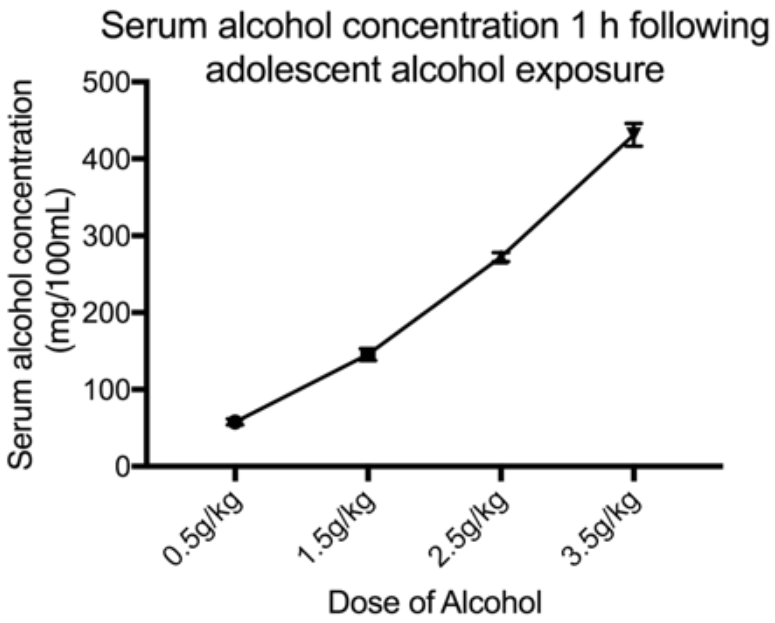


Figure s1 Serum alcohol concentration 1 h following the last gavage of alcohol in adolescence. All data was analysed using a one-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=5, *p < 0.05; **p < 0.01, *** p < 0.001.

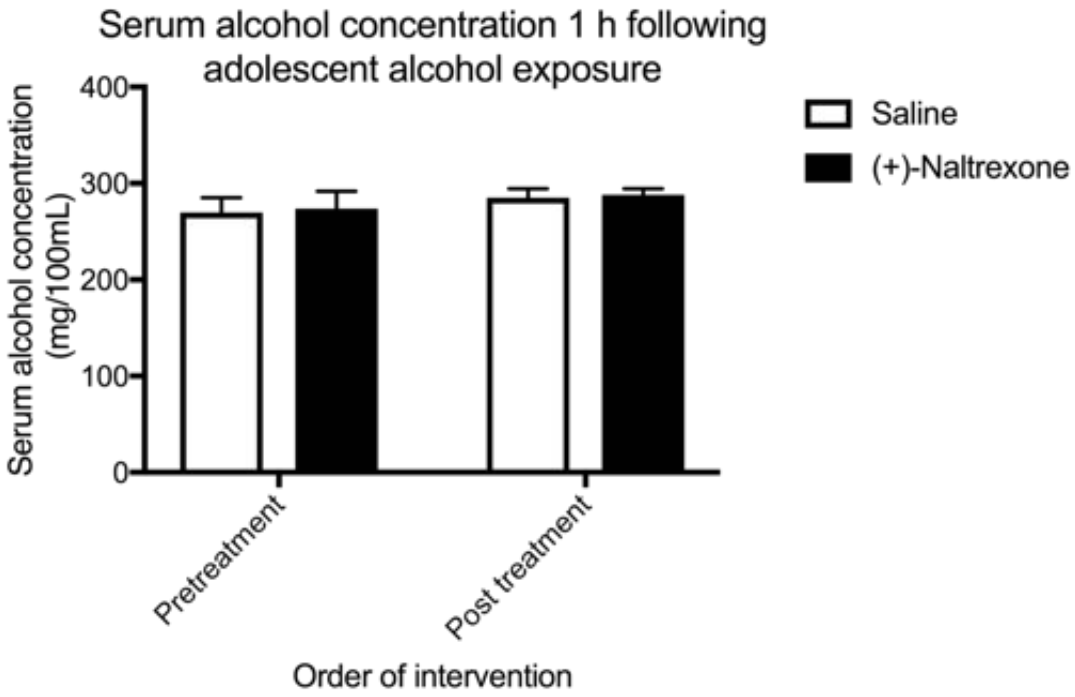


Figure s2 Attenuating TLR4 either before or after adolescent alcohol exposure has no effect on serum alcohol concentration. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=5, *p < 0.05; **p < 0.01, *** p < 0.001.

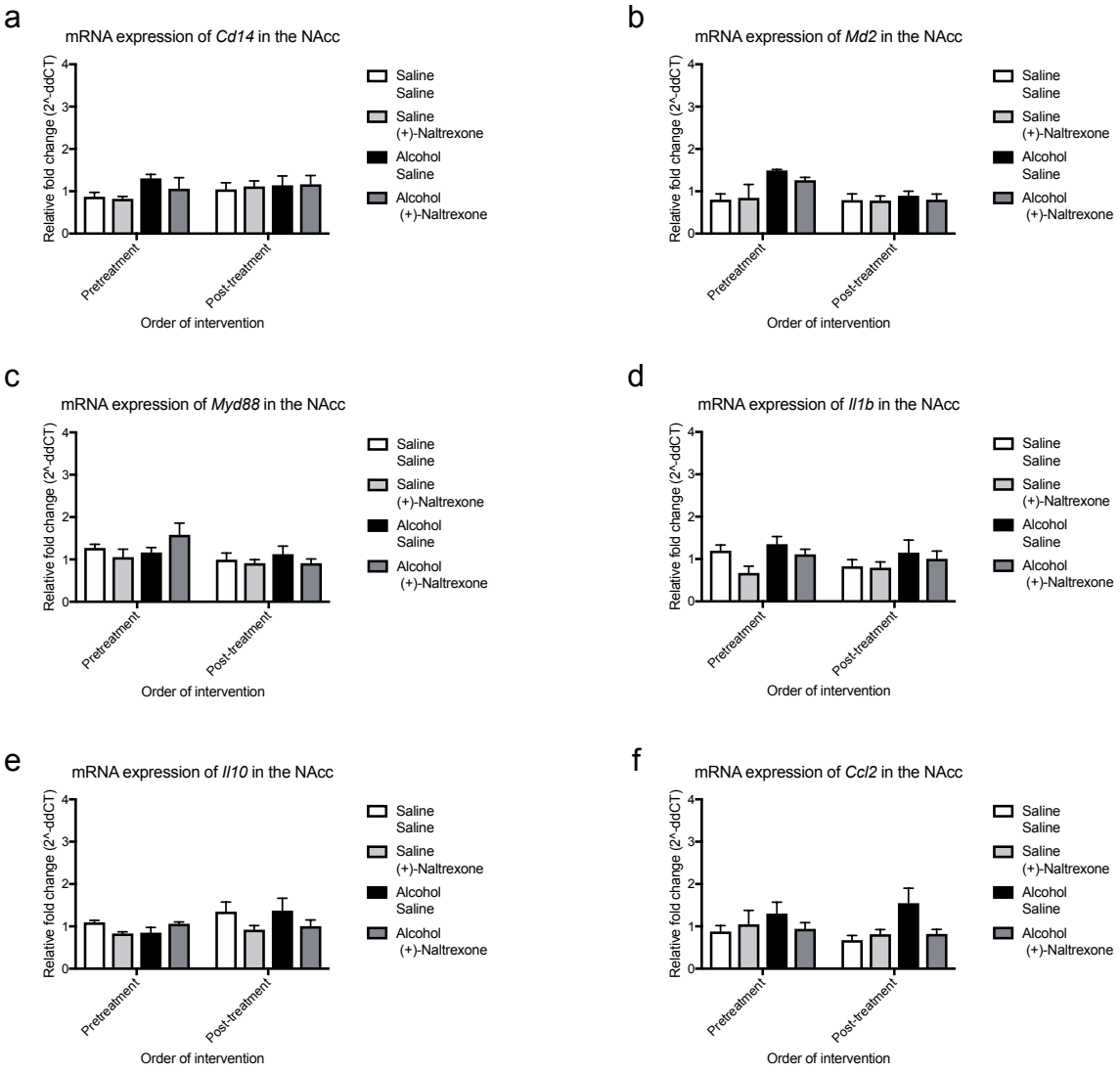
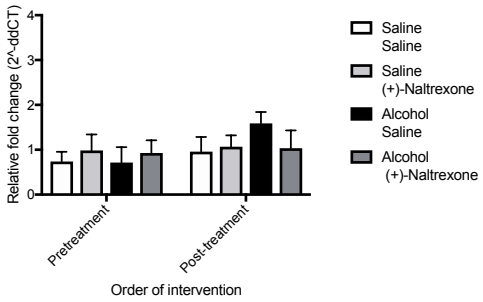
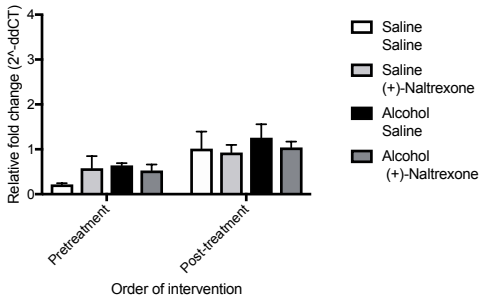


Figure s3 Attenuating TLR4 either before or after adolescent alcohol exposure has no effect on the level of expression of genes relating to the TLR4 pathway nucleus accumbens of adult mice. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

a


mRNA expression of *Drd1* in the NAcc
Relative fold change (2^-ddCT)
0
1
2
3
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

b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Relative fold change (2^-ddCT)
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1
2
3
4
Pretreatment
Post-treatment
Order of intervention
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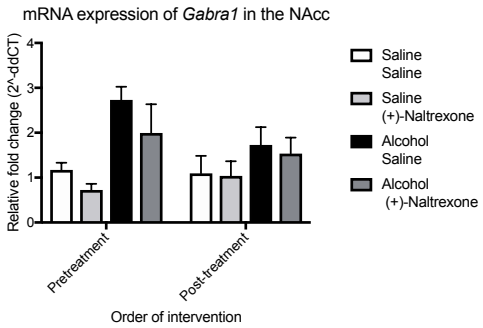
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

c


mRNA expression of *Gabra1* in the NAcc
Relative fold change (2^-ddCT)
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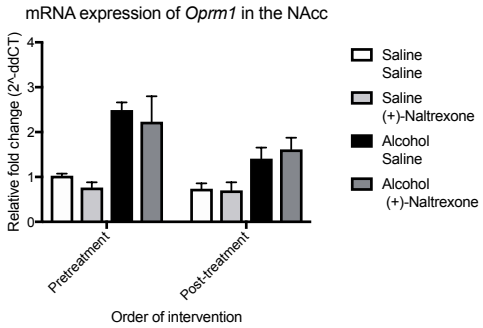
1
2
3
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

d


mRNA expression of *Oprm1* in the NAcc
Relative fold change (2^-ddCT)
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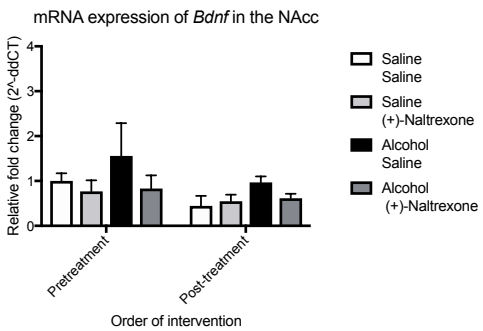
1
2
3
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

e


mRNA expression of *Bdnf* in the NAcc
Relative fold change (2^-ddCT)
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1
2
3
4
Pretreatment
Post-treatment
Order of intervention
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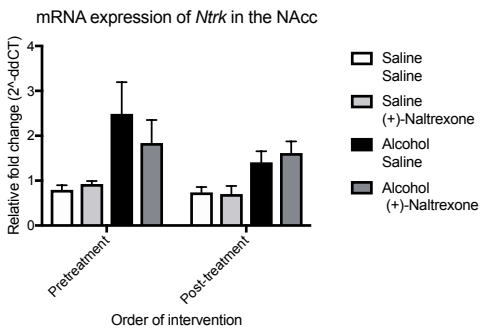
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

f


mRNA expression of *Ntrk* in the NAcc
Relative fold change (2^-ddCT)
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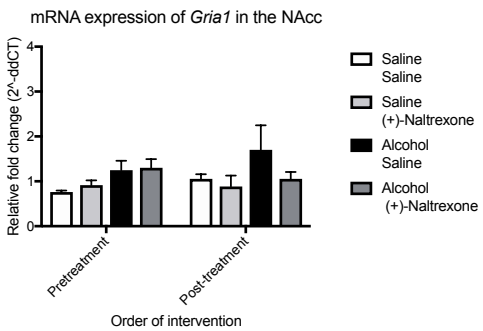
1
2
3
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

g


mRNA expression of *Gria1* in the NAcc
Relative fold change (2^-ddCT)
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2
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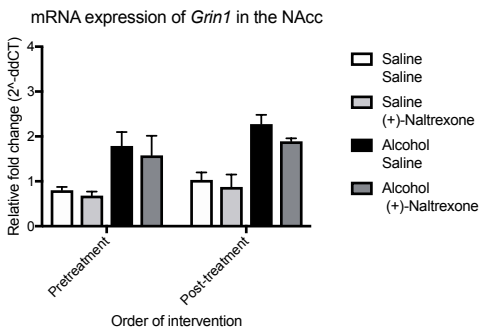
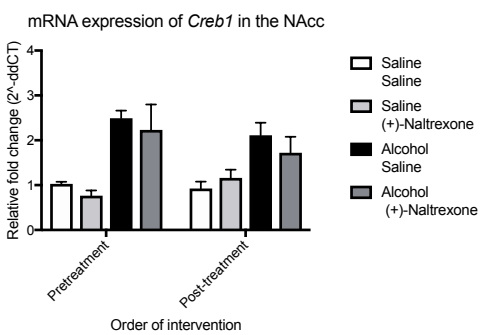
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

h


mRNA expression of *Grin1* in the NAcc
Relative fold change (2^-ddCT)
0
1
2
3
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

i


mRNA expression of *Creb1* in the NAcc
Relative fold change (2^-ddCT)
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1
2
3
4
Pretreatment
Post-treatment
Order of intervention
Saline
Saline
Saline
(+)-Naltrexone
Alcohol
Saline
Alcohol
(+)-Naltrexone

Figure s4 Attenuating TLR4 either before or after adolescent alcohol exposure has no effect on the level of expression of genes relating to alcohol reward and plasticity in the nucleus accumbens of adult mice. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

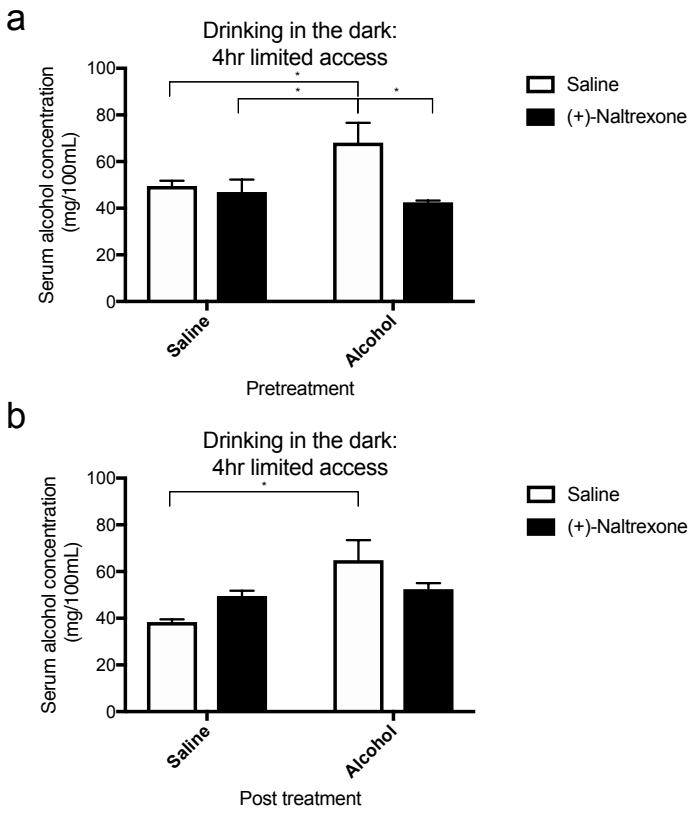

Figure s5 Attenuating TLR4 either before (a) or after (b) adolescent alcohol exposure reduces blood alcohol concentration following drinking in the dark in adulthood. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=5, *p < 0.05; **p < 0.01, *** p < 0.001.

5.8.3 Statistics for supplementary figures

Figure s1 Blood ethanol concentration one hour following the last gavage of alcohol in adolescence. All data was analysed using a one-way ANOVA with Tukey post hoc.

Dose $F_{(3, 16)} = 201.5$, $p < 0.0001$

Multiple comparisons: all data point significantly different from each other $p < 0.0001$

Figure s2 Attenuating TLR4 either before or after adolescent alcohol exposure has no effect on blood alcohol concentration. All data was analysed using a two-way ANOVA with Tukey post hoc.

Treatment, $F_{(1, 4)} = 0.74$, $p = 0.44$

Order, $F_{(1, 4)} = 0.24$, $p = 0.65$

Interaction (treatment x order), $F_{(1, 4)} = 0.0083$, $p = 0.93$

Figure s3 Attenuating TLR4 either before or after adolescent alcohol exposure has no effect on the level of expression of genes relating to the TLR4 pathway nucleus accumbens of adult mice. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

Pretreatment

(a) *Cd14*: gavage ($F_{(1, 3)} = 2.07$, $p = 0.29$), intervention ($F_{(1, 3)} = 1.48$, $p = 0.35$), interaction ($F_{(1, 3)} = 1.32$, $p = 0.37$).

(b) *Md2*: gavage ($F_{(1, 3)} = 8.21$, $p = 0.10$), intervention ($F_{(1, 3)} = 0.28$, $p = 0.65$), interaction ($F_{(1, 3)} = 1.15$, $p = 0.40$).

(c) *Myd88*: gavage ($F_{(1, 3)}$ = 3.43, p = 0.21), intervention ($F_{(1, 3)}$ = 0.72, p = 0.49), interaction ($F_{(1, 4)}$ = 2.12, p = 0.28).

(d) *Il1b*: gavage ($F_{(1, 3)}$ = 7.19, p = 0.12), intervention ($F_{(1, 3)}$ = 4.96, p = 0.16), interaction ($F_{(1, 3)}$ = 0.06, p = 0.82).

(e) *Il10*: gavage ($F_{(1, 3)}$ = 0.009, p = 0.93), intervention ($F_{(1, 3)}$ = 0.13, p = 0.75), interaction ($F_{(1, 3)}$ = 46.89, p = 0.021).

(f) *Ccl2*: gavage ($F_{(1, 3)}$ = 0.22, p = 0.68), intervention ($F_{(1, 3)}$ = 18, p = 0.72), interaction ($F_{(1, 3)}$ = 1.3, p = 0.37).

Post-treatment

(a) *Cd14*: gavage ($F_{(1, 3)}$ = 0.066, p = 0.80), intervention ($F_{(1, 3)}$ = 0.48, p = 0.51), interaction ($F_{(1, 3)}$ = 0.039, p = 0.85).

(b) *Md2*: gavage ($F_{(1, 3)}$ = 0.20, p = 0.67), intervention ($F_{(1, 3)}$ = 0.48, p = 0.51), interaction ($F_{(1, 3)}$ = 0.75, p = 0.41).

(c) *Myd88*: gavage ($F_{(1, 3)}$ = 0.12, p = 0.74), intervention ($F_{(1, 3)}$ = 0.95, p = 0.35), interaction ($F_{(1, 4)}$ = 5.76, p = 0.043).

(d) *Il1b*: gavage ($F_{(1, 3)}$ = 2.74, p = 0.15), intervention ($F_{(1, 3)}$ = 0.18, p = 0.68), interaction ($F_{(1, 3)}$ = 1.11, p = 0.33).

(e) *Il10*: gavage ($F_{(1, 3)}$ = 0.51, p = 0.50), intervention ($F_{(1, 3)}$ = 0.047, p = 0.83), interaction ($F_{(1, 3)}$ = 0.016, p = 0.90).

(f) *Ccl2*: gavage ($F_{(1, 3)}$ = 0.18, p = 0.68), intervention ($F_{(1, 3)}$ = 0.031, p = 0.87), interaction ($F_{(1, 3)}$ = 1.63, p = 0.25).

Figure s4 Attenuating TLR4 either before or after adolescent alcohol exposure has no effect on the level of expression of genes relating to alcohol reward and plasticity in the nucleus accumbens of adult mice. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

Pretreatment

(a) *Drd1*: gavage ($F_{(1, 3)}$ = 0.80, p = 0.47), intervention ($F_{(1, 3)}$ = 5.25 p = 0.15), interaction ($F_{(1, 3)}$ = 0.0015, p = 0.97).

(b) *Drd2*: gavage ($F_{(1, 3)}$ = 0.12, p = 0.76), intervention ($F_{(1, 3)}$ = 6.18, p = 0.13), interaction ($F_{(1, 3)}$ = 1.25, p = 0.38).

(c) *Gabra1*: gavage ($F_{(1, 3)}$ = 28.11 p = 0.034), intervention ($F_{(1, 3)}$ = 4.58, p = 0.17), interaction ($F_{(1, 4)}$ = 0.095, p = 0.78).

(d) *Oprm1*: gavage ($F_{(1, 3)}$ = 24.9, p = 0.038), intervention ($F_{(1, 3)}$ = 0.72, p = 0.49), interaction ($F_{(1, 3)}$ = 0.00047, p = 0.99).

(e) *Bdnf*: gavage ($F_{(1, 3)}$ = 0.43, p = 0.58), intervention ($F_{(1, 3)}$ = 1.58, p = 0.33), interaction ($F_{(1, 3)}$ = 0.34, p = 0.62).

(f) *Ntrk2*: gavage ($F_{(1, 3)}$ = 4.69, p = 0.16), intervention ($F_{(1, 3)}$ = 2.12, p = 0.28), interaction ($F_{(1, 3)}$ = 7.28, p = 0.11).

(g) *Gria1*: gavage ($F_{(1, 3)}$ = 31.3, p = 0.031), intervention ($F_{(1, 3)}$ = 0.22, p = 0.69), interaction ($F_{(1, 3)}$ = 0.070, p = 0.82).

(h) *Grin1*: gavage ($F_{(1, 3)}$ = 22.83, p = 0.041), intervention ($F_{(1, 3)}$ = 0.29, p = 0.66), interaction ($F_{(1, 3)}$ = 0.03, p = 0.88).

(i) *Creb1*: gavage ($F_{(1, 3)}$ = 0.27, p = 0.70), intervention ($F_{(1, 3)}$ = 22, p = 0.041), interaction ($F_{(1, 3)}$ = 1.2, p = 0.69).

Post-treatment

(a) *Drd1*: gavage ($F_{(1, 3)}$ = 0.021, p = 0.69), intervention ($F_{(1, 3)}$ = 2.01, p = 0.29), interaction ($F_{(1, 3)}$ = 5.24, p = 0.15).

(a) *Drd2*: gavage ($F_{(1, 3)}$ = 0.018, p = 0.91), intervention ($F_{(1, 3)}$ = 0.09, p = 0.79), interaction ($F_{(1, 3)}$ = 0.68, p = 0.50).

(b) *Gabra1*: gavage ($F_{(1, 3)}$ = 0.086 p = 0.80), intervention ($F_{(1, 3)}$ = 7.49, p = 0.11), interaction ($F_{(1, 4)}$ = 0.095, p = 0.78).

(c) *Oprm1*: gavage ($F_{(1, 3)}$ = 0.26, p = 0.66), intervention ($F_{(1, 3)}$ = 7.59, p = 0.11), interaction ($F_{(1, 3)}$ = 0.75, p = 0.38).

(d) *Bdnf*: gavage ($F_{(1, 3)}$ = 0.48, p = 0.55), intervention ($F_{(1, 3)}$ = 4.01, p = 0.18), interaction ($F_{(1, 3)}$ = 1.28, p = 0.38).

(e) *Ntrk2*: gavage ($F_{(1, 3)}$ = 2.53, p = 0.25), intervention ($F_{(1, 3)}$ = 3.53, p = 0.20), interaction ($F_{(1, 3)}$ = 2.67, p = 0.24).

(f) *Gria1*: gavage ($F_{(1, 3)}$ = 1.55, p = 0.34), intervention ($F_{(1, 3)}$ = 1.08, p = 0.41), interaction ($F_{(1, 3)}$ = 0.67, p = 0.50).

(g) *Grin1*: gavage ($F_{(1, 3)}$ = 3.46, p = 0.20), intervention ($F_{(1, 3)}$ = 21.48, p = 0.043), interaction ($F_{(1, 3)}$ = 1.38, p = 0.36).

(h) *Creb1*: gavage ($F_{(1, 3)}$ = 0.17, p = 0.72), intervention ($F_{(1, 3)}$ = 8.24, p = 0.10), interaction ($F_{(1, 3)}$ = 2.17, p = 0.28).

Figure s5 Attenuating TLR4 either before (a) or after (b) adolescent alcohol exposure reduces blood alcohol concentration following drinking in the dark in adulthood. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Pretreatment*

Intervention, $F_{(1, 4)}$ = 6.94, p = 0.03

Gavage, $F_{(1, 4)}$ = 7.19, p = 0.029

Interaction, $F_{(1, 4)}$ = 8.30, p = 0.025

(b) *Post treatment*

Intervention, $F_{(1, 4)}$ = 5.1, p = 0.049

Gavage, $F_{(1, 4)}$ = 24.81, p = 0.0011

Interaction, $F_{(1, 4)}$ = 1.058, p = 0.33

5.9 Summary

The preceding manuscript highlighted that adolescent alcohol exposure sensitises the reward and TLR4 signalling pathways and leads to increases in the “liking” and “wanting” component of alcohol reward in adulthood (Table 2). Interestingly, however, there was no effect of adolescent alcohol exposure on elevated plus maze performance, indicating that this model was insufficient to induce persistent alterations to brain regions involved in stress and anxiety (anti-reward). The engagement, or lack thereof in differential brain regions may be due to relative resilience of the amygdala towards alcohol, requiring multiple alcohol cycles before it is engaged (He & Crews, 2008). Alternatively, the lack of anxiety differences may be attributable to the background strain of mouse used in the study (Balb/c) - which may be masking the effects of alcohol (Carola et al., 2002).

An important finding of this study was that a shorter adolescent alcohol exposure model could lead to long-lasting increases (sensitised) in the expression of reward- and TLR4-related mRNAs. Adolescent alcohol exposure potentiated the expression of genes relating to the “wanting” and “liking” component of alcohol reward (for example *Drd1* and *Oprm1*) and aspects of the TLR4 signalling pathway in adulthood. Interestingly, while both TLR4 pathways (TRIF and MyD88) exhibited increases in prototypical end products such as *Il1b* and *Ifnb* mRNA following adolescent alcohol exposure, only the TRIF pathway exhibited additional increases in the expression of upstream signalling products.

Attenuating TLR4-TRIF signalling either before or after exposure to alcohol prevented the increase in alcohol “liking” but not “wanting” in adulthood as inferred by the drinking in the dark and conditioned place preference respectively. While, there was no difference of (+)-Naltrexone on *Oprm1* (μ opioid receptor) expression, this drug may

influence other mediators of the “liking” component of reward such as endogenous cannabinoids, orexins or other opioid receptor systems. Future experiments will therefore be required to address this hypothesis. The differing effects of (+)-Naltrexone on the “liking” and “wanting” components of reward may additionally be due to the differences in neuroanatomical regions and psychological processes responsible for each behavioural test. For example, conditioned place preference is dependent on numerous cellular processes involving multiple brain regions (amygdala, hippocampus, insula, nucleus accumbens, ventral tegmental area) integrating aspects of memory and reward (Tzschentke, 2007). By contrast, drinking in the dark is dependent on amygdala, nucleus accumbens and ventral tegmental area; and is largely dependent on thirst and reward (for example, Cozzoli et al., 2012; Hendrickson et al., 2009). Given the neuroanatomical differences and psychological components required for both behaviours, it is hypothesised that TLR4 may be expressed more in areas associated with drinking in the dark rather than conditioned place preference. Consequently, antagonising TLR4-TRIF with (+)-Naltrexone would have a more pronounced effect on alcohol-drinking than conditioned place preference.

Key to this study was the use of a post treatment paradigm. Adolescents do not take prophylactic medication prior to binge drinking. Consequently, the therapeutic window to mitigate the detrimental effects of alcohol-induced TLR4 signalling on neurodevelopment occurs after alcohol has activated TLR4. Therefore, a post-treatment paradigm was implemented into this study. Interesting, results from the three- and four-way ANVOAs demonstrated there was no difference in terms of administering (+)-Naltrexone as a pre- or post-treatment for drinking in the dark, conditioned place preference or elevated plus maze indicating this drug works irrespective of whether alcohol has already activated TLR4. However, given this drug

was administered only 30 mins after alcohol exposure, conclusions relating to its efficacy are somewhat limited.

Collectively, this study highlights the importance of TLR4 in mediating the enduring effects of adolescent alcohol exposure. Specifically, TLR4 is pivotal to adolescent alcohol potentiated “liking” but not “wanting” components of alcohol reward in adulthood.

Table 2 A brief summary of behavioural data (main effects) from chapter 5

Behaviour	**Effect of adolescent alcohol**	**Effect of (+)-Naltrexone**
Liking		
Drinking in the dark	↑ intake	↓ intake
Wanting		
Conditioned place preference	↑ place preference	—
Anxiety		
Elevated plus maze	—	—

—, no effect

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Chapter 6: Long-term alcohol exposure, reward and TLR4

6.1 Introduction

Acute alcohol exposure activates TLR4 leading to altered reward-like behaviour in mice (chapter 3). The TLR4 pathway can remain in a sensitised state following acute alcohol use which subsequently influences the behavioural response to alcohol (chapter 5). Upon re-exposure to alcohol, the TLR4 response is amplified as is the "liking" and "wanting" components of reward thereby promoting further intake. Consequently, sensitisation leads to the resumption of drinking, which over prolonged periods causes the transition from impulsive drinking to compulsive drinking – a prerequisite for alcohol dependence (1.2.1 and 1.3.4). Repeated cycles of binge drinking followed by periods of deprivation results in neuroplastic and epigenetic events designed to limit the effects of alcohol on the brain. These processes desensitise the reward pathway and recruit stress, anxiety and pain pathways. This in turn promotes tolerance in the presence of, and, stress and anhedonia in the absence of alcohol (Koob & Le Moal, 2001). This further perpetuates alcohol consumption to alleviate these dysphoric sensations.

6.2 Study 3 aims and hypothesis

Chronic exposure to alcohol activates the TLR4 pathway causing robust increases in microglia and astrocyte reactivity and the expression of inflammatory transcription factors, cytokines and reactive oxygen-producing enzymes (Alfonso-Loeches et al., 2010; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009). As outlined in the sections 1.6 – 1.8, these immune molecules interact with classical reward and anti-reward neurotransmitters, alter plasticity and epigenetic processes and cause cell death which assists in the development of tolerance, dependence, anxiety and anhedonia (Cui et al., 2014). Specifically, research has shown that $TLR4^{-/-}$ mice are protected against

alcohol-induced cognitive deficits, memory and motor impairments, and exhibit reductions in the “wanting” component of reward following long-term exposure (Harris et al., 2017; Pascual et al., 2011). However, genetic knockout studies assessing chronic alcohol consumption are potentially confounded given TLR4s role in acute alcohol consumption (Harris et al., 2017; Liu et al., 2011; Appendix). The reduced intake may limit the neuroplastic alterations to brain regions mediating reward and anti-reward. Consequently, tolerance, anhedonia and anxiety may not develop in these mice. This confounds the interpretations of these studies as TLR4 may not be involved in these processes rather it limited the ability of these processes to occur. Further, the background strain of rodents used to generate $TLR4^{-/-}$ mice are alcohol preferring (C57BL/6J) (Harris et al., 2017; Pascual et al., 2011). Therefore, inherent genetic predispositions may be masking the effects of TLR4 during and following long-term alcohol use.

Only one study has briefly considered the relative role of the MyD88 or TRIF pathways in mediating the long-term effects of alcohol on reward; and no study has considered how the “liking” component of reward is modified following long-term alcohol use or, controlled for confounding variables such as thirst and taste. Consequently, the aims of this study were to determine:

1. the expression of reward, anti-reward and TLR4 signalling pathways following long-term alcohol exposure in non-alcohol preferring mice; and
    a. determine whether long-term use preferentially modifies the TRIF or MyD88 pathways;
2. how anti-reward and the “liking” and “wanting” components of alcohol reward are modified following long-term intake (controlling for thirst and taste) in non-alcohol preferring mice; and

3. whether attenuating the TLR4-TRIF signalling pathway prevents alterations to reward and anti-reward behaviours induced by long-term alcohol exposure.

Given that long-term alcohol exposure results in robust increases in immune mediators and TLR4 activity, it was hypothesised that both TLR4 signalling pathways are activated following long-term alcohol exposure and contribute to the presentation of reward and anti-reward behaviour.

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By signing the Statement of Authorship, each author certifies that the candidate's stated contribution to the publication is accurate; permission is granted for the candidate to include the publication in the thesis; and the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Chapter 7: (+)-Naltrexone has limited efficacy in modifying reward or anxiety-like behaviour following long-term alcohol use

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7.1 Abstract

Long-term alcohol use results in numerous neuroadaptations to reward, stress and immune pathways within the brain. In the presence of alcohol, these adaptions typically manifest as tolerance towards alcohol's rewarding effects and in the absence of alcohol cause anhedonia, anxiety and craving. Key to the development and appearance of these adaptions and behaviours is Toll-like receptor 4 (TLR4), an innate immune pattern recognition receptor. However, studies examining the role of TLR4 are conflicting - an effect potentially attributable to the use of global knockouts and the use of genetically predisposed alcohol-preferring rodents. Therefore, this study sought to characterise whether non-alcohol preferring mice exhibit signs of tolerance, anhedonia and anxiety on a behavioural and molecular level following long-term alcohol use and whether the antagonising the TLR4-TRIF signalling pathway alleviates adverse effects attributable to long-term alcohol use.

This study demonstrated that Balb/c, a non-alcohol preferring mouse strain, exhibit increased alcohol and decreased sucrose preference immediately following long-term alcohol use. This effect coincided with escalated alcohol and alcohol + quinine, reduced sucrose intake and some indications of anxiety-like behaviour during withdrawal. The alterations in behaviour following long-term alcohol use were potentially explained by differences in reward, stress and TLR4-related genes. Collectively suggesting non-alcohol preferring mice exhibit signs of dependence on a molecular and behavioural level following long-term alcohol use. Administration of (+)-Naltrexone, a biased TLR4-TRIF antagonist, failed to modify increases in alcohol, and deficits in sucrose intake immediately following long-term alcohol use and during withdrawal. Further, this drug failed to attenuate increases in anxiety-like behaviour during the withdrawal period. Crucially, (+)-Naltrexone did not alter the expression of reward, stress or TLR4-related genes indicating acute administration of this drug

following long-term use has limited efficacy. Given that TLR4$^{-/-}$ mice are protected against measures of reward and anti-reward during withdrawal it suggests the MyD88 pathway (alone or in combination with the TRIF pathway) may underlie the effects of long-term alcohol use.

Keywords: Toll-like receptor 4, TRIF, alcohol, withdrawal, tolerance, anhedonia, anxiety

7.2 Introduction

Alcohol dependence is a disabling psychiatric disorder characterised by periods of compulsive drinking, withdrawal, craving and relapse (Koob & Le Moal, 2001). Pivotal to the development and maintenance of these stages are positive and negative reinforcement mechanisms imbued by alcohol. The occasional consumption of alcohol is largely characterised by positive reinforcement and manifests as hedonia/reward (Berridge & Robinson, 2016; Nestler, 2005). By contrast, alcohol dependence is largely driven by negative reinforcement manifesting as anhedonia and anxiety (anti-reward); however, some degree of positive reinforcement remains (Wise & Koob, 2013).

The transition from positive to negative reinforcement is dependent on numerous neuroplastic events and processes. Of particular importance is the desensitisation of the reward pathway (ventral tegmental area (VTA) and nucleus accumbens). Under non-dependent circumstances, this pathway generates alcohol-induced hedonia (euphoria or liking) and confers motivational importance towards the drug (wanting) collectively creating the process of reward (Robinson & Robinson, 2013; Nestler, 2005). On a molecular level, hedonia is mediated by increased concentration of endorphins and cannabinoids within the nucleus accumbens following alcohol intake (Olive et al., 2001). The motivational/wanting component of reward is mediated by increases in accumbal dopamine (Weiss & Porrino, 2006; Weiss et al.,1993). However, prolonged exposure to alcohol desensitises the reward pathway reducing the extracellular concentration of endorphins and dopamine following alcohol consumption (Weiss et al., 1996; Diana et al., 1992; Rossetti et al., 1992; Hutchinson et al., 1988). Consequently, the individual becomes tolerant to the effects of alcohol. The liking component of reward is reduced and more alcohol is wanted in an attempt to reach the same hedonic sensation (Weiss et al., 1993). During periods of withdrawal, the concentration of dopamine is further reduced, creating a state of anhedonia (a reduced

ability to feel pleasure). Consequently, the individual experiences increased drive to consume alcohol (craving) to alleviate this sensation – perpetuating relapse (Berridge & Robinson, 2016; Weiss & Porrino, 2006).

In addition, pathways responsible for stress and pain are also activated following long-term alcohol use. Of particular importance is the amygdala. This region contains high levels of stress (corticotrophin releasing hormone and dynorphin) and anti-stress (neuropeptide Y) peptides, which under basal conditions are succinctly regulated to ensure homeostatic functioning of the individual. However, prolonged alcohol consumption dysregulates this system, with elevated and depressed levels of stress and anti-stress peptides respectively. This effect is particularly apparent in the absence of alcohol and is thought to induce the negative behaviours associated with withdrawal such as anxiety, pain and craving (Gilpin et al., 2015; Koob & Le Moal, 2001).

The mechanisms behind reward desensitisation and the engagement of the stress pathway have been inferred by studies examining alcohol-preferring rodents (for example, Mayfield et al., 2016; McBride and Li, 1998). These rodents are genetically predisposed to consuming high levels of, and exhibiting exaggerated response towards alcohol. While these studies have served as an important foundation in establishing the mechanisms behind alcohol dependence, they are translationally only pertinent to humans who are genetically predisposed to developing an alcohol use disorder. While this population is hypothesised to encompass 50 per cent of those who go on to develop an alcohol use disorder, it highlights that other factors aside from genetic biases influence the response to alcohol (Prescott et al., 1999). Thus, to better understand and develop more translationally appropriate models, non-alcohol preferring rodents should also be used.

The importance of neuronal mechanisms in mediating the long-term effects of alcohol use cannot be understated. However, increasing evidence supports the involvement of the neuroimmune system in mediating many of the effects associated with long-term alcohol use (Crews et al., 2017). Particular emphasis has been placed on Toll-like receptor 4 (TLR4), an innate immune pattern recognition receptor (Montesinos et al., 2016a). Alcohol indirectly activates TLR4 culminating in the induction of its two signalling pathways: the MyD88 and TRIF pathways (Blanco et al., 2005). The MyD88 pathway leads to the activation of NFκB and MAPKs that result in the transcription of inflammatory cytokines such as IL-1β. By contrast, activation of TRIF culminates in the induction of IRF3 and type 1 interferons (Akira & Takeda, 2004). The activation of both TLR4 signalling pathways has been exclusively examined *in vitro* or *ex vivo* (Lawrimore & Crews, 2017; Fernandez-Lizarbe et al., 2009; Blanco et al., 2005). Studies are yet to determine whether both pathways are activated *in vivo* and if they contribute to alcohol-related behaviours.

Acute alcohol-induced TLR4 signalling results in a transient increase in immune mediators, which in addition to their immunomodulatory role, are hypothesised to act in a manner congruent with neurotransmitters (Lacagnina et al., 2016; Jacobsen et al., 2014). Long-term alcohol use results in higher expression of these mediators which further modify the function and behaviour of neurons and glia. For example, alcohol-induced TLR4 signalling regulates epigenetic processes relating to, (Montesinos et al., 2016b; Pascual et al., 2011) and the expression of transcription factors (CREB and NFκB) and signaling molecules (BDNF) involved in, plasticity (Montesinos et al., 2016b). These TLR4-induced processes are hypothesised to assist in the desensitisation and recruitment of the reward and stress pathways respectively thereby contributing to the emergence of alcohol dependence.

Furthermore, TLR4 influences the transcription of genes involved in reward and stress (Aurelian et al., 2016; June et al., 2015) and can directly influence GABAergic and CRF signalling in the central nucleus of the amygdala (Bajo et al., 2014; Ming et al., 2013), which sensitise mice to alcohol withdrawal (Breese & Knapp, 2016; Knapp et al., 2011). This raises the prospect that blockade of TLR4 during the withdrawal period may alleviate symptoms of anxiety and stress.

However, the behavioural effects of TLR4 on tolerance, anhedonia, anxiety and craving are only just beginning to be studied. For example, injection of LPS, a TLR4 agonist, perpetuated alcohol intake (Blednov et al., 2011), and sensitised mice to anxiety behaviour during withdrawal (Knapp et al., 2011; Breese et al., 2007). Furthermore, attenuating TLR4 protects against alcohol-induced cognitive impairments and anxiety following two weeks withdrawal from long-term alcohol use (Pla et al., 2016; 2014; Pascual et al., 2011) and reduces or has no effect on the resumption of alcohol intake following long-term use (Harris et al., 2017; Bajo et al., 2016; Pascual et al., 2011). However, studies assessing the impact of TLR4 predominately examine the protracted withdrawal period (2 weeks after alcohol cessation) and do not consider how TLR4 influences the acute withdrawal. Lastly, studies using $TLR4^{-/-}$ mice are inherently confounded, as there is evidence to suggest TLR4 is involved in the acute consumption of alcohol (Harris et al., 2017; Aurelian et al., 2016; June et al., 2015). Therefore, it is unclear whether $TLR4^{-/-}$ mice are protected against the effects of withdrawal because TLR4 is involved in anhedonic and stress behaviours or because these mice consumed less alcohol and thus these brain regions are not as engaged.

Consequently, the aims of this study were to determine whether non-alcohol preferring mice (Balb/c) develop symptoms congruent with alcohol dependence (tolerance,

anhedonia and anxiety) following long-term alcohol use and to determine whether alcohol activates TLR4 signalling pathways in reward and stress-associated brain regions (nucleus accumbens and amygdala respectively). Further, this studied aimed to determine whether attenuation of the TLR4-TRIF pathway could reduce adverse behaviours and molecular alterations associated with alcohol dependence.

7.3 Methods

7.3.1 Animals

Eight-week-old male Balb/c mice (obtained from the Laboratory Animal Services, University of Adelaide) were used for the following experiments. Mice were housed in a temperature (23 ± 3°C) and light/dark (12/12 h) controlled room, with food and water available *ad libitum*. Mice were acclimatised to their new environment for seven days and were handled for a further five days prior to experimentation. All animal care and experiments complied with the principles of the Australian Code of Practice for the care and use of animals for scientific purposes and was approved by the University of Adelaide's Animal Ethics Committee.

7.3.2 Drugs

Ethanol (99.5%) (herein referred to as alcohol) was purchased from Chemsupply (Gliman, SA, Australia). Sucrose and quinine were purchased from Sigma Aldrich (St Louis, MO, USA).

(+)-Naltrexone, a TLR4 antagonist, was synthesised and kindly supplied by Dr Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute of Alcohol Abuse and Alcoholism, Bethesda, MD, USA). (+)-Naltrexone was administered via intraperitoneal injections with doses ranging from 1

to 75 mg/kg (dose volume 10 ml/kg). Saline intraperitoneal injections were volume-matched.

7.3.3 Experimental design

7.3.3.1 Rationale of behavioural tests

Long-term alcohol exposure modifies many brain regions including those associated with reward (VTA and nucleus accumbens) and stress (amygdala and HPA axis). Repeated cycles of exposure and deprivation causes neuroadaptations within these brain regions resulting in tolerance and sensitisation of reward and stress pathways respectively. The behavioural consequence of which typically occur during and post alcohol intake. For example, to determine whether the reward pathway has undergone tolerance (desensitised), mice were tested immediately following the last drinking session before other motivational factors influencing intake become active. By contrast, to determine whether the reward and stress pathways are sensitised in the absence of alcohol (creating anhedonia and anxiety) mice were tested 48 h after the last drinking session (in withdrawal) (figure 1).

7.3.3.2 Experimental procedure

Following 36 days of intermittent alcohol exposure, mice were tested immediately (T_0) or were left for 48 h (T_{48}) before testing (figure 1). Mice immediately tested underwent the two-bottle choice test to examining hedonic-like behaviour (liking) as inferred by alcohol or sucrose intake. Quinine intake and preference was additionally assessed to determine whether these mice had altered gustatory or olfactory senses (figure 1a). Mice undergoing 48 h of withdrawal were subjected to alcohol, alcohol + quinine, quinine or sucrose two-bottle choice, elevated plus maze or open field test (figure 1b). All mice received two injections of (+)-Naltrexone (or saline) 48 h and 24 h before behavioural testing. This overlapped with the last alcohol drinking session in mice

tested immediately (T_0). Previous studies demonstrated an effect of light cycle on the presentation of reward-like behaviour, to control for this potential variable, all testing occurred during the rodents' dark cycle (ZT12).

7.3.3.3 Long-term alcohol exposure

Following acclimatisation, mice were individually housed. After a further week of acclimatisation, mice were presented with two bottles at the beginning of their dark cycle for 24 h. One bottle contained 5 per cent (v/v) alcohol and the other water. After 24 h, bottles were removed, weighed and replaced by a single bottle of water randomly allocated to either side of the cage lids for the next 24 h. Following three drinking sessions with 5 per cent alcohol, the concentration was increased to 10 per cent for the remainder of the study. This intermittent exposure cycle was repeated for a total of 36 days. This method of alcohol exposure was used as repeated cycles of alcohol intake following periods of deprivation is thought to promote higher levels of intake and withdrawal symptoms in mice (Ron & Barak, 2016).

The amount of alcohol consumed was calculated by the difference in bottle weights before and after drinking sessions. This enabled the amount of alcohol per bodyweight (grams/kilogram) and the preference ratio (alcohol intake obtained ÷ (total eight-hour water + alcohol intake)) to be calculated for each mouse and averaged for each treatment group. An empty cage with two bottles of either alcohol, alcohol + quinine, quinine, sucrose or water was used to determine the rate of evaporation. The rate of evaporation was subtracted from the results.

7.3.3.4 Behavioural tests

7.3.3.4.1 Elevated plus maze

To infer basal anxiety-like and exploratory behaviour, mice underwent the elevated plus maze (Walf & Frye, 2007). The elevated plus maze consisted of two areas characterised by a relatively dark environment and an open area.

Apparatus

The maze is made of black PVC and consists of four arms: two open and two closed. All arms were 30 cm long and 5 cm wide. The two enclosed arms had walls 25 cm high.

Procedure

Mice were acclimatised to the behavioural testing room 30 min prior to experimentation after which they were placed into the centre of the elevated plus maze with their head facing towards the open arm and they were allowed to explore the apparatus for 10 min. The time spent, distance travelled and the number of immobile episodes was recorded using a Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA). Mice underwent a pre- and post-test before long-term alcohol exposure and 48 hrs into withdrawal respectively. This was implemented to reduce inter-mouse variability – an effect more apparent in shorter behavioural tests. The difference between the two tests was used to infer exploratory and anxiety behaviour.

7.3.3.4.2 Open field test

To reinforce observations made by the elevated plus maze, the open field test was performed. This test offers additional measure of anxiety and exploratory behaviour (Gould et al., 2009).

Apparatus

The open field test occurs within an opaque square box (40 cm long x 40 cm high x 40 cm wide).

Procedure

Mice were acclimatised to the behavioural testing room 30 min prior to experimentation after which they were placed into the centre of the field and were allowed to explore the apparatus for 10 min. The relatively shorter test time emphasises exploratory behaviour, response to novelty and thus anxiety-like behaviour (Gould et al., 2009). The time spent in the centre, on the sides of the apparatus, immobile time and distance travelled was recorded using a Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA). Mice underwent a pre- and post-test before long-term alcohol exposure and 48 h into withdrawal respectively. The difference between the two tests was used to infer exploratory and anxiety behaviour.

7.3.3.4.3 Two-bottle choice tests

Alcohol, alcohol + quinine, quinine and sucrose preference test

Two-bottle choice preference tests were used to infer tolerance to alcohol's rewarding effects, anhedonic-like behaviour, and the desire to consume alcohol again following the withdrawal period.

Immediately following the last alcohol exposure or 48 h after the last drinking session, mice were presented with two bottles – one containing water, and the other 12 per cent (v/v) alcohol, 12 per cent alcohol plus 0.1 mM quinine, 0.1 mM quinine or 1 per cent sucrose. The paradigm lasted for 2 and 24 h for mice undergoing the two-bottle choice test at T_0 and T_{48}, respectively. At the end of the test, bottles were removed, weighed and replaced with a single bottle randomised to either side of the cage.

Only mice undergoing the sucrose two-bottle choice test underwent a pre- and post-test to determine whether anhedonia occurred (as inferred by a reduction in intake and preference relative to the pretest).

7.3.4 Blood alcohol concentration assay

Serum alcohol concentration was measured using a commercial kit (ADH-NAD Reagent Multiple Test Vial; Sigma-Aldrich) and performed as per the manufacturer instructions. In brief, it estimates alcohol induced reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH in the presence of alcohol dehydrogenase. The reaction is observed by recording the absorbance of 340 nM by the solution. Blood was isolated from the tail vein following the intermittent exposure paradigm and the 2 and 24 h two-bottle choice tests. An additional cohort of mice underwent the intermittent exposure paradigm and received the injections of saline or 60mg/kg of (+)-Naltrexone during the withdrawal period. 30 mins after the last injection, mice were gavaged with 3g/kg of alcohol and tail blood was isolated 20, 40, 60 and 180 min later. This experiment was designed to assess whether (+)-Naltrexone modified the pharmacokinetics of alcohol following long-term exposure.

7.3.5 RNA isolation, reverse transcription and quantitative PCR (qPCR)

The nucleus accumbens and the amygdala region were isolated using micropunches (Kai Medical, Seki City, Japan) from whole brains and submerged in RNAlater® ICE (ThermoFisher Scientific, Waltham, MA, USA) prior to performing RNA isolation. RNA was isolated using Maxwell® 16 LEV simply RNA Tissue Kit (Promega, Madison, WI, USA) as per manufacturer instructions. RNA was quantified using spectrophotometric analysis, with the quality of RNA verified by the OD260/280 ratio. Isolated RNA (900 ng) was reverse transcribed into cDNA using iScript™ cDNA reverse transcription kit (BioRad, Hercules, CA, USA), as per manufacturer instructions.

Gene expression was assessed using iTaq^{TM} Universal SYBR® Green Supermix, as per manufacturer instructions. Real time PCR was performed using the CFX96 Touch^{TM} Real-Time PCR Detection System (BioRad). Mouse *Bdnf*, *Cd14*, *Creb1*, *Crh*, *Crhr1*, *Crhr2*, *Drd1*, *Drd2*, *Gabra1*, *Gabra2*, *Gapdh*, *Gria1*, *Grin1*, *hmgb1*, *ifnb*, *il1b*, *il10, Md2*, *Myd88*, *Npy*, *Npyr1*, *Npyr2*, *Ntrk2*, *Oprm1*, *Oprk1*, *Prkca*, *Prkce, Ucn*, *Th*, *Tirap*, *Tlr2*, *Tlr4*, *Tram* and *Trif* forward and reverse primers were synthesised by Integrated DNA Technologies Pty. Ltd. (Baulkham Hills, NSW, Australia). For primer sequences refer to supplementary materials (Table 1). The genes assessed for this study reflect mediators pertinent to plasticity, reward, stress and TLR4 pathways.

The relative difference in expression level of each of the genes of interest were normalised to the CT of GAPDH for both the test and control sample. The ΔCT of the test sample was normalised to the ΔCT a control sample (equal amount of cDNA from all samples), and then expressed as a ratio ($2^{-\Delta\Delta CT}$). The primer sequence can be found in the supplementary materials.

Only mice undergoing 48 h of deprivation of alcohol were used for the following qPCR experiments as preliminary experiments noted there were no difference in the expression of TLR4 pathway genes between mice immediately tested and those undergoing 48 h of withdrawal.

7.3.6 Statistical analysis

All data was analysed using Graphpad Prism 6.0h (Graphpad Software, Inc. San Diago, CA, USA) and R Studio (RStudio Team, 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA).

Experiment 1: Alcohol, sucrose and quinine drinking behaviour at T_0 and T_{48} was analysed using a two-tail t-test (alcohol vs water) (figures 2 – 3). The elevated plus maze and open field test was analysed two-way ANOVA (arm x solution) with Tukey post hoc (figure 4).

Experiment 2: qPCR results were analysed using a two-tail t-test (alcohol vs water) (figures 5 – 6).

Experiment 3: Alcohol, sucrose and quinine drinking behaviour at T_0 and T_{48} analysed two-way ANOVA (dose x solution) with Tukey post hoc. Elevated plus maze and open field test were measured using a three-way ANOVA (arm x solution x dose) (figures 7 – 9).

Experiment 4: qPCR results were analysed using a two-way ANOVA (solution x treatment) (figures 10 – 11).

All summary values presented as mean ± standard error of mean (SEM). p-values ≤ 0.05 were considered statistically significant.

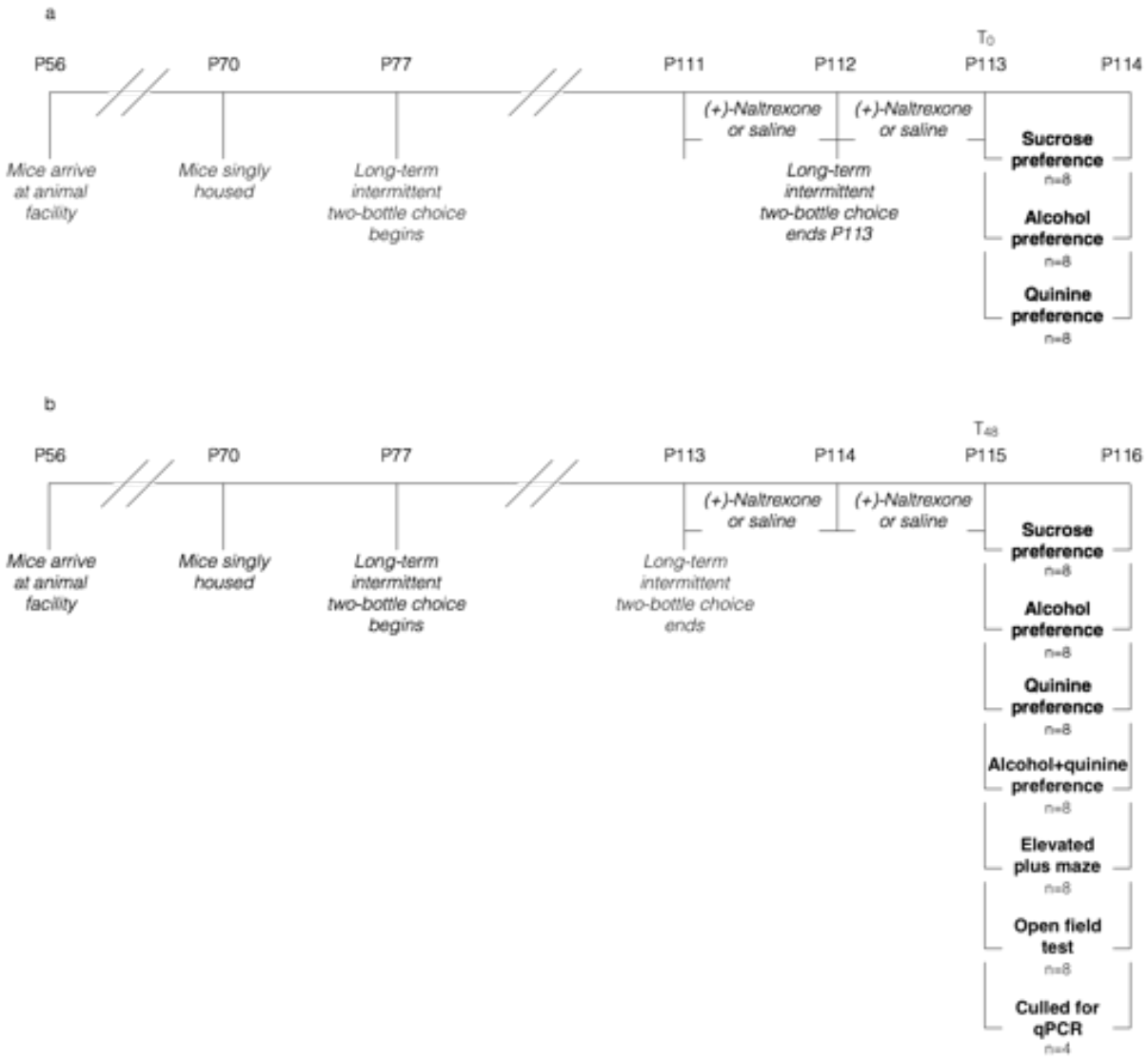

Figure 1 Timeline of behavioural experiments. Following 2 weeks of acclimatisation, mice were singly housed and underwent a 36 d intermittent two-bottle choice drinking paradigm. Alcohol and water was offered for 24 h followed by 24 h of water only. After 36 d of alcohol exposure, mice were immediately tested (a, T_0) or were withdrawn from alcohol for 48 h (b, T_{48}). Mice tested immediately underwent sucrose, alcohol or quinine two-bottle choice (T_0). Mice tested at T_{48} underwent sucrose, alcohol, quinine or alcohol + quinine two-bottle choice, elevated plus maze, open field test or were culled for qPCR.

7.4 Results

7.4.1 Experiment 1: Do non-alcohol preferring mice exhibit signs of tolerance, anhedonia and anxiety following long-term alcohol use?

To determine whether long-term alcohol exposure induces tolerance to rewarding stimuli in non-alcohol preferring mice, Balb/c mice underwent the two-bottle choice paradigm for 2 h immediately after the last drinking session (figure 2a - b). The two-tail t-test determined a significant effect of long-term drinking solution (water vs alcohol) on alcohol intake and preference (effect of solution, t = 1.92 df = 10, p = 0.049; and t = 2.65 df = 10, p = 0.028, respectively). Mice exposed to alcohol for 36 d had greater intake of, and preference for alcohol compared to mice who received water. Similarly, there was a significant effect of solution on sucrose intake and preference (effect of solution, t = 2.29 df = 10, p = 0.036; and t = 2.74 df = 10, p = 0.017, respectively) with mice exposed to alcohol exhibiting decreased intake and preference compared to those exposed to water (figure 2c – d). There was no difference in quinine intake or preference indicating the exposure to alcohol does not alter the sense of taste in Balb/c mice (effect of solution, t = 1.06 df = 10, p = 0.31; and t = 1.84 df = 10, p = 0.09, respectively) (figures 2e – f). Collectively, the results indicate mice exposed to alcohol long-term may be limited in their ability to generate reward to innately likable solutions, but are primed towards alcohol despite the decreased rewarding value.

To determine whether the reward pathway was sensitised following long-term use, mice underwent a 24 h two-bottle choice 48 h after the last drinking session. A two tail t-test determined a significant effect of solution on alcohol intake and preference (effect of solution, t = 6.02 df = 10, p = 0.027; and t = 15.02 df = 10, p = 0.0044, respectively) (Figure 3a – b). Mice exposed to alcohol exhibited greater intake and preference compared to those which received water.

However, the two-bottle choice is limited in its assessment of motivated behaviour, consequently alcohol was blended with quinine to create a more aversive substance to further infer motivation. Again, there was a significant effect of solution on alcohol + quinine intake and preference as inferred by two tail t-tests (effect of solution, t = 2.13 df = 10, p = 0.05; and t = 2.38 df = 10, p = 0.037, respectively) (figure 3e – f). Similarly, mice previously exposed to alcohol exhibited greater intake and preference to water controls. Quinine intake and preference was similar between the two groups (effect of solution, t = 0.13 df = 10, p = 0.90 and t = 0.47 df = 10, p = 0.65 respectively) (figure 3g – h). To infer whether anhedonia may be present and propagating alcohol intake, sucrose intake and preference was assessed (figure 3c – d). A two-tail t-test determined a significant effect of solution on sucrose intake but not preference (effect of solution, t = 2.36 df = 10, p = 0.032 and t = 1.92 df = 10, p = 0.080). Mice exposed to alcohol long-term exhibited a greater negative change (post-test intake – pretest intake) in sucrose intake but not preference compared to water control mice. The reduction in sucrose but increase in alcohol reinforce the suggestion that mice exposed to alcohol long-term may be anhedonic, an effect which may perpetuate the motivation to consume alcohol.

Anxiety was assessed using the elevated plus maze and open field test 48 h following the last drinking session (figure 4a – f). Mice spent more time, travelled further, and had more immobile episodes in the closed arm or sides of the elevated plus maze and open field test respectively (see supplementary materials). However, the effect of solution was inconsistent across the variables assessed in each test. For example, a two-way ANOVA calculated a significant effect of solution for immobile episodes in the elevated plus maze and open field test (effect of solution, $F_{(1, 7)}$ = 5, p = 0.076; and $F_{(1, 7)}$ = 10.4, p = 0.015, respectively). All other variables in the elevated plus maze and

open field test including time and distance had no effect of solution (see supplementary materials).

7.4.2 Experiment 2: Does long-term alcohol exposure alter the expression of molecular mediators of reward, stress and the TLR4-signalling pathway during withdrawal?

The alcohol-induced differences in sucrose and alcohol intake before and during withdrawal are potentially explained by alterations in the expression of reward, stress and anxiety-related genes. Consequently, qPCR was used to examine an array of genes relating to key mediators of reward and stress in the nucleus accumbens and the amygdala respectively (figure 5a – b). A two-tail t-test determined a significant effect of solution in the expression on *Th*, *Drd1* and *Gabra2* mRNA in the nucleus accumbens (effect of solution; *Th*, t = 10.62 df = 16, $p = 0.0004$; *Drd1*, t = 2.36 df = 16, $p = 0.05$; and *Gabra2*, t = 3.14 df = 16, $p = 0.013$, respectively) and *Crh*, *Crhr1, Gabra2* and *Npyr1* mRNA in the amygdala (effect of solution; *Crh*, t = 4.09 df = 16, $p = 0.0008$; *Crhr1*, t = 3.71 df = 16, $p = 0.0019$; *Gabra2*, t = 1.89 df = 16, $p = 0.014$; and *Npyr1*, t = 2.73 df = 16, $p = 0.021$, respectively). No differences were observed for *Crhr2, Urn*, *Npy*, *Npyr2*, *Oprm1*, *Oprk1*, *Gabra1*, *Prkca*, *Prkce*, *Fos* and *Creb1* mRNA in the amygdala and *Drd2*, *Oprm1*, *Gabra1*, *Prkca*, *Prkce, Fos* and *Creb1* mRNA in the nucleus accumbens (see supplementary materials for results of the statistical analysis).

Emerging evidence suggests the TLR4 pathway additionally regulates the effects of long-term alcohol use (Harris et al., 2017; Pascual et al., 2011). Therefore, the expression of this pathway was also assessed (figure 6a – b). There was a significant effect of solution on the expression of *Tlr4*, *Myd88, Hmgb1*, *Il1b* and *Tnfa* mRNA in the nucleus accumbens (effect of solution; *Tlr4*, t = 2.31 df = 16, $p = 0.05$; *Myd88*, t = 4.76

df = 16, *p* = 0.009; *Hmgb1*, t = 2.80 df = 16, *p* = 0.045; *Il1b*, t = 3.59 df = 16, *p* = 0.023; and *Tnfa*, t = 6.28 df= 16, *p* = 0.0033, respectively) as determined by a two tail t-test. Similarly, *Tlr4*, *Tlr2*, *Md2*, *Tram*, *Irf3*, *Il1b*, *Ifnb*, *Tnfa*, and *Hmgb1* mRNA in the amygdala was significantly altered by solution (effect of solution; *Tlr4*, t = 2.26 df = 16, *p* = 0.039; *Tlr2*, t = 2.52 df = 16, *p* = 0.038; *Md2*, t = 2.23 df = 16, *p* = 0.04; *Tram*, t = 2.86 df = 16, *p* = 0.01; *Irf3*, t = 2.23 df = 16, *p* = 0.04; *Il1b*, t = 2.32 df = 16, *p* = 0.034; *Ifnb*, t = 3.17 df = 16, *p* = 0.0059; *Tnfa*, t = 2.40 df = 16, *p* = 0.028; and *Hmgb1*, t = 2.93 df = 16, *p* = 0.009, respectively). No differences were observed in the expression of *Tlr2*, *Cd14*, *Md2*, *Tirap*, *Tram*, *Ifnb* or *Hmgb1* mRNA in the nucleus accumbens and *Cd14*, *Myd88*, *Tirap and Trif* mRNA in the amygdala and (see supplementary material for results of the statistical analysis).

7.4.3 Experiment 3: Does (+)-Naltrexone modify tolerance, anhedonia and anxiety-behaviour following long-term alcohol use?

The results above highlight that in addition to alteration in the expression of reward, stress and anxiety-related genes, the TLR4 signalling pathway is modified by long-term alcohol exposure. Consequently, the aim of the second series of experiments was to ascertain whether TLR4 and its signalling pathways were associated with or causative in the effects generated by long-term alcohol exposure. Specifically, the TLR4-TRIF pathway was examined given previous human studies linking the expression of its downstream signalling proteins in the severity of alcohol dependence (Johnson et al., 2015) and attenuating the TLR4-TRIF pathway successfully reduced opioid craving and relapse in rats (Theberge et al., 2013). Therefore, (+)-Naltrexone, a biased TLR4-TRIF antagonist, was used for the following experiments.

There was a significant effect of solution (alcohol vs water) for all the following experiments except for quinine intake and preference 48 h post long-term exposure

(supplementary data). In line with earlier experiments, mice exposed to alcohol exhibited increased alcohol and reduced sucrose intake/preference (supplementary data).

To determine whether TLR4 influences tolerance, mice were administered with (+)-Naltrexone twice before undergoing a 2 h two-bottle choice test immediately following the last drinking session (figure 7). Overall, a two-way ANOVA determined an effect of (+)-Naltrexone's dose on alcohol intake and preference (effect of dose, $F_{(6, 42)} = 2.35$, $p = 0.005$; and $F_{(6, 42)} = 10.26$, $p = 0.019$, respectively). No interactive effects were observed (see supplementary materials) (figure 7a – b). The main effect of dose was largely attributable to the water control group as post hoc analysis determined mice receiving 60 - 75 mg/kg (+)-Naltrexone demonstrated reduced intake relative to the vehicle group for mice in the water but not alcohol group. Serum alcohol concentration quantification was attempted. However, all groups were below the threshold of detection (limit of quantification 100 – 700 mg/100mL).

There was a significant effect of (+)-Naltrexone dose on sucrose intake and preference (effect of dose, $F_{(6, 42)} = 9.90$, $p = 0.021$; and $F_{(6, 42)} = 10.26$, $p = 0.019$) with an interactive effect observed only for intake ($F_{(6, 42)} = 5.44$, $p = 0.0004$) (two-way ANOVA, figure 7c – d). Again, the difference in intake appears largely attributable to the water exposed mice. Post hoc analysis calculated a significant reduction in sucrose intake from 45 – 75 mg/kg of (+)-Naltrexone in mice exposed to water but not alcohol. Significant reductions in sucrose preference were only observed at 75 mg/kg of (+)-Naltrexone in water and alcohol-exposed mice. No other post hoc differences were observed for mice exposed to alcohol. Quinine intake and preference was not significantly influenced by (+)-Naltrexone's dose (effect of dose, $F_{(6, 42)} = 1.05$, $p = 0.41$; and $F_{(6, 42)} = 2.27$, $p = 0.06$ respectively) (figure 7e – f). Collectively, the results

indicate, (+)-Naltrexone was unable to modify alcohol or sucrose intake and preference, suggesting it is unable to alter behaviours associated with alcohol tolerance.

Previous work demonstrated (+)-Naltrexone attenuated heroin craving and relapse (Theberge et al., 2013). Based on this study, mice received two injections of (+)-Naltrexone over the 48 h withdrawal period, at the end of which they underwent a 24 h two-bottle choice using sucrose or alcohol (figure 8). There was no significant effect of (+)-Naltrexone's dose on sucrose or alcohol intake and preference (effect of dose; sucrose intake, $F_{(6, 42)} = 0.83$, $p = 0.55$; preference $F_{(6, 42)} = 0.59$, $p = 0.73$; and alcohol intake, $F_{(6, 42)} = 0.89$, $p = 0.51$; preference $F_{(1, 7)} = 42.86$, $p = 0.60$) (figure 8a – d). No interactive effects were observed for any variable (see supplementary materials). A separate cohort of mice treated with 60 mg/kg (+)-Naltrexone or vehicle were assessed for quinine and alcohol + quinine intake and preference (Figure s4). Only one dose of (+)-Naltrexone was selected to reduce the number of mice required for the experiments given that no differences were observed in alcohol intake or preference. There was no effect of drug (saline vs. (+)-Naltrexone) on these variables (see supplementary materials). Further, serum alcohol concentrations were below threshold of detection. Collectively, (+)-Naltrexone did not modify behavioural manifestations of relapse or anhedonia suggesting acute attenuation of TLR4-TRIF does not modify alcohol-induced alterations to the reward pathway.

(+)-Naltrexone was additionally screened for its ability to reverse alterations to anxiety-like behaviour (figure 9). There was a significant effect of arm and test area for all variables of the elevated plus maze and the open field test respectively (see supplementary materials). Mice spent more time, travelled further, and had immobile episodes in the closed arm or side of the elevated plus maze and open field test

respectively (see supplementary materials). Again, there was a significant effect of solution for immobile episodes for both the elevated plus maze and open field test (see supplementary). However, solution did not modify any other variable associated with these tests. Furthermore, there was no significant effect of (+)-Naltrexone's dose for any variable of the elevated plus maze or open field test (see supplementary) indicating (+)-Naltrexone did not modify the presentation of anxiety-like behaviour.

7.4.4 Experiment 4: Does (+)-Naltrexone modify genes associated with reward, anti-reward and TLR4?

qPCR was used to investigate why (+)-Naltrexone failed to modify behaviour associated with reward and anxiety before and during withdrawal. In the nucleus accumbens, there was a significant effect of drug (saline vs (+)-Naltrexone) but not solution on the expression of *Cd14*, *Tlr4* and *Hmgb1* mRNA (effect of drug, $F_{(1, 3)}$ = 23.58, p = 0.04; $F_{(1, 3)}$ = 492.7, p = 0.02; and $F_{(1, 3)}$ = 28.6, p = 0.03, respectively) as determined by two-way ANOVAs. In contrast, only *Md2* expression in the amygdala exhibited a significant effect of drug (effect of drug, $F_{(1, 3)}$ = 7.07, p = 0.03). The remaining genes were not influenced by (+)-Naltrexone or saline (see supplementary materials). No TLR4-related gene exhibited both a solution and drug effect in either brain region. The remaining TLR4 pathway genes are presented in the supplementary material.

However, a two-way ANOVA determined a significant effect of solution and drug on the expression of *Gabra2* mRNA in the nucleus accumbens and amygdala (NAcc: effect of solution, $F_{(1, 3)}$ = 35.54, p = 0.027; effect of drug, $F_{(1, 3)}$ = 24.31, p = 0.039) (amygdala: effect of solution, $F_{(1, 8)}$ = 23.13, p = 0.0013; effect of drug, $F_{(1, 8)}$ = 5.87, p = 0.047) (figure 11a – b). There was no interactive effect in either brain region (NAcc, $F_{(1, 3)}$ = 2.48, p = 0.13; Amygdala, $F_{(1, 8)}$ = 4.48, p = 0.067). Post hoc analysis

determined the (+)-Naltrexone + alcohol significantly reduced the expression of *Gabra2* mRNA compared to and saline + alcohol in the nucleus accumbens. The expression of *Th* in the nucleus accumbens was significantly altered by solution and drug with an interaction observed between the two variables (effect of solution, $F_{(1, 3)}$ = 25.2, p = 0.038; effect of drug $_{(1, 3)}$ = 24.31, p = 0.038; and interaction, 21.37, p = 0.044). Post hoc analysis determined saline + alcohol, (+)-Naltrexone + alcohol and (+)-Naltrexone + water cohorts exhibited significant reductions in *Th* expression compared to the saline + water cohort.

7.4.5 Figures

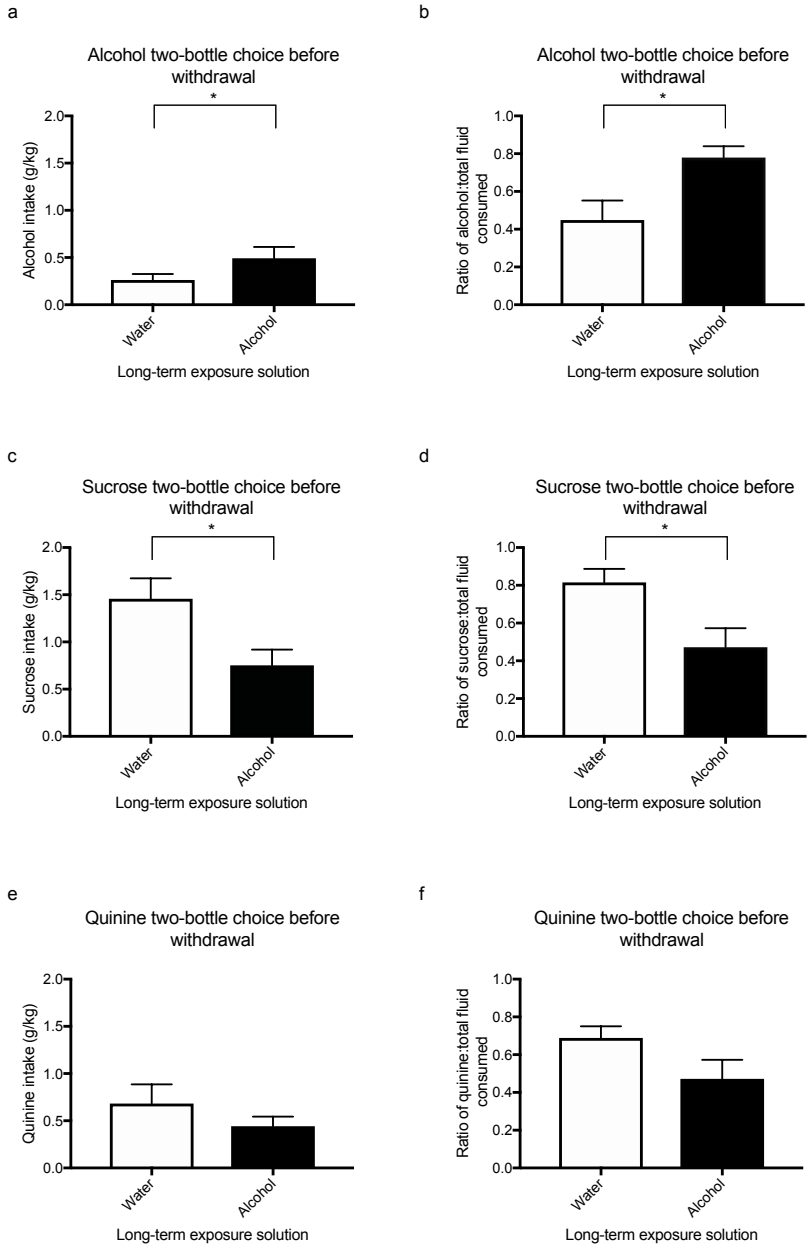

Figure 2 Long-term exposure to alcohol affected the intake and preference of alcohol (a – b), sucrose (c – d) but not quinine (e – f) before withdrawal in non-alcohol preferring mice (Balb/c). The intake and preference for alcohol and sucrose were significantly increased and decreased immediately following long-term alcohol exposure. However, long-term alcohol exposure has no effect on quinine intake and preference. All data was analysed using a paired two-tail t-test. Summary values represented as mean±SEM; n=8, *$p < 0.05$.

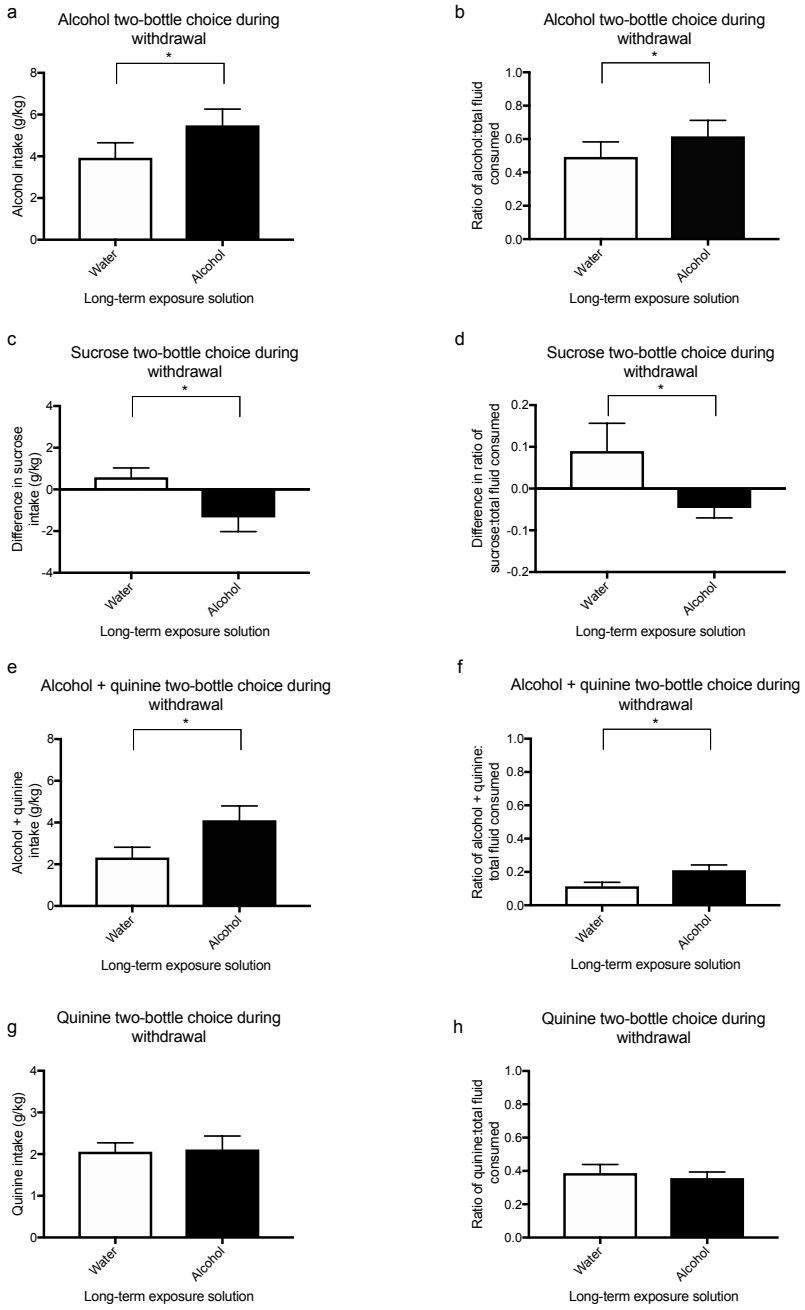

Figure 3 Long-term exposure to alcohol affected the intake and preference of alcohol (a – b), sucrose (c – d), alcohol + quinine (e – f) but not quinine (g – h) during withdrawal in non-alcohol preferring mice (Balb/c). The intake and preference for alcohol and sucrose were significantly increased and decreased following long-term alcohol exposure respectively. Further, long-term alcohol exposure increased alcohol + quinine intake and preference compared to control mice. However, long-term alcohol exposure has no effect on quinine intake and preference. All data was analysed using a paired two-tail t-test. Summary values represented as mean±SEM; n=8, *$p < 0.05$.

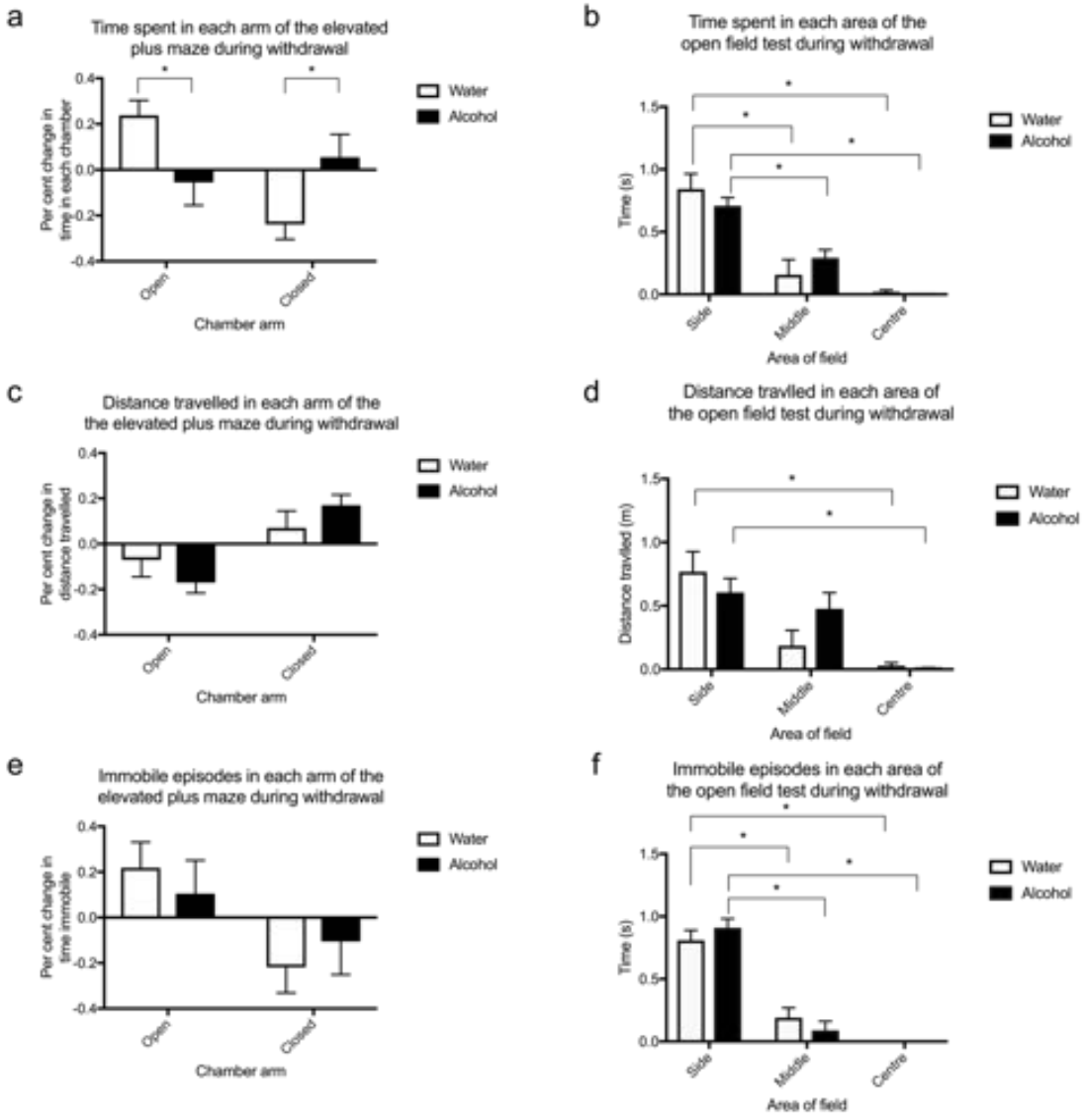

Figure 4 Long-term exposure to alcohol did not alter time spent (a – b) or distance (c – d) but increased immobile episodes (e – f) in the elevated plus maze and open field test) in non-alcohol preferring mice (Balb/c). Long-term exposure to alcohol did not alter the time spent or distance travelled in the open or closed arms of the elevated plus maze, or the sides, middle or centre of the open field test. However, alcohol increased the number of immobile episodes in the light and dark area of the elevated plus maze and the sides of the open field test. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8; elevated plus maze *$p < 0.05$; open field test *$p < 0.01$.

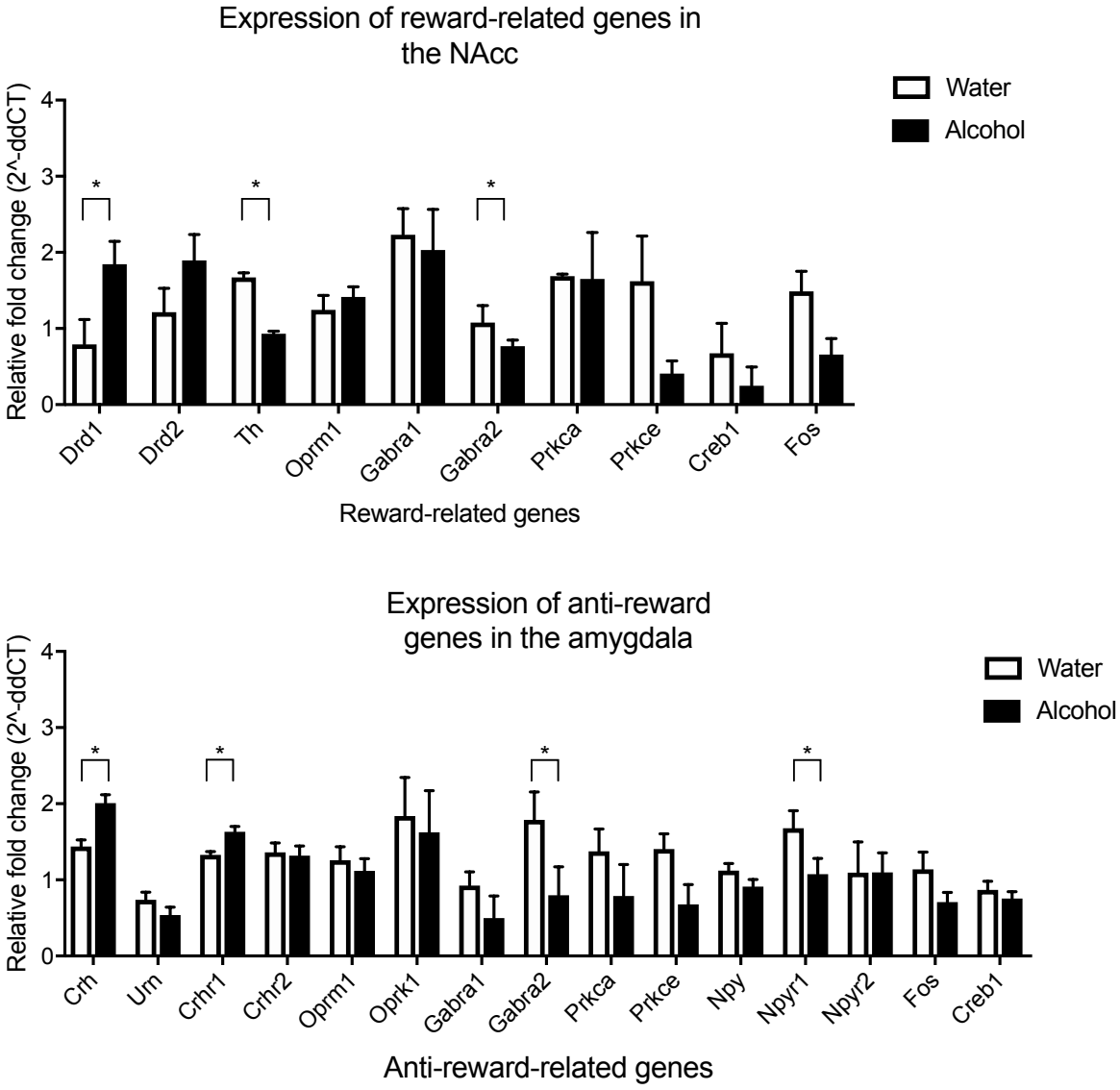

Figure 5 Long-term exposure to alcohol selectively altered the expression of reward and stress-related genes in the nucleus accumbens (a) and amygdala (b) during withdrawal in non-alcohol preferring mice (Balb/c). The expression of *Drd1* mRNA was increased, and *Gabra2* and *Th* mRNA was decreased in the nucleus accumbens following alcohol exposure. Further, the amygdala exhibited increases in *Crh*, *Crhr1* and *Gabra2* and decreases in *Npyr1* mRNA following alcohol exposure. All data was analysed using a paired two-tail t-test. Summary values represented as mean±SEM; n=4, *$p < 0.05$.

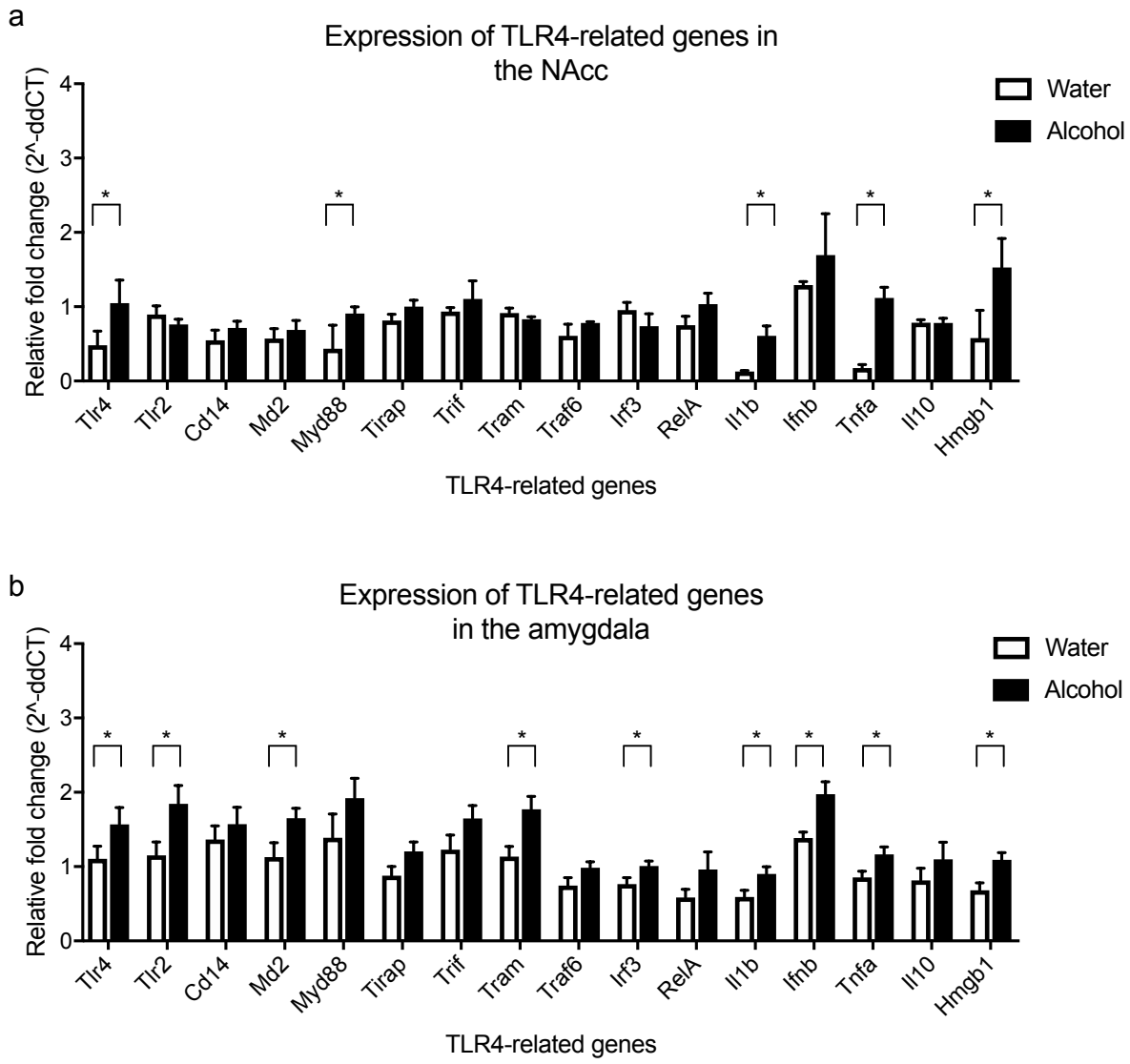

Figure 6 Long-term exposure to alcohol increased the expression of TLR4-related genes in the nucleus accumbens (a) and amygdala (b) during withdrawal in non-alcohol preferring mice (Balb/c). The expression of *Tlr4*, *Myd88, Il1b*, *Tnfa* and *Hmgb1* mRNA were significantly increased nucleus accumbens following alcohol exposure. Similarly, the amygdala exhibited increases in *Tlr4, Tlr2, Md2*, *Tram, Irf3, Il1b, Ifnb*, *Tnfa* and *Hmgb1* mRNA following alcohol exposure. All data was analysed using a paired two-tail t-test. Summary values represented as mean±SEM; n=4, *$p < 0.05$.

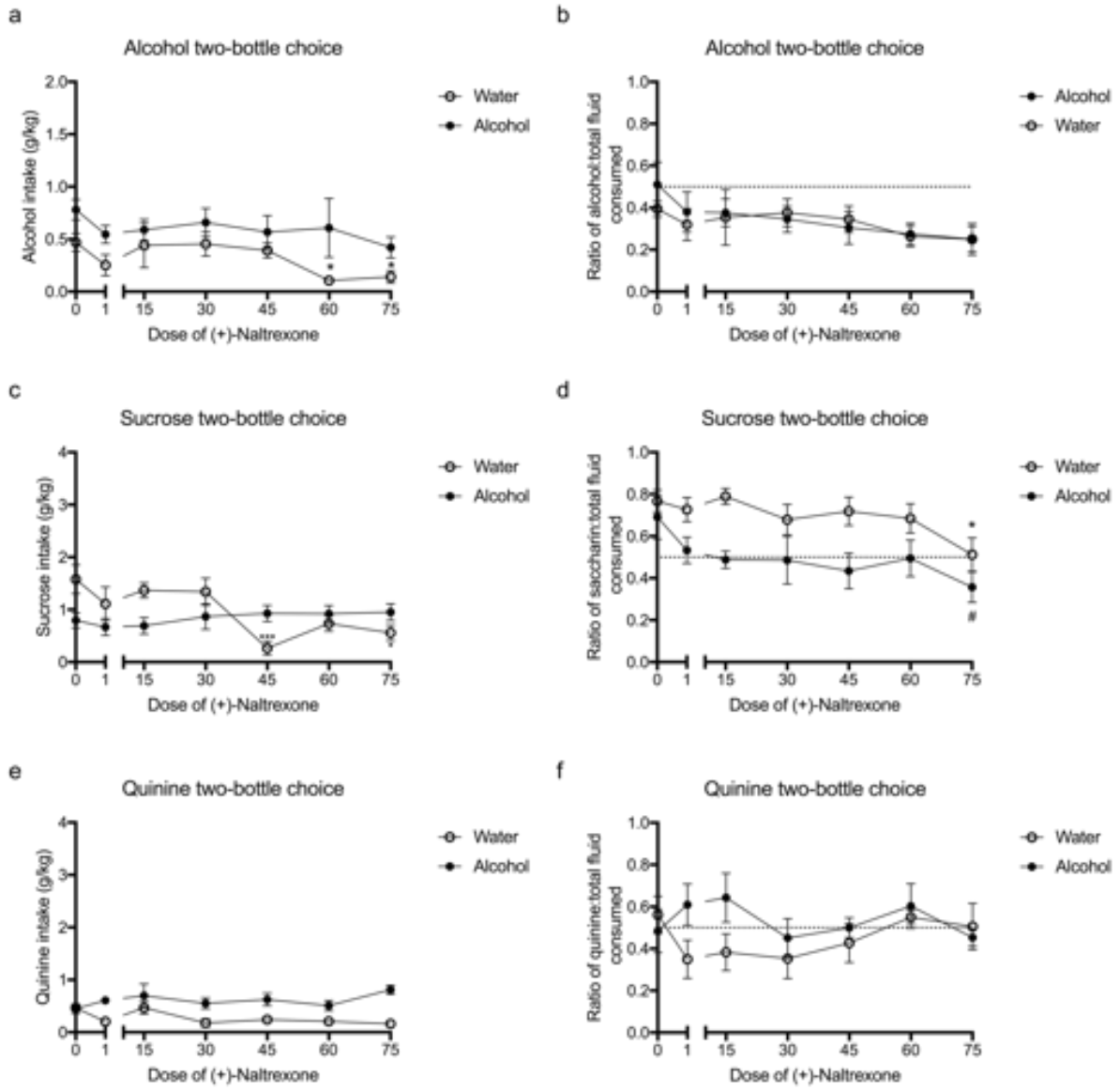

Figure 7 (+)-Naltrexone did not affect the intake and preference for alcohol (a – b), sucrose (c – d) or quinine (e – f) before withdrawal in alcohol-exposed mice. While there was a main effect of (+)-Naltrexone's dose on the intake and preference for alcohol and sucrose post hoc analysis determined this affect was apparent in water-exposed mice only. However, (+)-Naltrexone had no effect on quinine intake and preference. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8; alcohol group #$p < 0.05$ difference compared to the absence of (+)-Naltrexone (x = 0); water group *$p < 0.05$ difference compared to the absence of (+)-Naltrexone (x = 0).

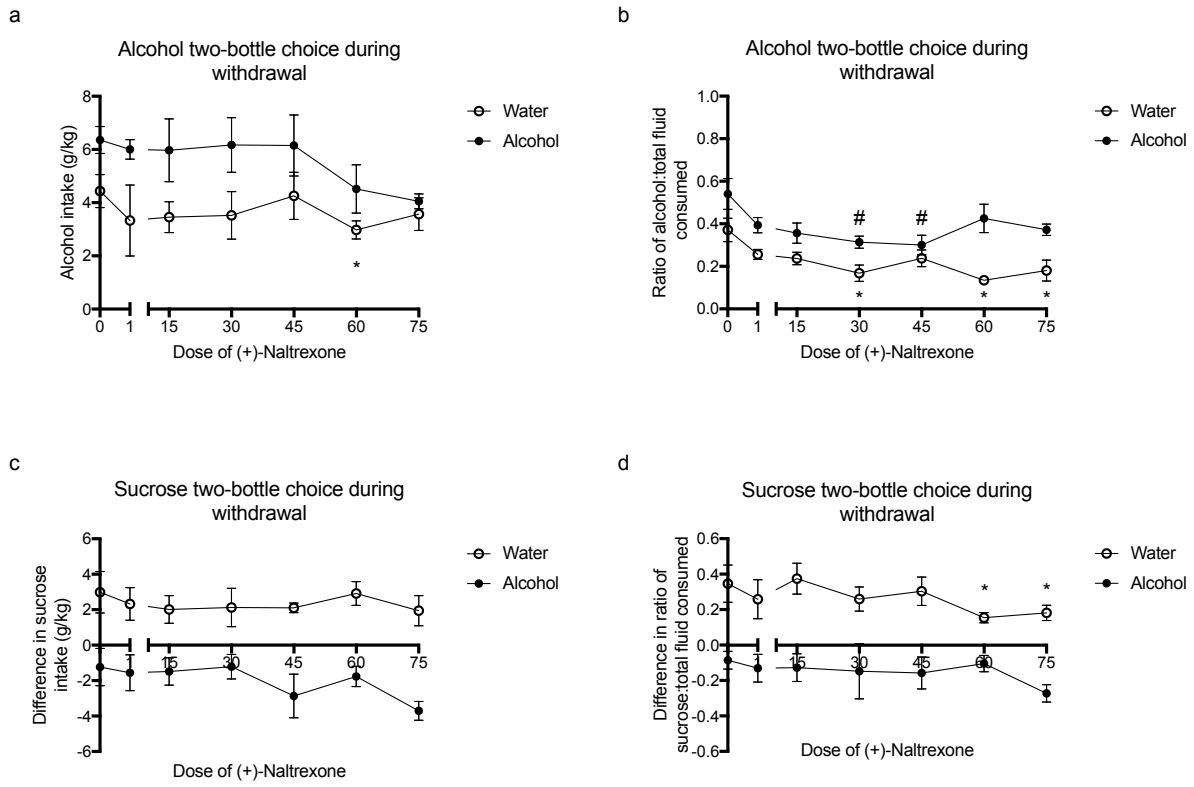

Figure 8 (+)-Naltrexone did not affect the intake and preference for alcohol (a – b), sucrose (c – d) or quinine (e – f) during withdrawal in alcohol-exposed mice. The intake and preference for alcohol and sucrose were not altered by (+)-Naltrexone dose. However, post hoc analysis determined significant reductions in alcohol preference at 30 – 45m/kg in alcohol-exposed and at 30, 60 and 75mg/kg in water-exposed mice. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8; alcohol group #$p < 0.05$ difference compared to the absence of (+)-Naltrexone (x = 0); water group *$p < 0.05$ difference compared to the absence of (+)-Naltrexone (x = 0).

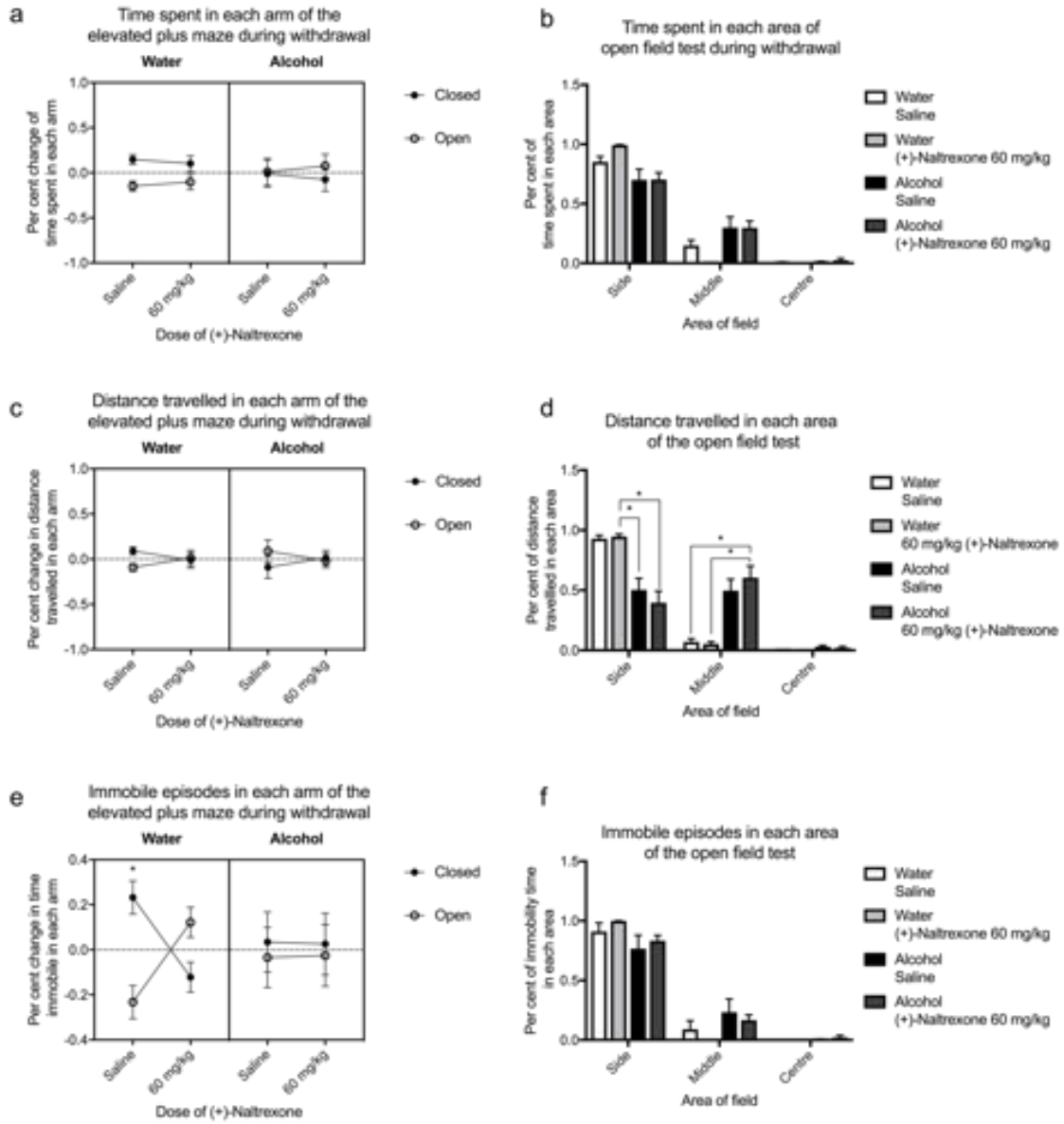

Figure 9 (+)-Naltrexone did not alter the time spent (a – b), distance (c – d) or immobility time (e – f) in the arms or area of the elevated plus maze and open field test respectively during withdrawal. (+)-Naltrexone did not modify the time spent, distance travelled or time immobile in each arm or area of the elevated plus maze or open field test at any dose in alcohol or water exposed mice. All data was analysed using a three-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8; e *p < 0.05 between arms; d *p < 0.05 between groups.

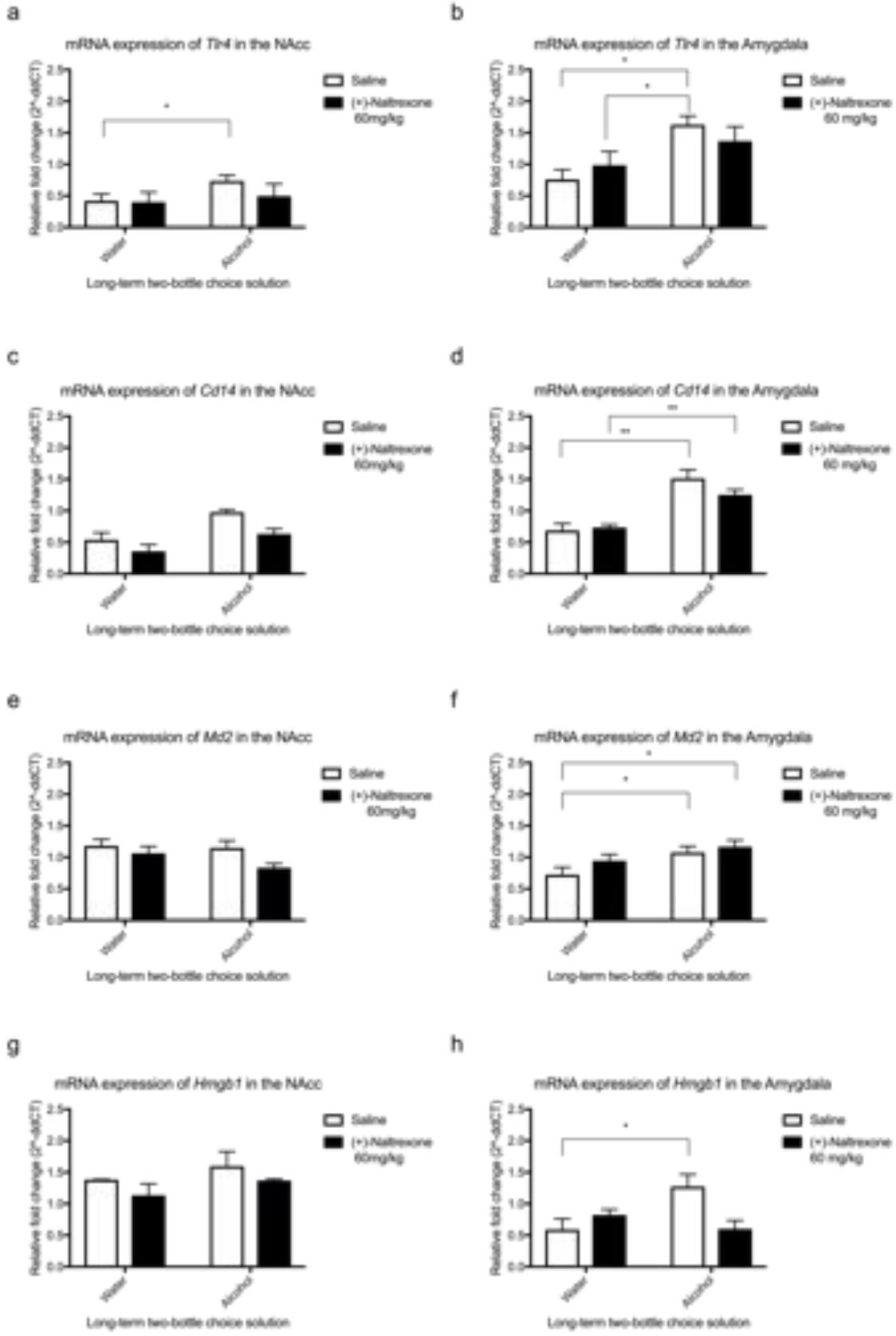

Figure 10 (+)-Naltrexone modified the expression of TLR4, its co-receptors and endogenous agonist in the nucleus accumbens (a, c, e, g) and amygdala (b, d, f, h) during withdrawal. There was a main effect of drug (saline vs (+)-Naltrexone) on the expression of *Tlr4, Cd14* and *Hmgb1* mRNA in nucleus accumbens but not the

amygdala 48 h following the last long-term drinking session. *Md2* mRNA in the amygdala but not nucleus accumbens was significantly modified by drug. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *$p < 0.05$; **$p < 0.01$.

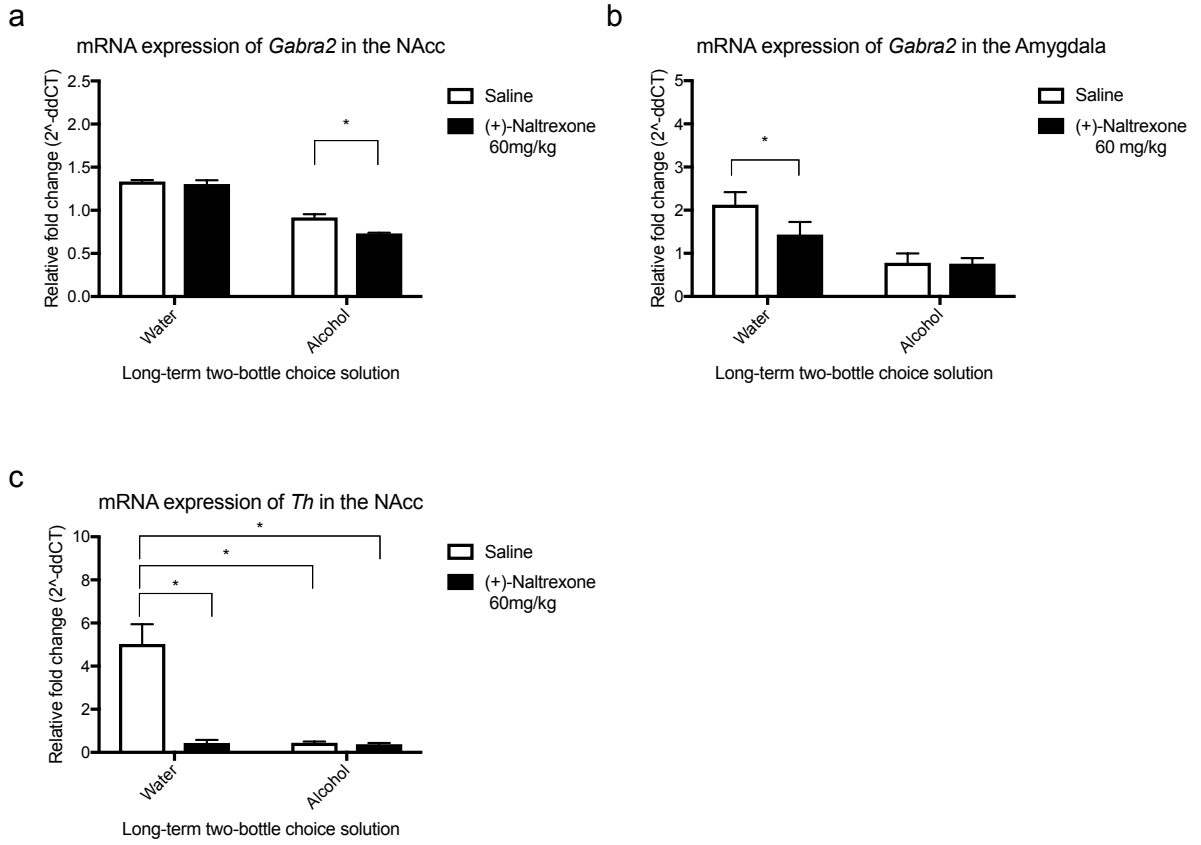

Figure 11 (+)-Naltrexone altered the expression of *Gabra2* (a – b) and *Th* (c) mRNA during withdrawal from alcohol. (+)-Naltrexone augmented the alcohol-induced reduction in *Gabra2 mRNA* expression in the nucleus accumbens (+)-Naltrexone additionally decreased *Gabra2* mRNA expression in water-exposed mice in the amygdala. (+)-Naltrexone reduced the expression of *Gabra2* and *Th* mRNA compared to control mice (saline + water). All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, $^*p < 0.05$.

7.5 Discussion

Long-term alcohol use results in numerous neuroadaptations to reward, stress and immune pathways within the brain. These adaptions result in tolerance to the rewarding effects of alcohol and anhedonia and anxiety in the absence of alcohol. The role of the neuroimmune system, specifically TLR4 in mediating these behaviours are becoming increasingly appreciated. Therefore, this study sought to determine the impact of TLR4 on the manifestation of tolerance, anhedonia and anxiety in Balb/c mice. The current study demonstrates that Balb/c, a non-alcohol preferring strain of mouse, can exhibit behaviours consistent with symptoms of alcohol dependence including tolerance, anhedonia, the motivation to consume alcohol and some aspects of anxiety during withdrawal. These effects were inferred by increased alcohol preference and immobility time, decreased sucrose preference immediately following and 48 h after the last drinking session. These effects coincided with alterations to a select number of reward-, stress- and immune-related genes. This study further indicated that acute attenuation of the TLR4-TRIF pathway (by administration of (+)-Naltrexone) had limited efficacy reducing alcohol preference, immobility time and sucrose deficits. Given that $TLR4^{-/-}$ mice are protected against tolerance, anhedonia and anxiety (Montesinos et al., 2016b; Pascual et al., 2011) it suggests either the MyD88 or both TLR4 pathways mediate these behaviours following long-term alcohol use.

The published alcohol research literature predominately uses rodents genetically predisposed to consume high levels of alcohol and exhibit exaggerated symptoms during withdrawal. Using these strains to elucidate and infer the genetic basis of dependence is extremely useful (McBride & Li, 1998). However, given the diverse nature of alcohol dependence, it is important to consider whether, and how other strains of rodents can exhibit features of dependence without necessarily having an

inherent genetic bias. This may assist in isolating common pathways causing dependence or identify a different a subgroup of individuals with dependence. Consequently, the first aim of this study was to characterise the effects of long-term alcohol exposure on reward and anxiety behaviours in non-alcohol preferring mice. Results presented in this study indicate that following long-term alcohol use, Balb/c mice increase alcohol intake. This potentially suggests the formation of tolerance towards alcohol; as more alcohol is required to achieve the desired pharmacological effects. An alternative interpretation is that Balb/c mice “like” alcohol more and thus will consume more when tested. However, the reduced intake of sucrose, an innately likable solution supports the former conclusion. Further, these effects were unlikely due to altered sense of taste as quinine preference was unchanged. This exposure paradigm additionally elicited anhedonic behaviour during withdrawal as inferred by a reduction in sucrose intake and escalated alcohol intake when re-tested. Adulterating alcohol to make it more aversive using quinine failed to reduce the amount consumed indicating that the motivation to consume alcohol is increased regardless of the taste. Importantly, these behaviours are consistent with alcohol-preferring mice, indicating commonalities between the different strains (Mayfield et al., 2016; McBride & Li, 1998). It is likely the increased to desire to consume alcohol observed in this study, stemmed from alterations to reward-related genes with a decreased and increased expression of tyrosine hydroxylase and dopamine D1 receptors respectively. This suggests, dopamine production is reduced despite the system being in a dopamine sensitive state. Further, the downregulation of *Gabra2* mRNA suggest the system lacks inhibitory control conferred by GABA, increasing the likelihood of engaging in impulsive behaviours. There were no alterations to the μ opioid receptor gene, *Oprm1*, suggesting the induction of tolerance may not be reflected by the genes examined or alterations may be occurring at the translation or post-translation stage. Lastly, there

were no alterations to genes regulating plasticity indicating that unlike alcohol-preferring mice, Balb/c mice are potentially more resilient to the effects of alcohol.

By contrast, there was little effect of alcohol on anxiety-like behaviour as inferred by performance in the elevated plus maze and open field test despite an up regulation of genes associated with stress (CRF) and down-regulation of genes associated anti-stress (NPY). Again, the behavioural and molecular responses towards alcohol were limited compared to alcohol-preferring mice. However, it is unclear whether the lack of effects observed with Balb/c mice is because they are resilient to the effects of alcohol or because they (likely) consumed less alcohol than alcohol preferring mice thus reducing the potential neuroadaptations occurring in response to long-term alcohol intake. Further, the lack of effect of alcohol may be attributable to the genetic background of Balb/c mice which renders them particularly “sensitive” to anxiety (Carola et al., 2002). Despite anxiety being a crucial personality trait associated with developing dependence (Smith & Randall, 2012), it suggests that non-alcohol preferring mice can exhibit some signs of dependence with the greatest effects observed in reward-related behaviours.

Given the emerging role of the neuroimmune system in mediating reward and anxiety following long-term alcohol use, the expression of TLR4 was examined (Pascual et al., 2011). TLR4 was selected as it is a unique immune receptor capable of integrating signals from stress hormones and danger signals to generate immune responses, both of which are present during and following long-term alcohol use (Gárate et al., 2013; Yu et al., 2006). Further, TLR4 regulates: alcohol-induced cell death which propagates neuroinflammation; the expression of stress hormones which sensitise withdrawal behaviours; and epigenetic events which may contribute to the enduring behavioural deficits such as anxiety and reward following chronic alcohol exposure (Montesinos et

al., 2016b; Alfonso-Loeches et al., 2012; 2010; Pascual et al., 2011). These processes interact with reward and anti-reward behaviours. For example, epigenetic process may contribute to the persistent upregulation of cytokines and chemokines in the absence of an overt TLR4 signal. These cytokines regulate the stress response system which in turn, sensitise mice to alcohol withdrawal and anxiety-like behaviour (Breese et al., 2007). Given TLR4s importance to many of the processes following long-term alcohol use, it was hypothesised that acutely blocking TLR4 may reduce symptoms of withdrawal, such as anhedonia and anxiety. However, it is unclear whether this is driven by MyD88 or TRIF pathways.

Results from this study suggest long-term alcohol use increased the mRNA pertaining to aspects of the MyD88 and TRIF signalling pathways (*Il1b*,*Tnfa* and *Ifnb*) a finding largely in accordance with studies examining alcohol-preferring mice (Pascual et al., 2016; 2015). Interestingly, unlike genes relating to reward and stress which exhibited limited alterations following alcohol exposure, almost all aspects of the TLR4 pathway were upregulated in the amygdala including co-receptors, signalling proteins and cytokines. The difference in TLR4 response between the amygdala and the nucleus accumbens is potentially attributable to the number of glial cells, the primary immunocompetent cell within the CNS, innervating each area (Grabert et al., 2016; Doorn et al., 2015). These findings contrast studies by other researchers (He & Crews, 2008; Whitman et al., 2013) who demonstrate that the amygdala is relatively resilient towards the immunological effects of alcohol. Differences in the long-term drinking paradigm, the time tissue was collected post exposure and rodent species used may underscore the differing results.

The TLR4-TRIF pathway was examined as aspects of this pathway are associated with dependence in humans and attenuation of this pathway blocked heroin (a TLR4

agonist) craving and relapse (Johnson et al., 2015; Theberge et al., 2013). (+)-Naltrexone, a biased TLR4-TRIF antagonist (Wang et al., 2016), was used to investigate the effects of this pathway on alcohol-induced behaviours. (+)-Naltrexone failed to alleviate the behavioural manifestations of tolerance, anhedonia and craving for alcohol as inferred by the two-bottle choice test results. Specifically, (+)-Naltrexone did not alter alcohol intake and preference before and during withdrawal suggesting it cannot modify tolerance or wanting of alcohol in mice exposed to alcohol. This finding parallels that of Harris et al., (2017) who demonstrated (+)-Naloxone, a chemically similar compound, did not alter alcohol intake following prior alcohol exposure. Furthermore, (+)-Naltrexone increased the sucrose deficit indicating a worsened symptom of anhedonia. This effect is consistent with previous findings demonstrating siRNA knock down of TLR4 reduces the expression of tyrosine hydroxylase – a key enzyme underlying dopaminergic transmission (Aurelian et al., 2016).

Furthermore, (+)-Naltrexone failed to modify the performance in the elevated plus maze or open field test indicating it was unable to modify anxiety-like behaviour during withdrawal. This finding contrasts previous studies suggesting TLR4$^{-/-}$ mice are protected against withdrawal induced cytokine increases and anxiety behaviour following long-term alcohol exposure (Montesinos et al., 2016b; Pascual et al., 2011). Given TLR4$^{-/-}$ mice are protected it suggests that both the MyD88 and TRIF signalling pathways are required for molecular and behavioural alterations following long-term alcohol use.

To determine why (+)-Naltrexone had limited efficacy on reward- and anxiety-like behaviour, genes relating to the TLR4, reward and stress pathways were assessed. (+)-Naltrexone reduced alcohol-induced alterations to *Gabra2* mRNA in the nucleus accumbens and the amygdala. No other gene reported a significant effect of both

alcohol and (+)-Naltrexone. While GABA receptors are associated with alcohol dependence (Edenberg et al., 2004; Soyka et al., 2008), it is unclear whether the reduction in *Gabra2* mRNA would be beneficial following long-term alcohol use as this period is typically characterised by a lack of inhibitory control.

The limited efficacy of (+)-Naltrexone is unlikely attributable to an altered metabolism of alcohol, as blood alcohol concentrations were similar between saline and (+)-Naltrexone-treated mice following a bolus dose of alcohol. However, the pharmacokinetics of (+)-Naltrexone may underscore its limited efficacy. In mice, (+)-Naltrexone has a short half-life and is metabolised to Naltrexone-glucuronide, a potential agonist at TLR4 given its glucuronide moiety (Lewis et al., 2013). Therefore, it is unclear whether (+)-Naltrexone could attenuate a TLR4 signal following long-term use of alcohol, which is characterised by high levels of circulating cytokines and endogenous danger signals. In addition, long-term alcohol use engages multiple immune pathways (Crews et al., 2017). For example, HMGB1 signals via TLR2, 4 and 5 leading to activation of many immune pathways and processes (Das et al., 2016; Yu et al., 2006). Therefore, targeting one component of the innate immune system is unlikely to attenuate the global neuroimmune response. Future experiments should increase the duration and frequency of (+)-Naltrexone and use broad glial targeted anti-inflammatory agents to determine whether the immune system is of functional importance in the presentation of anxiety, anhedonia and tolerance following long-term alcohol exposure.

The results suggest that non-alcohol preferring mice can exhibit signs of dependence including tolerance towards alcohol; increased motivation to consume alcohol during withdrawal; anhedonia; and some but not all aspects of anxiety-like behaviour. This effect is potentially attributable to the altered expression of genes relating to reward,

stress and TLR4 pathways. However, attenuating the TLR4-TRIF pathway via (+)-Naltrexone did not modify any aspect of reward or anxiety before and during withdrawal. Further, there was no alteration at a gene level suggesting acute blockade of TLR4 during withdrawal may have limited efficacy. Since $TLR4^{-/-}$ mice are protected against the effects of acute and prolonged withdrawal it raises the possibility MyD88 or both TLR4 pathways are required for reward and anxiety behaviours following long-term alcohol use.

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7.8 Supplementary material

Table 1 Primer sequence used in qPCR

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Cd14 – Cluster of differentiation 14 antigen	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT
Creb1 – cAMP responsive element binding protein 1 transcript variant 1, 2, 3, 4, 6, 7, 8	ATCTGGAGCAGACAACCAGC	TGAGCTGCTGGCATGGATAC
Crh – Corticotrophin releasing hormone	TCTGATCCGCATGGGTGAAG	AGCAACACGCGGAAAAAGTT
Crhr1 - Corticotrophin releasing hormone receptor 1	TGCCAGGAGATTCTCAACGAA	AAAGCCGAGATGAGGTTCCAG
Crhr2 - Corticotrophin releasing hormone receptor 2	TACCGAATCGCCCTCATTGT	CCACGCGATGTTTCTCAGAAT
Drd1 – Dopamine receptor D1 transcript variant 1	GTTGAGTCCAGGGGTTTTGGG	ACTTTTCGGGGATGCTGCC
Drd2 – Dopamine receptor D2	GTGAACAGGCGGAGAATGGA	TGGGAGGGATGGGGCTATAC
Gabra1 – Gamma aminobutyric acid A receptor, subunit alpha 1	CCTGCTTCCTAGCTTGCGTT	AACCGATCCTTGTAACTCTGCT

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Gabra2 – Gamma aminobutyric acid A receptor, subunit alpha 2 variant X1	GCAGCAGAGACCATACATTGC	GCAGCAGAGACCATACATTGC
Gapdh – Glyceraldehyde-3-phosphate dehydrogenase transcript variant 1	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Gria1 – Glutamate receptor, ionotropic, AMPA1 (alpha 1), transcript variant 1	AGGGGAATGTGGAAGCAAGG	CCAGCCTCCAATCAGGATG
Grin1 – Glutamate receptor, ionotropic, NMDA1 (zeta 1), transcript variant 1, 2, 3	CCTATGACAAGCGCGGA	AGCAGAGCCGTCACATTCTT
Hmgb1 – High mobility group box 1, transcript variant 1	CCATTGGTGATGTTGCAAAG	CTTTTTCGCTGCATCAGGTT
Ifnb – Interferon beta 1, fibroblast	TGGGAGATGTCCTCAACTGC	CCAGGCGTAGCTGTTGTACT
Il1b – Interleukin 1 beta	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Il10 – Interleukin 10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Md2 – Lymphocyte antigen 96 transcript variant 1, 2	CGCTGCTTTCTCCCATATTGA	CCTCAGTCTTATGCAGGGTTCA
Myd88 – Myeloid differentiation primary response gene 88	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCA

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
Npy – Neuropeptide Y	TGGACTGACCCTCGCTCTAT	TAGTGTCGCAGAGCGGAGTA
Npyr1 – Neuropeptide Y receptor 1	GAATGAAGTGGCAAAACAGGTCC	AGCTCAGCACTCTGATGAACA
Npyr2 – Neuropeptide Y receptor 2	CGCAAGAGTCAATACAGCCAAG	GATCAGGGGGCAACTCACC
Oprm1 – Opioid receptor, mu 1, transcript variant 1C, 1M, 1U	TCCGACTCATGTTGAAAAACCC	CCTTCCCCGGATTCCTGTCT
Oprk1 – Opioid receptor kappa transcript variant 1	TCCCCAACTGGGCAGAATC	GACAGCGGTGATGATAACAGG
Th – Tyrosine hydroxylase	CCTTCCGTGTGTTTCAGTGC	TCAGCCAACATGGGTACGTG
Tlr4 – Toll-like receptor 4	GCCTTTCAGGGAATTAAGCTCC	GATCAACCGATGGACGTGTAAA
Tram – TRIF-related adaptor molecule	CGATCAAGACGGCCATGAGTC	CTCGTCGGTGTCATCTTCTGC
Tirap – Toll-interleukin 1 receptor domain-containing adaptor protein variant 1	CTCATTTTCCCCAACCGTGC	CTCGGGATCTGTGTTTCGGT
Trif – Toll-like receptor adaptor molecule 1	AACCTCCACATCCCCTGTTTT	GCCCTGGCATGGATAACCA
Urn – Urocortin transcript variant 1	ATCCGAATCTGCGATGGAGC	GTGAGGTCGATGGACAGTGG

7.8.1 Statistics for in-text figures

Figure 4 Long-term exposure to alcohol did not alter time spent (a – b) or distance (c – d) but increased immobility time (e – f) in the elevated plus maze and open field test) in non-alcohol preferring mice (Balb/c).

(a) *Elevated plus maze time*

Effect of arm, $F_{(1, 7)} = 2.5$, $p = 0.17$

Effect of solution, $F_{(1, 7)} = 5.56$, $p = 0.065$

Interaction (arm x solution), $F_{(1, 7)} = 5.72$, $p = 0.062$

(b) *Open field test time*

Effect of area, $F_{(2, 14)} = 24.52$, $p < 0.0001$

Effect of solution, $F_{(1, 7)} = 0.87$, $p = 0.38$

Interaction (arm x solution), $F_{(2, 14)} = 1.44$, $p = 0.27$

(c) *Elevated plus maze distance*

Effect of arm, $F_{(1, 7)} = 5.0$, $p = 0.075$

Effect of solution, $F_{(1, 7)} = 2.08$, $p = 0.21$

Interaction (arm x solution), $F_{(1, 7)} = 2.53$, $p = 0.17$

(d) *Open field test distance*

Effect of area, $F_{(2, 14)} = 18.11$, $p < 0.0001$

Effect of solution, $F_{(1, 7)} = 0.24$, $p = 0.64$

Interaction (arm x solution), $F_{(2, 14)} = 7.32$, $p = 0.0015$

(e) *Elevated plus maze time immobile*

Effect of arm, $F_{(1, 7)} = 4.89$, $p = 0.078$

Effect of solution, $F_{(1, 7)} = 2.83$, $p = 0.15$

Interaction (arm x solution), $F_{(1, 6)} = 0.28$, $p = 0.62$

(f) *Open field test immobile time*

Effect of area, $F_{(2, 14)} = 39.62$, $p < 0.0001$

Effect of solution, $F_{(1, 7)} = 10.4$, $p = 0.0146$

Interaction (arm x solution), $F_{(2, 14)} = 6.29$, $p = 0.0033$

Figure 5 Long-term exposure to alcohol selectively altered the expression of reward and stress-related genes in the nucleus accumbens (a) and amygdala (b) during withdrawal in non-alcohol preferring mice (Balb/c).

Nucleus accumbens (a)

Drd2, t = 0.41 df = 4, $p = 0.71$

Oprm1, t = 0.48 df = 4, $p = 0.66$

Gabra1, t = 0.86 df = 4, $p = 0.45$

Prkca, t = 0.93 df = 4, $p = 0.40$

Prkce, t = 1.42 df = 3, $p = 0.25$

Fos, t = 2.46 df = 4, $p = 0.07$

Creb1, t = 0.92 df = 4, $p = 0.41$

Amygdala (b)

Ucn, t = 1.39 d f= 4, $p = 0.18$

C*rhr2*, t = 0.24 df = 4, $p = 0.82$

Oprm1, t = 0.58 df = 4, $p = 0.57$

Okr1, t = 0.29 df = 4, *p* = 0.77

Npy, t = 1.60 df= 4, *p* = 0.13

Npyr1, t = 0.81 df= 4, *p* = 0.43

Npyr2, t = 0.0038 df= 4, *p* = 0.99

Gabra1, t = 1.90 df = 4, *p* = 0.13

Prkca, t = 1.62 df = 4, *p* = 0.31

Prkce, t = 2.21 df = 4, *p* = 0.092

Fos, t = 1.64 df = 4, *p* = 0.12

C*reb1*, t = 0.79 df= 4, *p* = 0.44

Figure 6 Long-term exposure to alcohol increased the expression of TLR4-related genes in the nucleus accumbens (a) and amygdala (b) during withdrawal in non-alcohol preferring mice (Balb/c).

Nucleus accumbens (a)

Tlr2, t = 0.98 df = 4, *p* = 0.38

Cd14, t = 1.0 df = 4, *p* = 0.37

Md2, t = 1.0 df = 4, *p* = 0.37

Tirap, t = 0.56 df = 4, *p* = 0.60

Tram, t = 1.09 df = 4, *p* = 0.34

Irf3, t = 1.11 df = 4, *p* = 0.33

Rela, t = 0.31 df = 4, *p* = 0.77

Ifnb, t = 0.39 df = 4, *p* = 0.72

Il10, t = 0.081 df = 4, *p* = 0.94

Hmgb1, t = 0.10 df = 4, *p* = 0.92

Amygdala (b)

Cd14, t = 0.70 df = 16, p = 0.49

Myd88, t = 1.27 df = 16, p = 0.22

Tirap, t = 1.87 df = 16, p = 0.08

Trif, t = 1.59 df =16. p = 0.13

Rela, t = 1.43 df = 16, p = 0.17

Il10, t = 1.01 df = 16, p = 0.33

Figure 7 (+)-Naltrexone did not affect the intake and preference for alcohol (a – b), sucrose (c – d) or quinine (e – f) before withdrawal in alcohol-exposed mice.

(a) *Alcohol intake*

Effect of solution, $F_{(1, 7)}$ = 2.88, p = 0.02

Effect of dose, $F_{(6, 42)}$ = 9.90, p = 0.021

Interaction (dose x solution), $F_{(6, 42)}$ = 5.44, p = 0.0004

(b) *Alcohol preference*

Effect of solution, $F_{(1, 7)}$ = 1.46, p = 0.22

Effect of dose, $F_{(6, 42)}$ = 10.26, p = 0.019

Interaction (dose x solution), $F_{(6, 42)}$ = 1.04, p = 0.42

(c) *Sucrose intake*

Effect of solution, $F_{(1, 7)}$ = 2.88, p = 0.02

Effect of dose, $F_{(6, 42)}$ = 9.90, p = 0.021

Interaction (dose x solution), $F_{(6, 42)}$ = 5.44, p = 0.0004

(d) *Sucrose preference*

Effect of solution, $F_{(1, 7)} = 1.46$, $p = 0.22$

Effect of dose, $F_{(6, 42)} = 10.26$, $p = 0.019$

Interaction (dose x solution), $F_{(6, 42)} = 1.04$, $p = 0.42$

(e) *Quinine intake*

Effect of solution, $F_{(1, 7)} = 306.5$, $p < 0.0001$

Effect of dose, $F_{(6, 42)} = 1.05$, $p = 0.41$

Interaction (dose x solution), $F_{(6, 42)} = 2.92$, $p = 0.02$

(f) *Quinine preference*

Effect of solution, $F_{(1, 7)} = 0.66$, $p = 0.45$

Effect of dose, $F_{(6, 42)} = 2.27$, $p = 0.059$

Interaction (dose x solution), $F_{(6, 42)} = 1.35$ $p = 0.26$

Figure 8 (+)-Naltrexone did not affect the intake and preference for alcohol (a – b), sucrose (c – d) or quinine (e – f) during withdrawal in alcohol-exposed mice.

(a) *Alcohol intake*

Effect of solution, $F_{(1, 7)} = 11.87$, $p = 0.014$

Effect of dose, $F_{(6, 42)} = 0.89$, $p = 0.51$

Interaction (dose x solution), $F_{(7, 42)} = 0.72$, $p = 0.63$

(b) *Alcohol preference*

Effect of solution, $F_{(1, 7)} = 5.38$, $p = 0.0005$

Effect of dose, $F_{(6, 42)} = 42.86$, $p = 0.006$

Interaction (dose x solution), $F_{(6, 42)} = 1.37$, $p = 0.26$

(c) *Sucrose intake*

Effect of solution, $F_{(1, 7)} = 77.25$, $p < 0.0001$

Effect of dose, $F_{(6, 42)} = 0.83$, $p = 0.55$

Interaction (dose x solution), $F_{(6, 42)} = 0.67$, $p = 0.67$

(d) *Sucrose preference*

Effect of solution, $F_{(1, 7)} = 115.1$, $p < 0.0001$

Effect of dose, $F_{(6, 42)} = 0.59$, $p = 0.73$

Interaction (dose x solution), $F_{(6, 42)} = 0.47$, $p = 0.82$

Figure 9 (+)-Naltrexone did not alter the time spent (a – b), distance (c – d) or immobility time (e – f) in the arms or area of the elevated plus maze and open field test respectively during withdrawal.

(a) Elevated plus maze Time

Effect of dose, $F_{(6, 196)} = 0.0026$, $p > 0.99$

Effect of solution, $F_{(1, 196)} = 0.0026$, $p = 0.96$

Effect of arm, $F_{(1, 196)} = 83.24$, $p < 0.0001$

Interaction (dose x solution), $F_{(6, 196)} = 0.0026$, $p > 0.99$

Interaction (dose x arm), $F_{(6, 196)} = 1.52$, $p = 0.17$

Interaction (arm x solution), $F_{(1, 196)} = 12.43$, $p = 0.0005$

Interaction (dose x arm x solution), $F_{(6, 196)} = 1.16$, $p = 0.33$

(b) Open field test time

Effect of test area, $F_{(2, 84)} = 310.7$, $p < 0.0001$

Effect of solution, $F_{(1, 84)} = 0.029$, $p = 0.87$

Effect of treatment, $F_{(1, 84)} = 0.0019$, $p = 0.97$

Interaction (test area x solution), $F_{(2, 84)} = 21.48$, $p < 0.0001$

Interaction (test area x arm), $F_{(2, 84)} = 2.32$, $p = 0.10$

Interaction (solution x treatment), $F_{(6, 196)} = 0.016$, $p = 0.90$

Interaction (test area x solution x treatment), $F_{(6, 196)} = 2.19$, $p = 0.12$

(c) Elevated plus maze Distance

Effect of dose, $F_{(6, 196)} = 0.0020$, $p = 0.98$

Effect of solution, $F_{(1, 196)} = 1.7$, $p = 0.20$

Effect of arm, $F_{(1, 196)} = 16.99$, $p < 0.0001$

Interaction (dose x solution), $F_{(6, 196)} = 0.0020$, $p = 0.98$

Interaction (dose x arm), $F_{(6, 196)} = 4.37$, $p = 0.004$

Interaction (arm x solution), $F_{(1, 196)} = 0.23$, $p = 0.63$

Interaction (dose x arm x solution), $F_{(6, 196)} = 1.64$, $p = 0.13$

(d) Open field test distance

Effect of test area, $F_{(3, 112)} = 27.32$, $p < 0.0001$

Effect of solution, $F_{(1, 112)} = 6.50$, $p = 0.012$

Effect of treatment, $F_{(1, 112)} = 5.06$, $p = 0.027$

Interaction (test area x solution), $F_{(3, 112)} = 9.33$, $p < 0.0001$

Interaction (test area x arm), $F_{(3, 112)} = 0.76$, $p = 0.52$

Interaction (solution x treatment), $F_{(1, 112)} = 0.96$, $p = 0.33$

Interaction (test area x solution x treatment), $F_{(3, 112)} = 0.43$, $p = 0.73$

(e) Elevated plus maze immobility time

Effect of dose, $F_{(6, 196)} = 0.20$, $p = 0.98$

Effect of solution, $F_{(1, 196)} = 1.38$, $p = 0.24$

Effect of arm, $F_{(1, 196)} = 11.82$, $p = 0.0007$

Interaction (dose x solution), $F_{(6, 196)} = 0.20$, $p = 0.98$

Interaction (dose x arm), $F_{(6, 196)} = 4.70$, $p = 0.0002$

Interaction (arm x solution), $F_{(1, 196)} = 7.14$, $p = 0.0083$

Interaction (dose x arm x solution), $F_{(6, 196)} = 5.19$, $p < 0.0001$

(f) Open field test immobility time count

Effect of test area, $F_{(3, 112)} = 82.47$, $p < 0.0001$

Effect of solution, $F_{(1, 112)} = 12.65$ $p = 0.0006$

Effect of treatment, $F_{(1, 112)} = 0.0014$, $p = 0.97$

Interaction (test area x solution), $F_{(3, 112)} = 5.65$, $p = 0.0012$

Interaction (test area x arm), $F_{(3, 112)} = 0.0866$, $p = 0.97$

Interaction (solution x treatment), $F_{(1, 112)} = 0.015$, $p = 0.90$

Interaction (test area x solution x treatment), $F_{(3, 112)} = 0.16$, $p = 0.92$

7.8.2 Supplementary figures

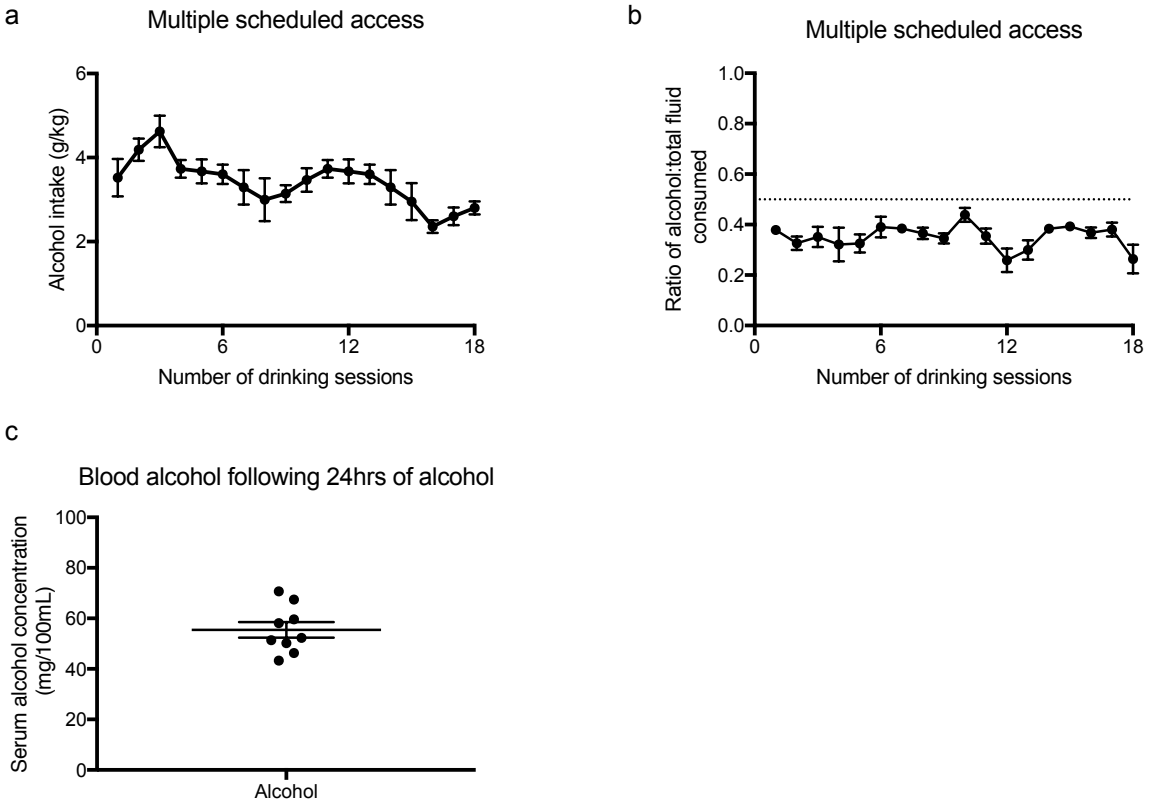


Figure s1 Average alcohol intake (a) and preference (b) for each drinking session during the intermittent alcohol access protocol and the average serum alcohol concentration (c) following the last drinking session in Balb/c mice. Summary values represented as mean±SEM; n=8-20

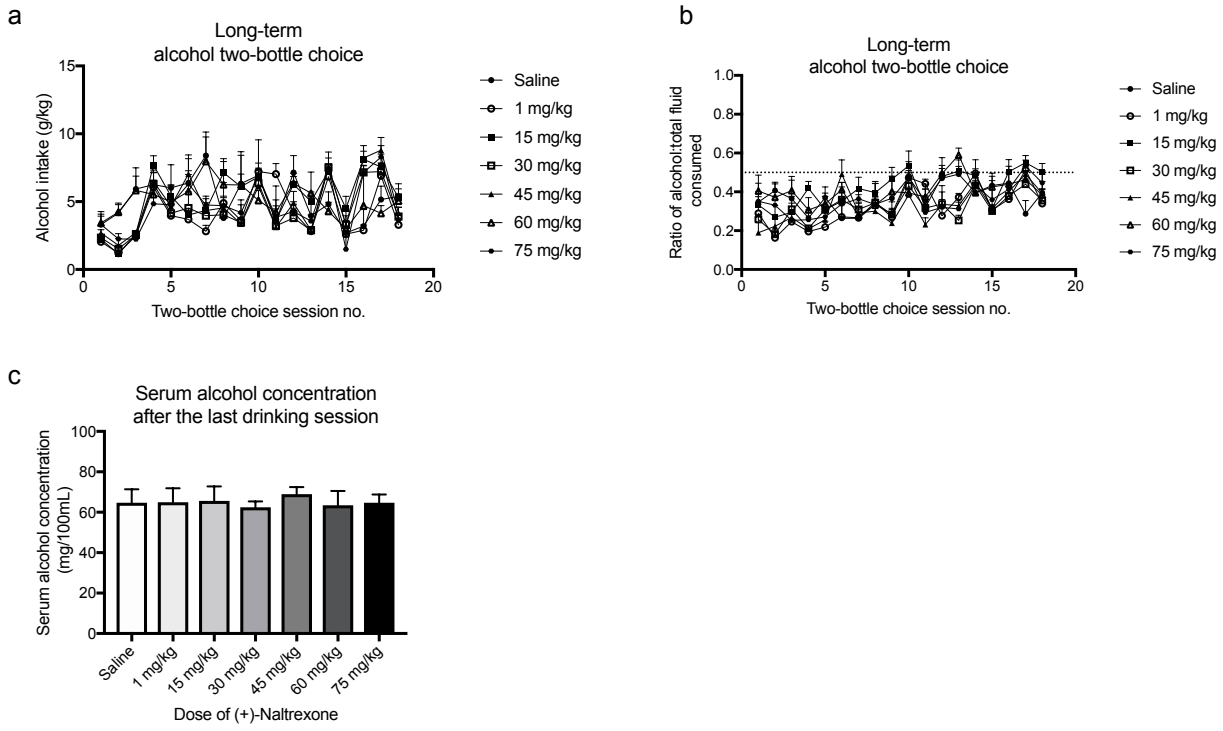


Figure s2 Average alcohol intake (a) and preference (b) for each drinking session during the intermittent alcohol access protocol and the average serum alcohol concentration (c) following the last drinking session in saline or (+)-Naltrexone grouped mice. Mice anticipated to receive saline or (+)-Naltrexone did not exhibit differences in the intake, preference or serum alcoholl concentration following the last drinking session. All data was analysed using a two-way ANOVA with Tukey post hoc (a – b) or one-way ANOVA with Tukey post hoc (c). Summary values represented as mean±SEM; n=16-24.

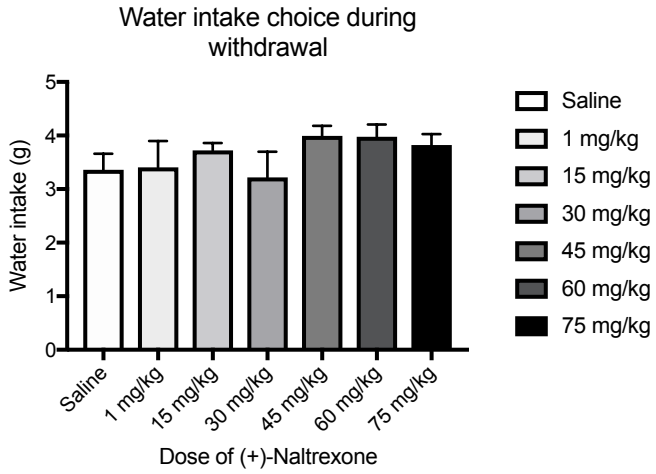


Figure s3 Water intake during withdrawal in saline or (+)-Naltrexone grouped mice. Mice receiving saline or (+)-Naltrexone did not differ in their intake of water during the withdrawal period. All data was analysed using a one-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8.

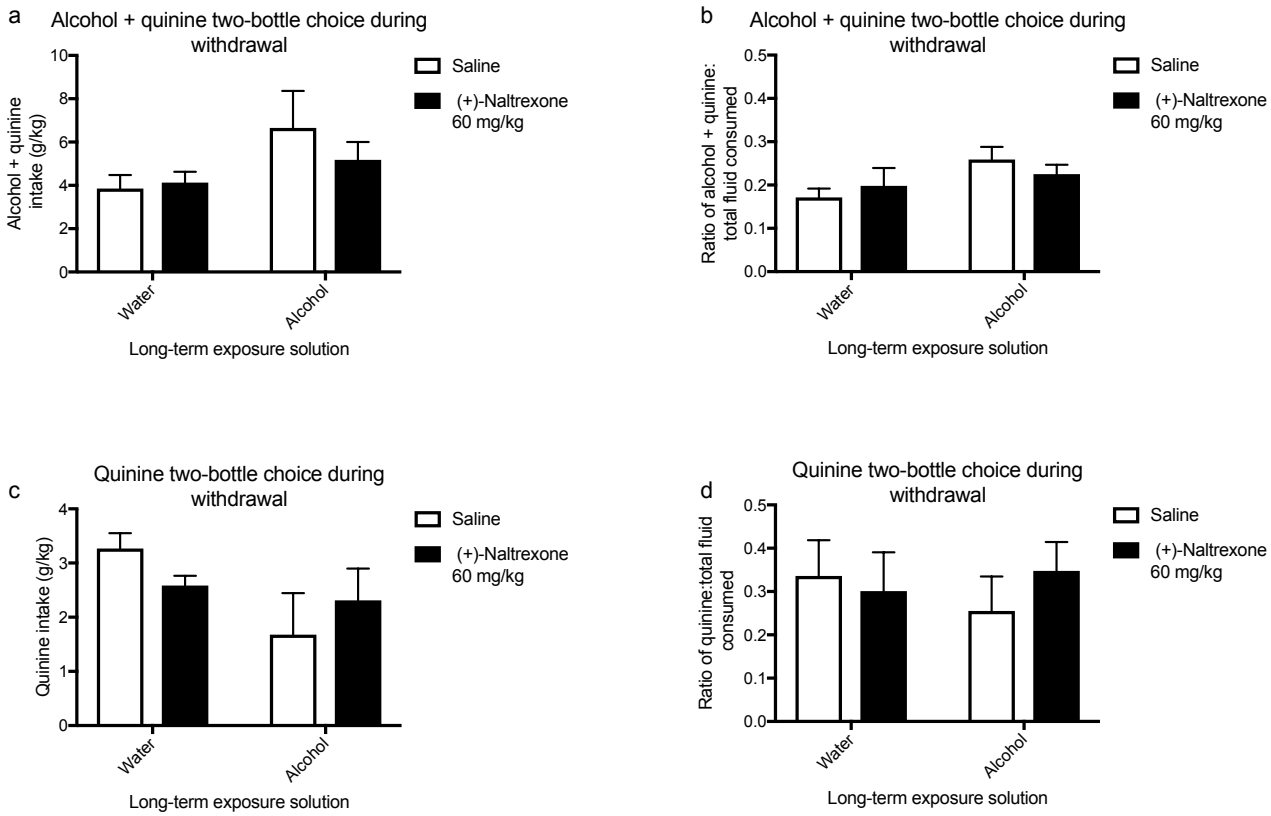
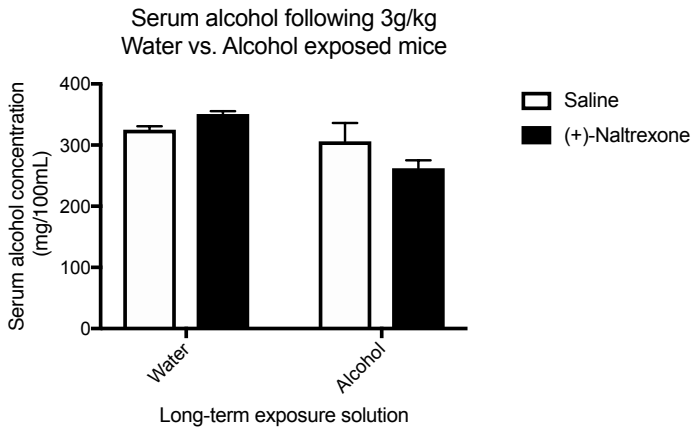


Figure s4 (+)-Naltrexone did not alter the intake and preference of alcohol + quinine (a – b) or quinine (c – d). The intake and preference for alcohol + quinine or quinine was similar between mice treated with saline or (+)-Naltrexone during withdrawal. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=8.

a



b

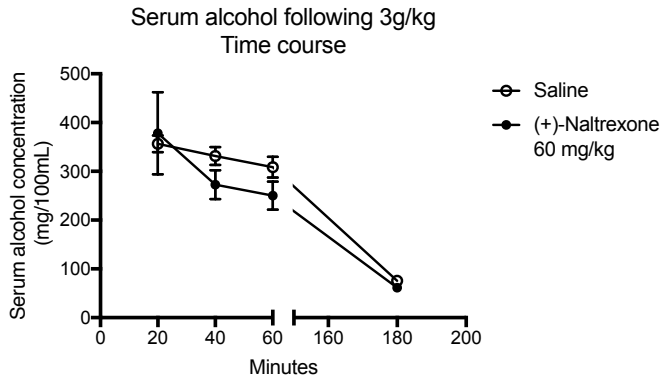


Figure s5 (+)-Naltrexone does not alter the pharmacokinetics of alcohol (a – b). Mice exposed to alcohol long-term exhibited lower serum alcohol concentrations compared water control mice after 60 minutes. However, (+)-Naltrexone did not alter the serum alcohol concentration in water or alcohol-exposed mice (a). Similarly, mice exposed to alcohol long-term did not differ in the rate of alcohol metabolism following injections of saline or (+)-Naltrexone (b). All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=4.

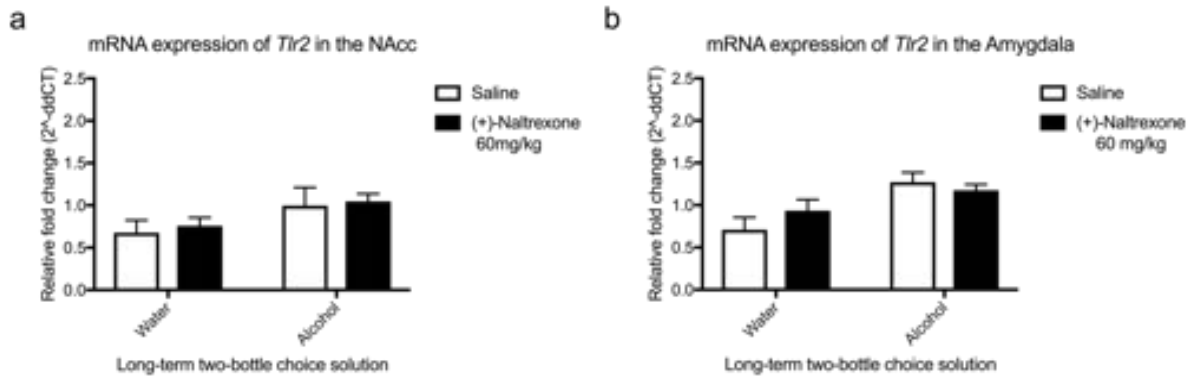


Figure s6 Neither alcohol or (+)-Naltrexone modified the expression of *Tlr2* mRNA in the nucleus accumbens (a) or amygdala (b) following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4.

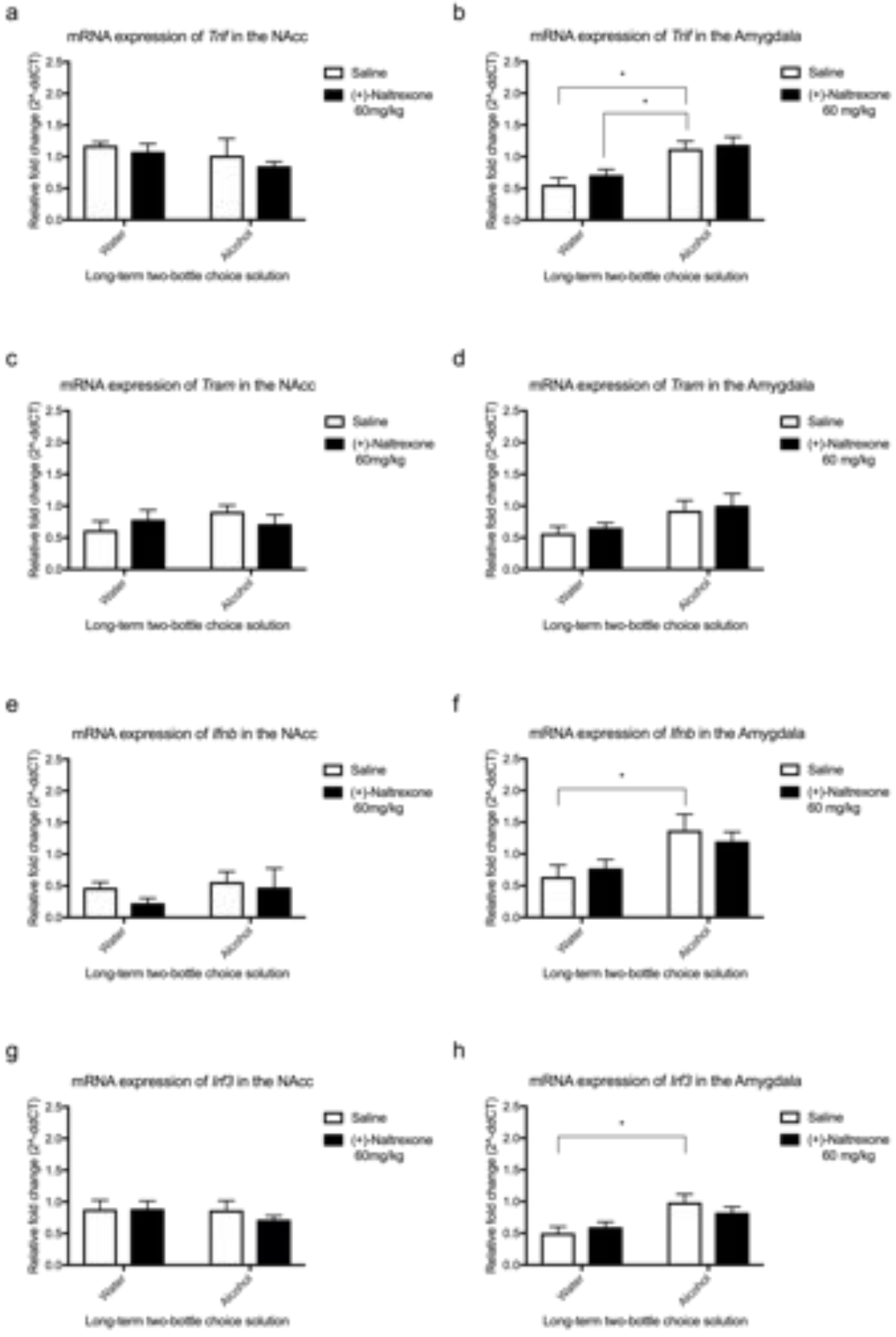
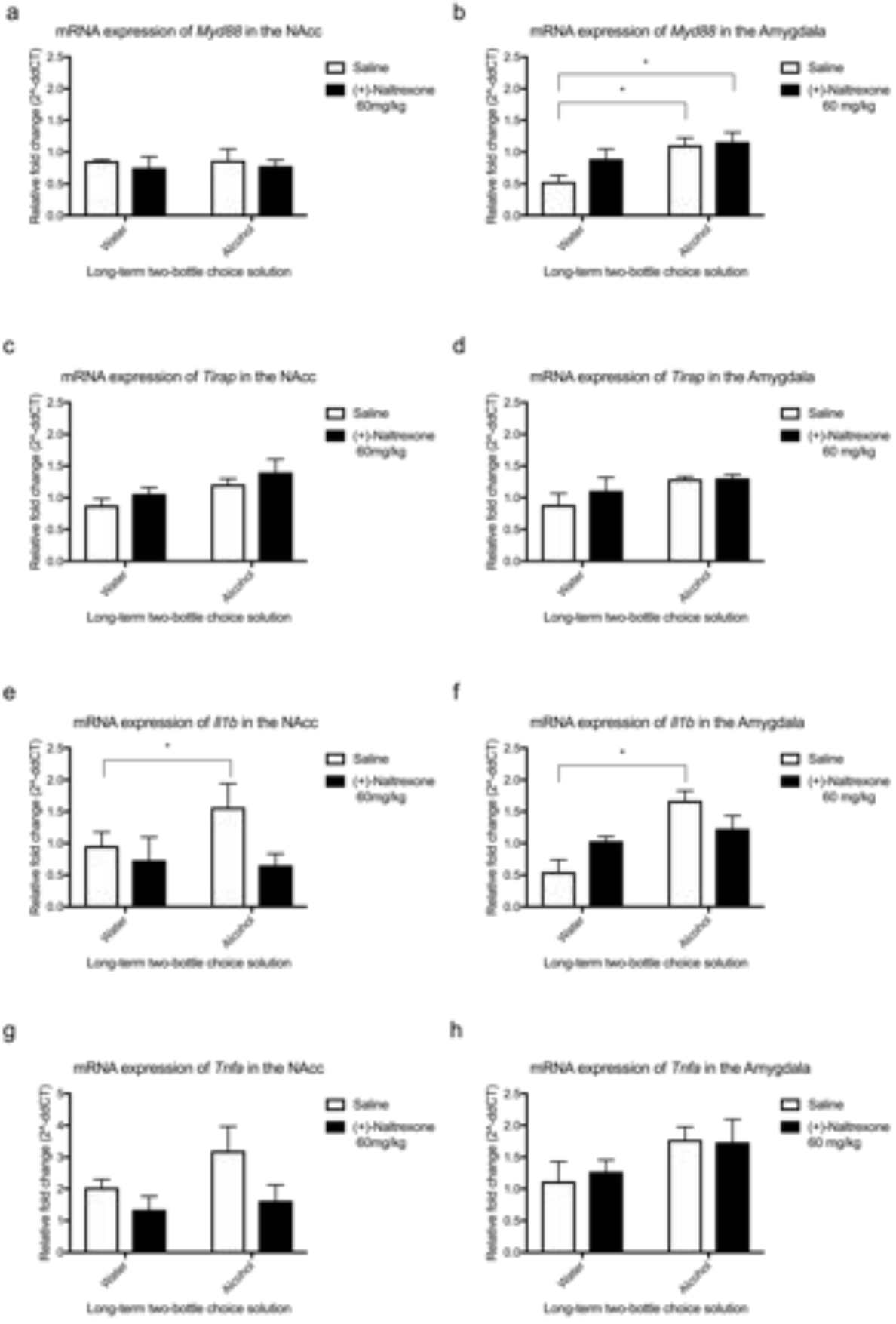


Figure s7 Alcohol but not (+)-Naltrexone modified the expression of TRIF-related mRNA in the amygdala (b, d, f) but not nucleus accumbens (a, c, e) following 48 h of withdrawal. There was a main effect of solution but not drug on the expression

of *Trif*, *Tram*, *Ifnb* and *Irf3* mRNA in the amygdala following withdrawal. Post hoc analysis determined the expression of *Trif*, *Ifnb* and *Irf3* mRNA was increased in the alcohol + saline cohort compared to the water + saline cohort. However, the expression TRIF-related genes were not modified by solution or drug in the nucleus accumbens. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, $*p < 0.05$.


a
mRNA expression of Myd88 in the NAcc
Relative fold change (2^-ddCT)
Saline
(+)-Naltrexone 60mg/kg
Water
Alcohol
Long-term two-bottle choice solution
b
mRNA expression of Myd88 in the Amygdala
Saline
(+)-Naltrexone 60 mg/kg
c
mRNA expression of Tirap in the NAcc
d
mRNA expression of Tirap in the Amygdala
e
mRNA expression of Il1b in the NAcc
f
mRNA expression of Il1b in the Amygdala
g
mRNA expression of Tnfa in the NAcc
h
mRNA expression of Tnfa in the Amygdala

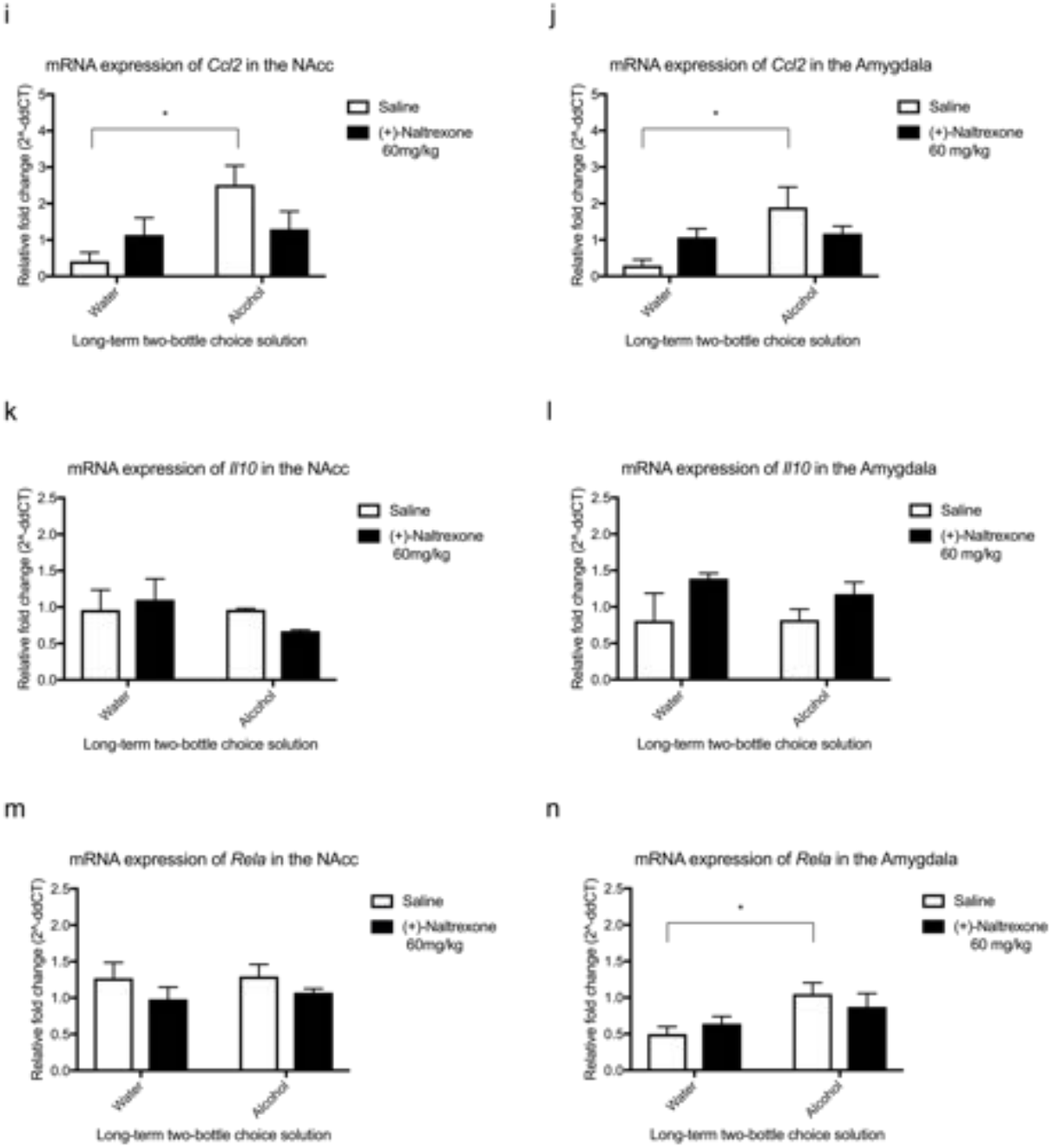


Figure s8 Alcohol but not (+)-Naltrexone modified the expression of MyD88-related mRNA in the amygdala (b, d, f, h, j, l, n) but not nucleus accumbens (a, c, e, g, I, k, m) following 48 h of withdrawal. There was a main effect of solution but not drug on the expression of *Myd88*, *Tirap*, *Il1b*, *Tnfa*, *Ccl2* and *Rela* mRNA in the amygdala following withdrawal. The expression of these genes were not modified by solution or drug in the nucleus accumbens. Post hoc analysis determined the expression of *Myd88* and *Rela* mRNA in the amygdala and *Il1b* and *Ccl2* mRNA in the amygdala and nucleus accumbens was significantly increased in the alcohol + saline

cohort compared to the water + saline cohort. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *$p < 0.05$.

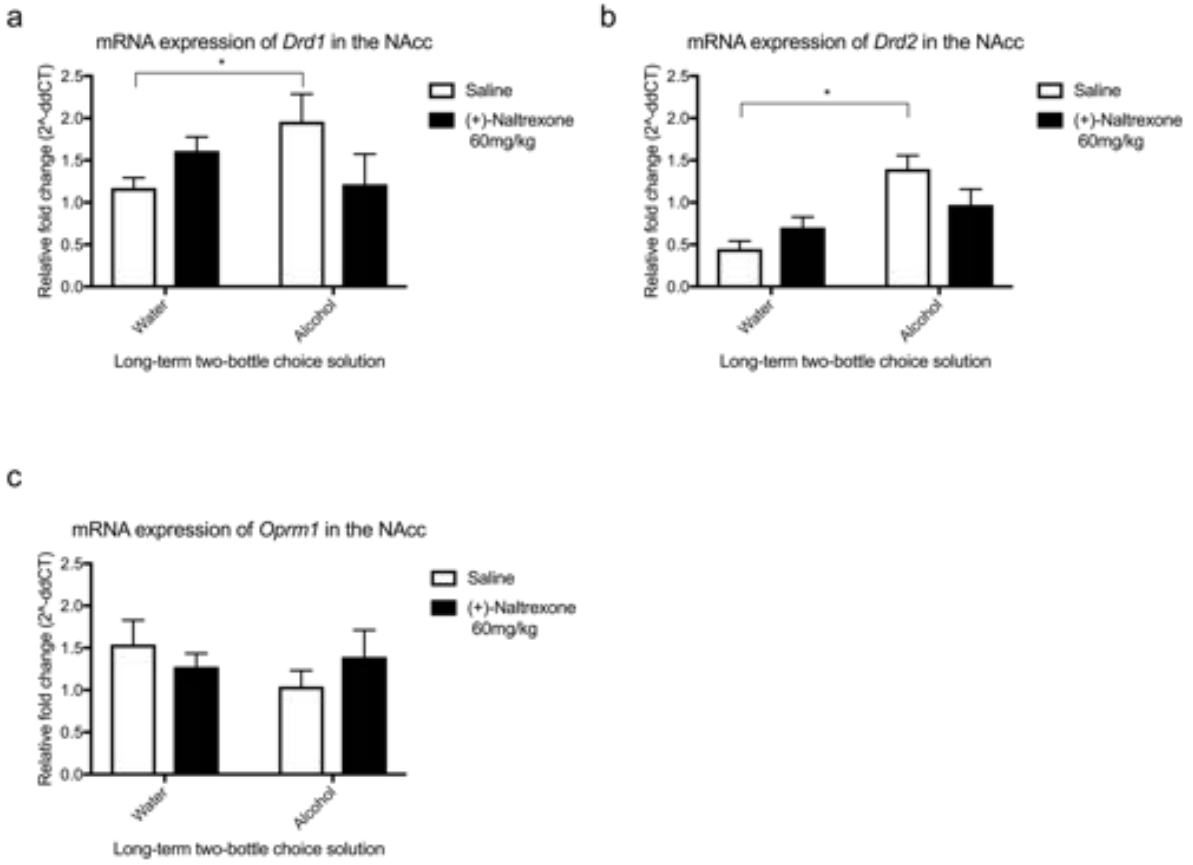


Figure s9 There was no effect of solution or drug on the expression of reward-related genes in the nucleus accumbens following 48 h of withdrawal. There was no main effect of solution or drug on the expression of *Drd1*, *Drd2* and *Opmr1*. However, post hoc analysis determined alcohol + saline mice exhibited increased expression of *Drd1* and *Drd2* mRNA compared to water + saline mice. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *$p < 0.05$.

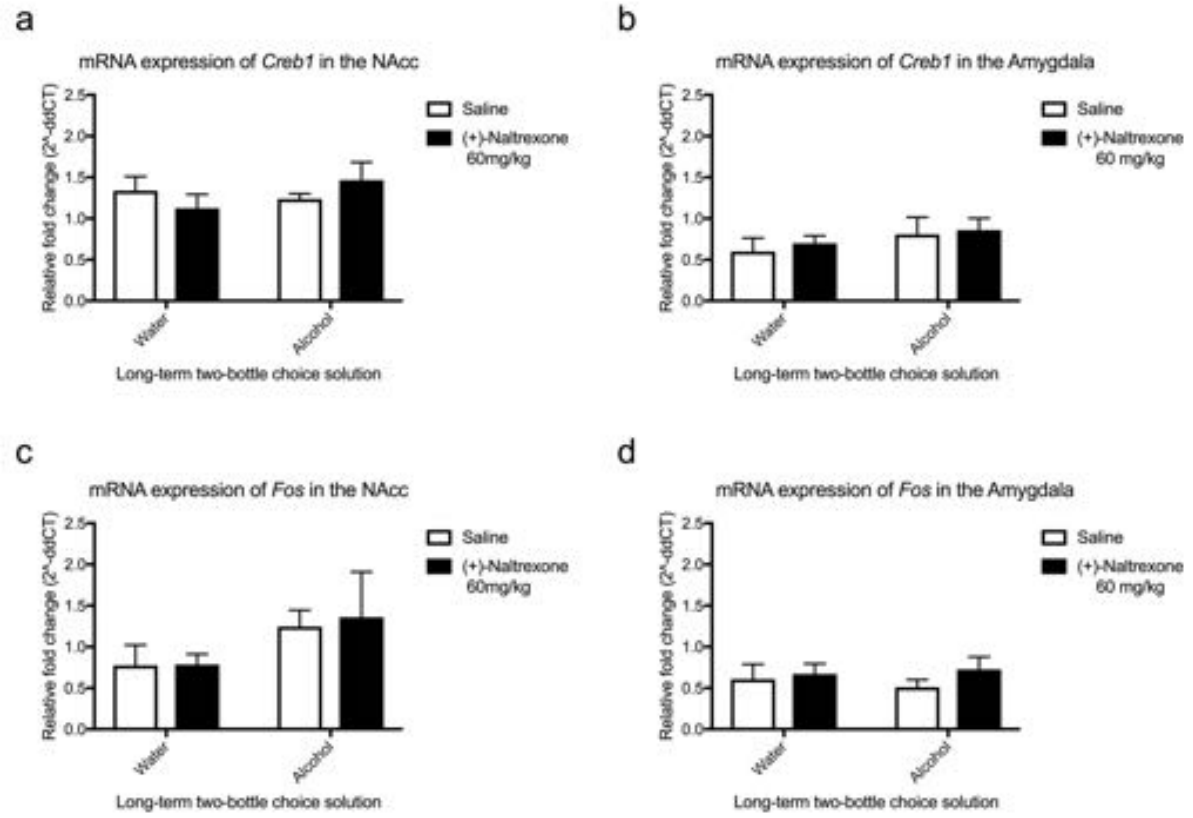


Figure s10 There was no effect of solution and drug on the expression of plasticity-related genes in the nucleus accumbens (a, c) and amygdala (b, d) following 48 h of withdrawal. There were no main effects or post hoc differences in the expression of *Creb1* or *Fos* mRNA in the amygdala or nucleus accumbens following withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4.

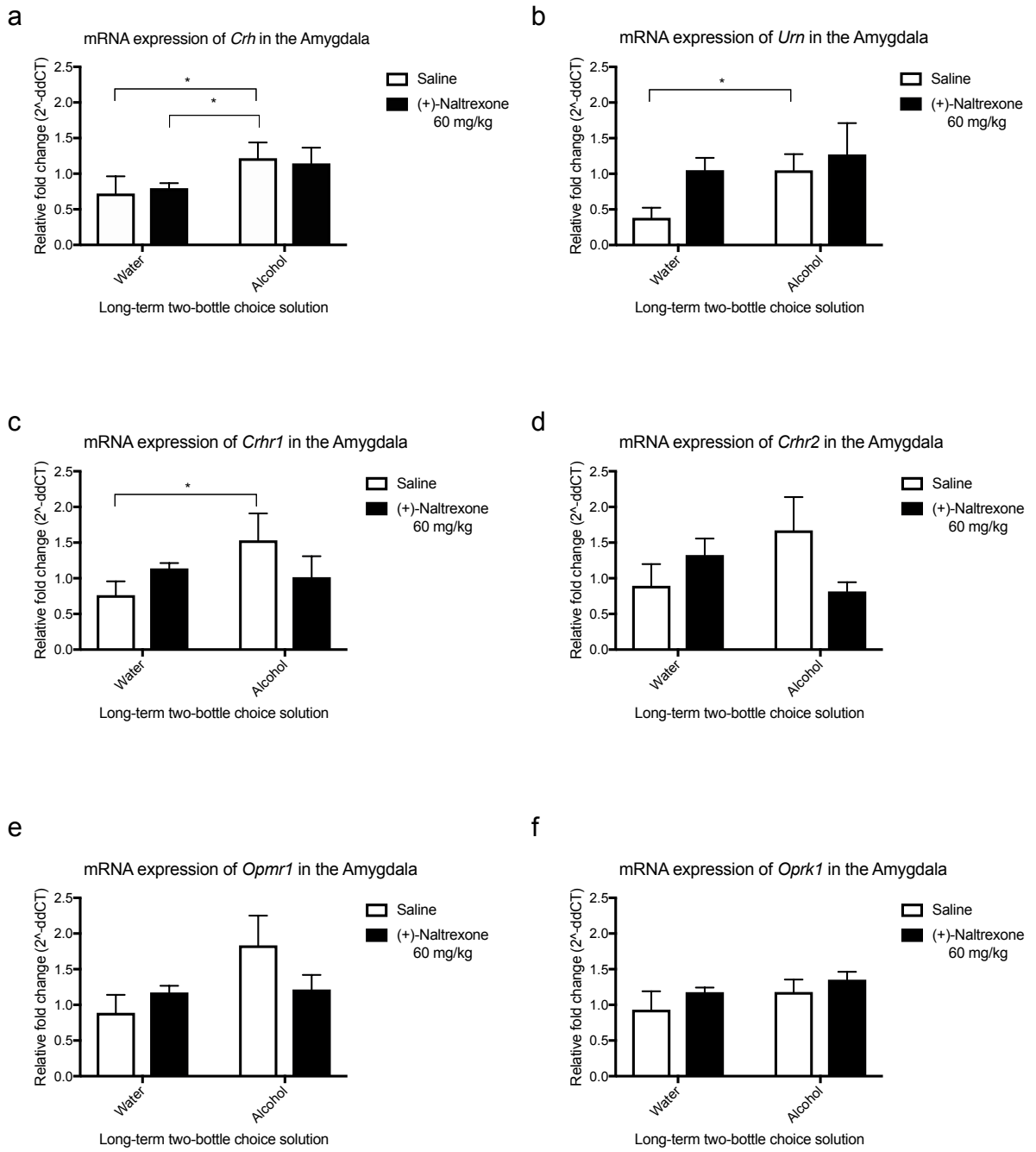


Figure s11 There was no effect of solution or drug on the expression of stress-related genes in the amygdala following 48 h of withdrawal. There was no main effect of solution or drug on the expression of *Crh*, *Crhr1*, *Crhr2*, *Urn*, *Opmr1* and *Oprk1*. However, post hoc analysis determined the expression of *Crh*, *Crhr1* and *Urh* mRNA was increased in alcohol + saline cohorts compared to water + saline cohorts. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, **p* < 0.05.

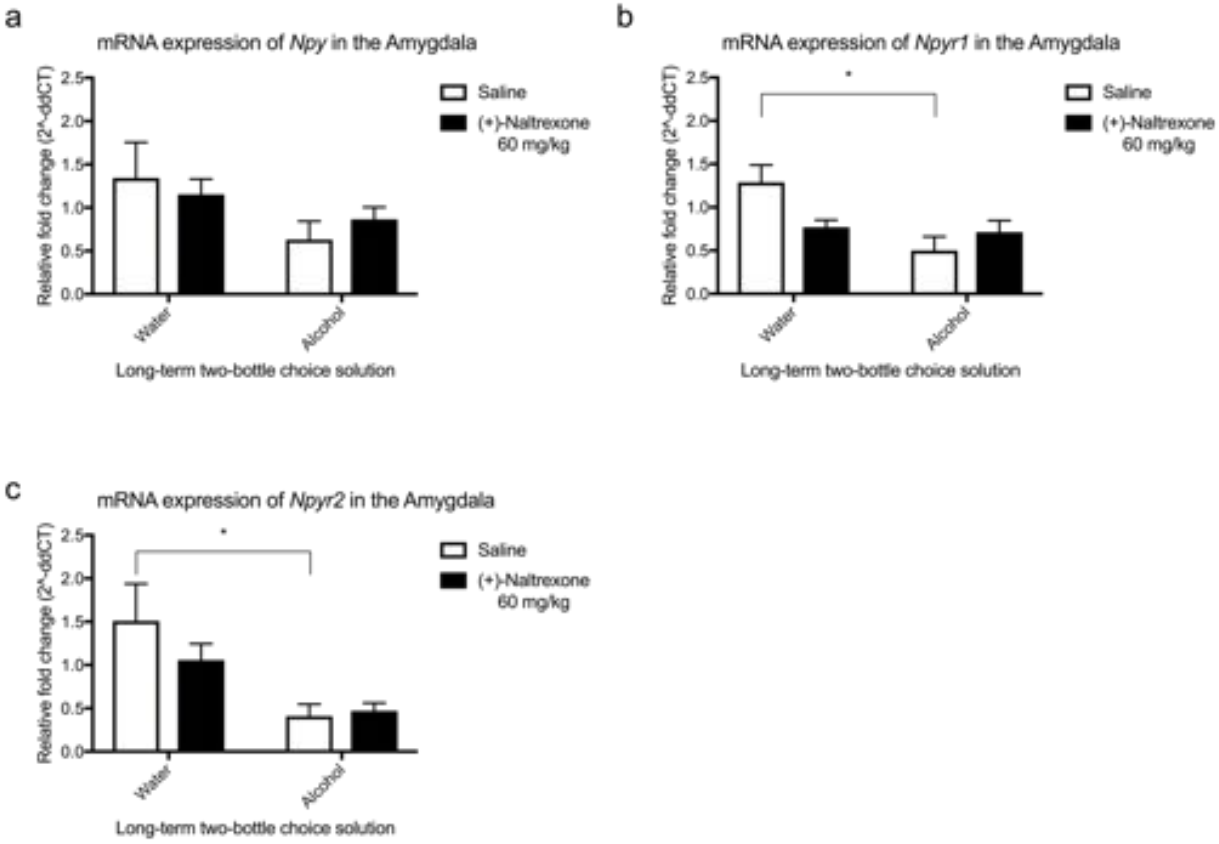


Figure s12 Alcohol but not (+)-Naltrexone modified the expression of anti-stress-related mRNA in the amygdala following 48 h of withdrawal. There was a main effect of solution but not drug on the expression of *Npy*, *Npyr1* and *Npyr2* mRNA following withdrawal. Post hoc analysis determined alcohol + saline cohorts exhibited reduced expression of *Npyr1* and *Npyr2* mRNA compared to water + saline cohorts. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, $*p < 0.05$.

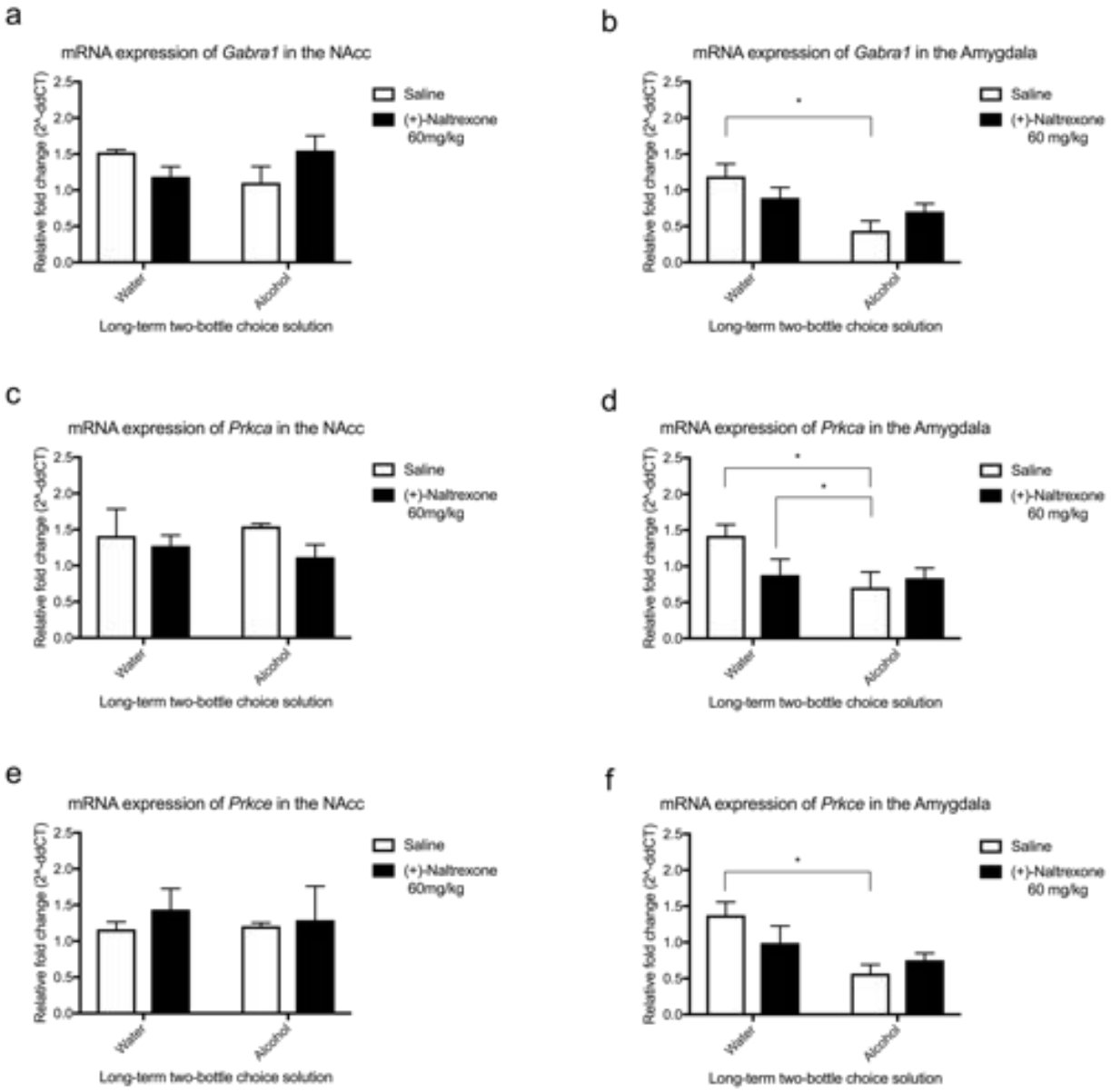


Figure s13 Alcohol but not (+)-Naltrexone modified the expression of GABA-related mRNA in the amygdala (b, d, f) but not nucleus accumbens (a, c, e) following 48 h of withdrawal. There was a main effect of solution on the expression of *Gabra1*, *Prkca* and *Prkce* mRNA in the amygdala but not nucleus accumbens following withdrawal. Post hoc analysis determined alcohol + saline cohorts exhibited significantly reduced expression of *Gabra1*, *Prkca* and *Prkce* mRNA in the amygdala but not nucleus accumbens compared to water + saline cohorts. All data was analysed using a two-way ANOVA with Bonferonni post hoc. Summary values represented as mean±SEM; n=4, *$p < 0.05$.

7.8.3 Supplementary figure statistics

Figure s2 Alcohol intake (a) and preference (b) and average serum alcohol concentration (c) following the last drinking session in saline or (+)-Naltrexone grouped mice. All data was analysed using a two-way ANOVA with Tukey post hoc (a – b) or a one-way ANOVA with Tukey post hoc (c).

(a) *Intake*

Effect of solution $F_{(17, 102)} = 20.62$, $p < 0.0001$

Effect of dose $F_{(17, 102)} = 0.73$, $p = 0.63$

Interaction $F_{(17, 102)} = 3.64$, $p < 0.0001$

(b) *Preference*

Effect of solution $F_{(17, 102)} = 6.67$, $p < 0.0001$

Effect of dose $F_{(17, 102)} = 3.23$, $p = 0.012$

Interaction $F_{(17, 102)} = 1.87$, $p < 0.0001$

(c) *Serum ethanol concentration*

Effect of dose $F_{(6, 13)} = 0.14$, $p = 0.98$

Figure s3 Water intake during withdrawal in saline or (+)-Naltrexone grouped mice. All data was analysed using a one-way ANOVA with Tukey post hoc.

Effect of dose $F_{(6, 41)} = 0.96$, $p = 0.46$

Figure s4 (+)-Naltrexone does not alter the intake and preference of alcohol + quinine l (a – b) or quinine (c – d). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Alcohol + quinine intake*

Effect of solution $F_{(1, 7)} = 2.71$, $p = 0.15$

Effect of treatment $F_{(1, 7)} = 0.36$, $p = 0.57$

Interaction $F_{(1, 7)} = 1.25$, $p = 0.31$

(b) *Alcohol + quinine preference*

Effect of solution $F_{(1, 7)} = 7.04$, $p = 0.038$

Effect of treatment $F_{(1, 7)} = 0.028$, $p = 0.87$

Interaction $F_{(1, 7)} = 0.76$, $p = 0.42$

(c) *Quinine intake*

Effect of solution $F_{(1, 7)} = 2.46$, $p = 0.17$

Effect of treatment $F_{(1, 7)} = 0.002$, $p = 0.97$

Interaction $F_{(1, 7)} = 4.44$, $p = 0.08$

(d) *Quinine preference*

Effect of solution $F_{(1, 7)} = 0.024$, $p = 0.88$

Effect of treatment $F_{(1, 7)} = 0.11$, $p = 0.75$

Interaction $F_{(1, 7)} = 2.14$, $p = 0.19$

Figure s5 (+)-Naltrexone does not alter the average alcohol alcohol concentration reached following a bolus gavage of alcohol (a – b). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Peak blood alcohol concentration*

Effect of solution, $F_{(1, 4)} = 61.82$, $p = 0.0043$

Effect of drug, $F_{(1, 4)} = 1.42$, $p = 0.31$

Interaction (solution x drug), $F_{(1, 4)} = 8.05$, $p = 0.066$

(b) *Time course of blood alcohol concentration*

Effect of time, $F_{(3, 9)} = 23.8$, $p = 0.0001$

Effect of drug, $F_{(3, 9)} = 0.63$, $p = 0.48$

Interaction (solution x drug), $F_{(3, 9)} = 1.54$, $p = 0.27$

Figure s6 Neither alcohol or (+)-Naltrexone modified the expression of *Tlr2* mRNA in the nucleus accumbens or amygdala following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Nucleus accumbens Tlr2*

Effect of solution, $F_{(1, 3)} = 0.009$, $p = 0.93$

Effect of treatment, $F_{(1, 3)} = 4.92$, $p = 0.08$

Interaction, $F_{(1, 3)} = 0.095$, $p = 0.79$

(b) *Amygdala Tlr2*

Effect of solution, $F_{(1, 3)} = 4.0$, $p = 0.08$

Effect of treatment, $F_{(1, 3)} = 4.0$, $p = 0.08$

Interaction, $F_{(1, 3)} = 5.43$, $p = 0.05$

Figure s7 Alcohol but not (+)-Naltrexone modified the expression of TRIF-related mRNA in the amygdala (b, d, f) but not nucleus accumbens (a, c, e) following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Nucleus accumbens Trif*

Effect of solution, $F_{(1, 3)} = 3.25$, $p = 0.21$

Effect of treatment, $F_{(1, 3)} = 2.81$, $p = 0.24$

Interaction, $F_{(1, 3)} = 0.021$, $p = 0.89$

(b) *Amygdala Trif*

Effect of solution, $F_{(1, 3)} = 26.85$, $p = 0.008$

Effect of treatment, $F_{(1, 3)} = 0.70$, $p = 0.43$

Interaction, $F_{(1, 3)} = 0.10$, $p = 0.76$

(c) *Nucleus accumbens Tram*

Effect of solution, $F_{(1, 3)} = 0.41$, $p = 0.59$

Effect of treatment, $F_{(1, 3)} = 0.015$, $p = 0.91$

Interaction, $F_{(1, 3)} = 1.89$, $p = 0.30$

(d) *Amygdala Tram*

Effect of solution, $F_{(1, 3)} = 16.12$, $p = 0.0039$

Effect of treatment, $F_{(1, 3)} = 0.30$, $p = 0.60$

Interaction, $F_{(1, 3)} = 0.00057$, $p = 0.98$

(e) *Nucleus accumbens Ifnb*

Effect of solution, $F_{(1, 3)} = 0.77$, $p = 0.47$

Effect of treatment, $F_{(1, 3)} = 0.55$, $p = 0.53$

Interaction, $F_{(1, 3)} = 1.21$, $p = 0.39$

(f) *Amygdala Ifnb*

Effect of solution, 7.41, $p = 0.027$

Effect of treatment, $F_{(1, 3)} = 0.0076$, $p = 0.93$

Interaction, $F_{(1, 3)} = 0.99$, $p = 0.38$

(g) *Nucleus accumbens Irf3*

Effect of solution, $F_{(1, 3)} = 1.81$, $p = 0.30$

Effect of treatment, $F_{(1, 3)} = 1.06$, $p = 0.41$

Interaction, $F_{(1, 3)} = 0.15$, $p = 0.73$

(h) *Amygdala Irf3*

Effect of solution, $F_{(1, 3)} = 10.35$, $p = 0.012$

Effect of treatment, $F_{(1, 3)} = 0.048$, $p = 0.83$

Interaction, $F_{(1, 3)} = 0.167$, $p = 0.69$

Figure s8 Alcohol but not (+)-Naltrexone modified the expression of MyD88-related mRNA in the amygdala (b, d, f, h, j, l, n) but not nucleus accumbens (a, c, e, g, l, k, m) following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Nucleus accumbens Myd88*

Effect of solution, $F_{(1, 3)} = 0.38$, $p = 0.60$

Effect of treatment, $F_{(1, 3)} = 3.56$, $p = 0.20$

Interaction, $F_{(1, 3)} = 0.0020$, $p = 0.97$

(b) *Amygdala Myd88*

Effect of solution, $F_{(1, 3)} = 7.22$, $p = 0.028$

Effect of treatment, $F_{(1, 3)} = 2.35$, $p = 0.16$

Interaction, $F_{(1, 3)} = 1.54$, $p = 0.25$

(c) *Nucleus accumbens Tirap*

Effect of solution, $F_{(1, 3)} = 4.16$, $p = 0.18$

Effect of treatment, $F_{(1, 3)} = 6.35$, $p = 0.13$

Interaction, $F_{(1, 3)} = 0.00054$, $p = 0.98$

(d) *Amygdala Tirap*

Effect of solution, $F_{(1, 3)} = 4.17$, $p = 0.08$

Effect of treatment, $F_{(1, 3)} = 0.89$, $p = 0.37$

Interaction, $F_{(1, 3)} = 0.68$, $p = 0.43$

(e) *Nucleus accumbens Il1b*

Effect of solution, $F_{(1, 3)} = 0.63$, $p = 0.51$

Effect of treatment, $F_{(1, 3)} = 2.12$, $p = 0.28$

Interaction, $F_{(1, 3)} = 2.20$, $p = 0.28$

(f) *Amygdala Il1b*

Effect of solution, $F_{(1, 3)} = 29.66$, $p = 0.0006$

Effect of treatment, $F_{(1, 3)} = 0.015$, $p = 0.91$

Interaction, $F_{(1, 3)} = 6.09$, $p = 0.039$

(g) *Nucleus accumbens Tnfa*

Effect of solution, $F_{(1, 3)} = 14.66$, $p = 0.062$

Effect of treatment, $F_{(1, 3)} = 3.01$, $p = 0.22$

Interaction, $F_{(1, 3)} = 0.66$, $p = 0.50$

(h) *Amygdala Tnfa*

Effect of solution, $F_{(1, 3)} = 0.091$, $p = 0.77$

Effect of treatment, $F_{(1, 3)} = 0.10$, $p = 0.76$

Interaction, $F_{(1, 3)} = 4.62$, $p = 0.06$

(i) *Nucleus accumbens Ccl2*

Effect of solution, $F_{(1, 3)} = 3.62$, $p = 0.19$

Effect of treatment, $F_{(1, 3)} = 0.67$, $p = 0.49$

Interaction, $F_{(1, 3)} = 63.65$, $p = 0.015$

(j) *Amygdala Ccl2*

Effect of solution, $F_{(1, 3)} = 5.3$, $p = 0.051$

Effect of treatment, $F_{(1, 3)} = 0.0043$, $p = 0.94$

Interaction, $F_{(1, 3)} = 5.62$, $p = 0.045$

(k) *Nucleus accumbens Il10*

Effect of solution, $F_{(1, 3)} = 1.19$, $p = 0.39$

Effect of treatment, $F_{(1, 3)} = 0.14$, $p = 0.74$

Interaction, $F_{(1, 3)} = 1.50$, $p = 0.35$

(l) *Amygdala Il10*

Effect of solution, $F_{(1, 3)} = 2.58$, $p = 0.25$

Effect of treatment, $F_{(1, 3)} = 0.30$, $p = 0.64$

Interaction, $F_{(1, 3)} = 55.82$, $p = 0.017$

(m) *Nucleus accumbens Rela*

Effect of solution, $F_{(1, 3)} = 0.13$, $p = 0.75$

Effect of treatment, $F_{(1, 3)} = 3.07$, $p = 0.22$

Interaction, $F_{(1, 3)} = 0.25$, $p = 0.67$

(n) *Amygdala Rela*

Effect of solution, $F_{(1, 3)} = 4.9$, $p = 0.058$

Effect of treatment, $F_{(1, 3)} = 0.037$, $p = 0.85$

Interaction, $F_{(1, 3)} = 1.98$, $p = 0.20$

Figure s9 There was no effect of solution or drug on the expression of reward-related genes in the nucleus accumbens following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Drd1*

Effect of solution, $F_{(1, 3)} = 2.65$, $p = 0.24$

Effect of treatment, $F_{(1, 3)} = 0.10$, $p = 0.77$

Interaction, $F_{(1, 3)} = 8.22$, $p = 0.10$

(b) *Drd2*

Effect of solution, $F_{(1, 3)} = 234.4$, $p = 0.0042$

Effect of treatment, $F_{(1, 3)} = 0.21$, $p = 0.69$

Interaction, $F_{(1, 3)} = 2.66$, $p = 0.23$

(c) *Oprm1*

Effect of solution, $F_{(1, 3)} = 1.40$, $p = 0.36$

Effect of treatment, $F_{(1, 3)} = 0.074$, $p = 0.81$

Interaction, $F_{(1, 3)} = 1.31$, $p = 0.37$

Figure s10 There was no effect of solution and drug on the expression of plasticity-related genes in the nucleus accumbens (a, c) and amygdala (b, d) following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Nucleus accumbens Creb1*

Effect of solution, $F_{(1, 3)} = 3.58$, $p = 0.19$

Effect of treatment, $F_{(1, 3)} = 0.0050$, $p = 0.95$

Interaction, $F_{(1, 3)} = 2.07$, $p = 0.27$

(b) *Amygdala Creb1*

Effect of solution, $F_{(1, 3)} = 1.06$, $p = 0.33$

Effect of treatment, $F_{(1, 3)} = 0.16$, $p = 0.70$

Interaction, $F_{(1, 3)} = 0.029$, $p = 0.87$

(c) *Nucleus accumbens Fos*

Effect of solution, $F_{(1, 3)} = 7.75$, $p = 0.11$

Effect of treatment, $F_{(1, 3)} = 0.086$, $p = 0.79$

Interaction, $F_{(1, 3)} = 0.028$, $p = 0.88$

(d) *Amygdala Fos*

Effect of solution, $F_{(1, 3)} = 0.012$ $p = 0.92$

Effect of treatment, $F_{(1, 3)} = 0.97$, $p = 0.35$

Interaction, $F_{(1, 3)} = 0.69$, $p = 0.43$

Figure s11 There was no effect of solution or drug on the expression of stress-related genes in the amygdala following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Crh*

Effect of solution, $F_{(1, 3)} = 2.5$, $p = 0.15$

Effect of treatment, $F_{(1, 3)} = 0.00054$, $p = 0.99$

Interaction, $F_{(1, 3)} = 0.16$, $p = 0.70$

(b) *Urn*

Effect of solution, $F_{(1, 3)} = 4.67$, $p = 0.06$

Effect of treatment, $F_{(1, 3)} = 2.20$, $p = 0.18$

Interaction, $F_{(1, 3)} = 0.60$, $p = 0.46$

(c) *Crhr1*

Effect of solution, $F_{(1, 3)} = 2.37$, $p = 0.16$

Effect of treatment, $F_{(1, 3)} = 0.038$, $p = 0.85$

Interaction, $F_{(1, 3)} = 4.15$, $p = 0.076$

(d) *Crhr2*

Effect of solution, $F_{(1, 3)} = 0.17$, $p = 0.69$

Effect of treatment, $F_{(1, 3)} = 0.97$, $p = 0.35$

Interaction, $F_{(1, 3)} = 5.08$, $p = 0.054$

(e) *Oprm1*

Effect of solution, $F_{(1, 3)} = 3.13$, $p = 0.12$

Effect of treatment, $F_{(1, 3)} = 0.39$, $p = 0.55$

Interaction, $F_{(1, 3)} = 2.46$, $p = 0.15$

(f) *Oprk1*

Effect of solution, $F_{(1, 3)} = 1.49$, $p = 0.28$

Effect of treatment, $F_{(1, 3)} = 1.60$, $p = 0.24$

Interaction, $F_{(1, 3)} = 0.065$, $p = 0.80$

Figure s12 Alcohol but not (+)-Naltrexone modified the expression of anti-stress-related mRNA in the amygdala following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Npy*

Effect of solution, $F_{(1, 3)} = 5.20$, $p = 0.051$

Effect of treatment, $F_{(1, 3)} = 0.0081$, $p = 0.93$

Interaction, $F_{(1, 3)} = 1.56$, $p = 0.25$

(b) *Npyr1*

Effect of solution, $F_{(1, 3)} = 12.54$, $p = 0.0076$

Effect of treatment, $F_{(1, 3)} = 1.17$, $p = 0.31$

Interaction, $F_{(1, 3)} = 7.46$, $p = 0.026$

(c) *Npyr2*

Effect of solution, $F_{(1, 3)} = 10.72$, $p = 0.013$

Effect of treatment, $F_{(1, 3)} = 0.793$, $p = 0.40$

Interaction, $F_{(1, 3)} = 1.43$, $p = 0.26$

Figure s13 Alcohol but not (+)-Naltrexone modified the expression of GABA-related mRNA in the amygdala (b, d, f) but not nucleus accumbens (a, c, e) following 48 h of withdrawal. All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Nucleus accumbens Gabra1*

Effect of solution, $F_{(1, 3)} = 0.25$, $p = 0.67$

Effect of treatment, $F_{(1, 3)} = 0.039$, $p = 0.86$

Interaction, $F_{(1, 3)} = 5.38$, $p = 0.15$

(b) *Amygdala Gabra1*

Effect of solution, $F_{(1, 3)} = 14.12$, $p = 0.0056$

Effect of treatment, $F_{(1, 3)} = 0.010$, $p = 0.92$

Interaction, $F_{(1, 3)} = 3.19$, $p = 0.11$

(c) *Nucleus accumbens Prkca*

Effect of solution, $F_{(1, 3)} = 0.0051$, $p = 0.94$

Effect of treatment, $F_{(1, 3)} = 1.3$, $p = 0.37$

Interaction, $F_{(1, 3)} = 0.24$, $p = 0.67$

(d) *Amygdala Prkca*

Effect of solution, $F_{(1, 3)} = 4.28$, $p = 0.072$

Effect of treatment, $F_{(1, 3)} = 1.33$, $p = 0.28$

Interaction, $F_{(1, 3)} = 7.22$, $p = 0.028$

(e) *Nucleus accumbens Prkce*

Effect of solution, $F_{(1, 3)} = 0.12$, $p = 0.77$

Effect of treatment, $F_{(1, 3)} = 0.30$, $p = 0.64$

Interaction, $F_{(1, 3)} = 0.47$, $p = 0.56$

(f) *Amygdala Prkce*

Effect of solution, $F_{(1, 3)} = 12.84$, $p = 0.0072$

Effect of treatment, $F_{(1, 3)} = 0.48$, $p = 0.51$

Interaction, $F_{(1, 3)} = 4.78$, $p = 0.06$

7.9 Summary

Long-term alcohol use recruits and modifies many neurotransmitter systems in brain regions associated with reward and stress (Koob & Volkow, 2009). This in turn alters the “liking” and “wanting” components of reward and induces anxiety (Berridge & Robinson, 2016; Koob & Le Moal, 2001). In addition to these neuronal processes, the TLR4 pathway is activated inducing the expression of immune mediators (Alfonso-Loeches, et al., 2010). These mediators, specifically cytokines, sensitise rodents to, and mediate alcohol reward and anxiety behaviours following long-term use (Marshall et al., 2016a; Marshall et al., 2016b; Breese et al., 2007). It was therefore hypothesised that acute attenuation of TLR4 would reduce cytokine levels and in turn assist in alleviating anxiety and reward-related behaviours during withdrawal.

However, the preceding manuscript highlighted the lack of involvement of the TLR4-TRIF pathway in the altering the “liking” and “wanting” components of alcohol-induced reward following long-term use (Table 2). In brief, (+)-Naltrexone failed to reduce the potentiated alcohol intake and reduced sucrose intake induced by long-term exposure immediately proceeding the last drinking session. This suggests (+)-Naltrexone was unable to modify tolerance of the “liking” component of reward. In accordance with Chapter 3 however, mice exposed to water exhibited significant reductions in sucrose and alcohol intake following (+)-Naltrexone. The “wanting” component of alcohol reward was assessed 48 h after the last drinking session – a time where the motivation and “want” to consume alcohol is high. (+)-Naltrexone did not attenuate the subsequent intake of alcohol or alcohol + quinine or restore sucrose deficits suggesting it was unable to reduce the “wanting” component of reward and alleviate symptoms of anhedonia respectively. To ascertain why (+)-Naltrexone failed to modify these behaviours the serum alcohol concentration and expression of reward-, stress- and TLR4-related genes was assessed. (+)-Naltrexone did not modify peak serum alcohol

concentration compared to saline in water or alcohol exposed mice, nor did it modify the time-course of alcohol clearance. This suggests the lack of effect was not simply attributable to alterations in the pharmacokinetics of alcohol. Interestingly, mice exposed to alcohol long-term exhibit enhanced serum alcohol clearance suggesting more alcohol is required to achieve the same serum alcohol concentration in naïve mice. In contrast to previous chapters (3 and 5), there was no significant effect of (+)-Naltrexone on *Trif or Ifnb* mRNA expression in either the nucleus accumbens or amygdala. Further, there was no effect of both (+)-Naltrexone and alcohol on any gene except *Gabra2*. It is unlikely that a reduction in *Gabra2* mRNA following long-term alcohol use is likely to be beneficial to an individual given long-term alcohol exposure is characterised by a lack of inhibitory GABAergic control, an effect which underlies compulsivity and stress (Silberman et al., 2009; Koob, 2004). This effect may offer some explanation to the limited efficacy of (+)-Naltrexone following long-term alcohol use.

There are additional explanations why (+)-Naltrexone exhibited limited efficacy following long-term use. For example, prolonged use of alcohol results in numerous neuroplastic and epigenetic modifications which collectively desensitise and sensitise the reward pathway and stress pathways, respectively (Koob & Volkow, 2009). Consequently, these pathways may be somewhat more resilient to the effects of an acute pharmacological blockade, and would better respond to a chronic treatment regiment. This idea is further supported owing to the relatively fast metabolism of Naltrexone in mice (Malspeis et al., 1975). Consequently, the limited blockade of TLR4 is unlikely to result in large modifications to behaviour or genes. In contrast to rodents, the half-life of Naltrexone in humans is longer (on the order of several hours) and extended-release injections are available, further increasing Naltrexone's efficacy (Dunbar et al., 2006; Verebey et al., 1976). Therefore, in humans, Naltrexone may be

more successful in reducing the neuroimmune response following long-term alcohol use.

The high levels of immune mediators following long-term alcohol is likely due to DAMPs from apoptosing neurons and glia (Crews et al., 2017) and epigenetic modifications to immune-related genes increasing the likelihood of their transcription (Hennessy & McKernan, 2016; Montesinos et al., 2016). Consequently, multiple immune pathways are engaged and potentially contribute together to result in alcohol-related behaviours. Therefore, attenuating one receptor within this complex system is unlikely to successfully reduce all neuroimmune mediators (Crews et al., 2017). A better therapeutic strategy is to attenuate global, overlapping immune-related transcription factors such as NFκB. This is particularly relevant to humans, as post mortem studies have found that the NFκB system in the PFC is dysregulated among alcohol dependents (Ökvist et al., 2007). Therefore, restoring its activity to basal homeostatic levels may alleviate symptoms associated with long-term alcohol use.

In conclusion, this study highlights the limited efficacy of attenuating the TLR4-TRIF pathway on restoring and reducing “liking” and “wanting” components of reward of alcohol following long-term use.

Table 2 Brief summary of behavioural data (post hoc differences) from chapter 7

Behaviour	**Effect of (+)-Naltrexone Water exposed mice**	**Effect of (+)-Naltrexone Alcohol exposed mice**
Liking		
2 h alcohol exposure	↓ intake 60 - 75 mg/kg — preference	— intake — preference
2 h sucrose exposure	↓ intake 45 – 75 mg/kg ↓ preference 75 mg/kg	— intake ↓ preference 75 mg/kg
2 h quinine exposure	— intake — preference	— intake — preference
Wanting		
24 h alcohol exposure	↓ intake 60 mg/kg ↓ preference 30, 60, 75 mg/kg	— intake — preference
24 h alcohol + quinine exposure	— intake — preference	— intake — preference
24 h sucrose exposure	— intake ↓ preference 60 - 75 mg/kg	— intake — preference
Anxiety		
Elevated plus maze	— time, distance, immobile episodes	— time, distance, immobile episodes
Open field test	— time, distance, immobile episodes	— time, distance, immobile episodes

N/A, not applicable; ↑, increased intake/preference relative to saline; ↓ decreased intake/preference relative to saline; — no difference relative to saline

7.10 References

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Chapter 8: Discussion

8.1 Introduction

Occasional and long-term alcohol use confers substantial health and economic costs to both individuals and the society at large (Rehm & Shield, 2014; World Health Organisation, 2014). Despite this, alcohol use is normative among adults in western cultures (NIAAA, 2017), an effect attributed to the rewarding and anti-rewarding properties of alcohol (Wise & Koob, 2013; Koob & Nestler, 1997). Pivotal to the emergence, persistence and alterations to alcohol-induced reward and anti-reward is the neuroimmune system (Cui et al., 2014; Mayfield et al., 2013). Specifically, research has emphasised TLR4, an innate immune pattern recognition receptor, as a key contributor to the neuroimmune involvement in alcohol-induced reward and anti-reward behaviours (for example, Pascual et al., 2015; Liu et al., 2011). However, whether alcohol activates both TLR4 pathways (TRIF and MyD88) *in vivo* and the relative contribution of each pathway towards reward and anti-reward is still unknown.

Therefore, the aim of this thesis was to characterise the TLR4 signalling pathway following acute and chronic alcohol exposure and to determine whether attenuating the TLR4-TRIF pathway reduces reward and anti-reward behaviour in naïve, sensitised and alcohol-dependent mice. The work presented herein suggest both the MyD88 and TRIF pathways are activated by acute and chronic exposure but only the TRIF pathway remains elevated for prolonged periods of time post exposure (sensitised). Attenuating the activation of the TLR4-TRIF signalling pathway using (+)-Naltrexone, prevented alcohol reward behaviour in naïve mice (“liking” and “wanting”), partially prevented reward behaviour in sensitised mice (“liking” only), but had no effect on reward or anti-reward in dependent mice. Collectively, the results suggest (+)-Naltrexone may only prove beneficial for attenuating occasional drinking but is unlikely to alter drinking following long-term alcohol use.

8.2 Alcohol-induced TLR4 signalling

A growing body of evidence implicates TLR4 in the molecular and behavioral consequences of alcohol exposure as TLR4 genetic knockout, siRNA knockdown or pharmacological antagonism *in vitro* and *in vivo* reduces the rise in TLR4-related immune mediators following alcohol exposure (Pascual et al., 2015; Alfonso-Loeches et al., 2010; Blanco et al., 2005). Importantly, this phenomenon translates into humans. Post-mortem studies demonstrate increased expression of TLR4 and downstream signaling pathways in alcohol dependents compared to healthy controls (Crews et al., 2013; Liu et al., 2005; Ökvist et al., 2007). Further, the expression of TLR4 and HMGB1 (an endogenous agonist of TLR4) correlate with the estimated life-time alcohol intake in humans (Crews et al., 2013). However, few studies have considered whether the MyD88 or TRIF (or both) signaling pathways are triggered, or remain activated following alcohol exposure in rodents and humans.

It is important to discern which TLR4 pathway alcohol activates because there are specific behavioral outcomes associated with the MyD88 and TRIF pathways. For example, the expression of interferons, and their receptors (regulated by the TRIF pathway) correlates with alcohol dependence in humans (Johnson et al., 2015) whereas NFκB (a TRIF and MyD88 shared transcription factor) underlies much of the neuroplastic adaptations which have occurred in long-term alcohol users (Ökvist et al., 2007). In rodents, MyD88 and its prototypical product IL-1β, additionally regulate alcohol-induced motor impairment and sedation (Blednov et al., 2017b; Wu et al., 2012). Under alcohol naïve circumstances however, these proteins play little role in regulating alcohol intake (Blednov et al., 2017b). If a specific alcohol-related behavior preferentially results in or is caused by MyD88 or TRIF signaling in the brain, a therapeutic agent targeting the causative pathway whilst leaving the other pathway undisturbed would be ideal. This is pertinent for occasional and long-term alcohol

users, who often have compromised peripheral immune systems because of alcohol exposure (Muralidharan et al., 2014).

The studies presented herein are the first to assess whether acute and chronic alcohol upregulates either (or both) the MyD88 or TRIF pathways *in vivo*. In brevity, my research demonstrates that all aspects of the TLR4 pathway were upregulated by acute and chronic alcohol exposure including; endogenous agonists, co-receptors and aspects of the MyD88 and TRIF pathway. This suggests, like a general immune response towards LPS, both pathways are required to create a maximal immune response. It is interesting that acute exposure mirrors (albeit to a lesser extent) chronic exposure, suggesting that even a low amount of alcohol can activate the neuroimmune system. Further, these findings suggest that unlike LPS which undergoes tolerance following chronic exposure, alcohol (at an mRNA output measure) does not. Given we observed these increases in TLR4-related mRNA expression in brain regions typically associated with the "wanting" and "liking" of alcohol (nucleus accumbens, hypothalamus and amygdala) it was hypothesised that these immune mediators are interacting with neurons altering synaptic transmission, plasticity and function thereby influencing reward and anti-reward behaviours.

In contrast to acute and chronic exposure, alcohol exposure during adolescence preferentially elevated the expression of TLR4, its co-receptor (MD2) and aspects of the TRIF in adulthood. Interestingly, the MyD88 pathway did not exhibit elevations in mRNA. It is unclear how or why alcohol preferentially upregulates the TRIF pathway. However, Shen et al., (2008) in their work examining the effects of relative role of each TLR4 signaling pathway in macrophages found the TRIF pathway regulates the expression and release of most LPS-induced cytokines compared to the MyD88 pathway. The authors concluded that from an immunological standpoint, the TRIF

pathway has a much greater role in LPS-TLR4 signaling. By extending these results to the current studies, it is hypothesized that from a purely immunological viewpoint, the TRIF pathway remains elevated because it is more immunologically pertinent to alcohol. However, the effect observed by Shen et al., (2008) was cell specific, with differing results observed between macrophages and dendritic cells. This raises a crucial limitation of my present studies; the cell type(s) within which the mRNA expression changes occurred remains unknown.

It is important to identify the specific cell types underlying the alterations in alcohol-induced TLR4 signalling as each cell type would undoubtedly result in different behavioural and molecular outcomes. The results presented in the appendix aimed to partly address this by examining the alcohol-induced immune response in BV2 microglia-like cells. However, in stark contrast to previous studies (Lawrimore & Crews, 2017) alcohol was unable to induce an immune response. Methodological considerations such as the use of a chamber to maintain alcohol saturation may underscore the different results obtained by the two studies. This apparatus lessens the effects of alcohol evaporation maintaining a constant concentration of alcohol in the culture media. This is thought to maintain the alcohol-induced stress placed on cells which in turn, releases danger molecules (such as HMGB1) initiating a TLR4 response. However, my study did not use this apparatus and consequently alcohol evaporated relatively quickly from the culture media. This may have reduced the stress placed on these cells and consequently, the released danger molecules thus preventing a TLR4 response.

To date only one study has directly compared how an alcohol-induced immune signal differs between cell types. Lawrimore & Crews, (2017) evaluated an immune response following alcohol exposure in BV2 and SH-SY5Y cells, microglia- and neuronal-like

cells respectively. Following 24 h of alcohol exposure, TNFα, IL-1β and IRF-3 but not MAPKs or NFκB were elevated in BV2 cells. By contrast, SH-SY5Y cells, exhibited increases in IRF-3, MAPKs and NFκB following alcohol exposure. However, the expression of TNFα and IL-1β was unaltered in the latter. Neither cell type exhibited increases in MyD88 gene expression and the TRIF-IFNβ pathway was not widely explored despite the increases in IRF-3. Collectively, the results suggest that alcohol exposure potentially increases the TLR4-TRIF pathway in microglia (with some aspects of the MyD88 pathway upregulated). By contrast, while alcohol increases the expression of IRF-3, MAPKs and NFκB in neurons, the outcome is likely non-immune related. This concept is supported by *in vivo* findings. For example, in neurons, TLR4 activation induces the expression of tyrosine hydroxylase and CRF and alters GABA-related activity (Aurelian et al., 2016; June et al., 2015; Bajo et al., 2014). In glia, alcohol-induced TLR4 signalling increases the expression and release of inflammatory cytokines and reactive oxygen creating enzymes (Fernandez-Lizarbe et al., 2009; Blanco et al., 2005). While glia and neurons likely respond to long-term alcohol use, it is presently unclear the degree to which the shorter alcohol exposure models such as those described in chapters 3 and 5 engage microglia *in vivo*. Furthermore, it is presently unclear whether neurons *in vivo* express all proteins pertinent to the TRIF pathway (Okun et al., 2011) and if they remain in a primed state following alcohol exposure. Therefore, the prolonged increases in the TLR4-TRIF pathway (which signals to IRF3) are more likely to originate from microglia than neurons *in vivo*. However, the cell type underlying the acute effects of TLR4 activation remains a key question to be addressed by future experiments.

Examining how alcohol influences the TLR4 pathway from an mRNA perspective can provide us with crucial information regarding how transcription factors are working and can infer what genomic and epigenetic processes have occurred in response to alcohol

exposure. However; the degree to which a gene is up or downregulated does not necessarily correlate to the protein level changes (Gry et al., 2009; Greenbaum et al., 2003). Further, these alterations are often transient and are dependent on numerous transcription factors; not all of which are related to TLR4. Given the fundamental importance of proteins in signal transduction, not having this information limits the type of conclusions drawn from these studies. This is especially pertinent when considering the neuroimmune effects of alcohol. Four gavages of alcohol are required before neuroimmune-related protein changes occur (Qin et al., 2008). This highlights that a single dose of alcohol might initialise/prepare for a neuroimmune response. However, further immune-related stimulation may be required before the effect of this neuroimmune response translates into functional effectors. Interestingly, novel microRNAs are induced by low doses of alcohol and may additionally influence subsequent TLR4 immune responses towards alcohol (Lippai et al., 2013). Consequently, the neuroimmune system, specifically, the glial component, may not be as involved in the initial components of alcohol-related behaviour, but are gradually recruited and assist in the propagation of its effects. Instead, non-immunological TLR4 signalling such as those observed in GABAergic neurons (Bajo et al., 2014) may underlie the acute effects of alcohol.

This has further implications in the adolescent study and the concept of sensitisation. It is hypothesised that neuroimmune sensitisation following immune stimulation does not cause a persistent upregulation of protein. Rather, the cells remain in a phenotypically activated state with concurrent epigenetic processes increasing the likelihood of gene expression. Consequently, there may be an upregulation of neuroimmune genes following alcohol exposure during adolescence which persist through to adulthood. However, the functional outcomes attributable to the persistent upregulation of these inflammatory-related genes remains to be determined.

The quantification of protein was attempted using BD Bioscience and BioSearch ELISA kits. However, the limited sensitivity of these kits did not allow the detection of proteins from *ex vivo* samples. This is an outcome that has plagued many attempts to quantify scarce, but potent immune-related proteins in the CNS. Future development of cytokine detection tools, such as the spatial ELISA (Liu et al., 2017) may provide alternative methods that will allow the quantification of these critical protein levels at behaviourally meaningful concentrations.

8.3 (+)-Naltrexone's mechanism of action

Given the TLR4-TRIF pathway was up-regulated in all the studies, we sought to examine its functional importance in alcohol-induce reward and anti-reward behaviour. These studies are among the first to assess the relative importance of TLR4-TRIF signalling across a range of alcohol-related behaviours. The impact of this pathway was assessed using (+)-Naltrexone an enantiomer of the μ opioid receptor antagonist (-)-Naltrexone.

The most comprehensive pharmacological characterisation of (+)-Naltrexone was undertaken using microglia and macrophage-like cells (BV2 and RAW247.2 cells respectively). This work demonstrated (+)-Naltrexone selectively modifies the TLR4-TRIF-IRF3 pathway as inferred by a reduction in phosphorylated IRF-3 (Wang et al., 2016) but not NFκB, p38 or JNK levels following LPS (an effect which is mimicked in *ex vivo* hippocampal cells (Wu et al., 2012)). This further coincided with a reduction in IFNβ, NO, TNFα but not IL-1β production (Wang et al., 2016). Importantly, (+)-Naltrexone did not inhibit IFNγ or TNFα-induced NO production (which signal via IFNγ and TNF receptors respectively) indicating this effect was exclusive to TLR4 (Wang et al., 2016). However, results presented in this appendix suggest (+)-Naloxone, another TLR4 inhibitor, exhibit biased antagonism only at specific doses of LPS. It is thought

that at low concentrations of LPS, (+)-Naloxone inhibits both MyD88 and TRIF-dependent signalling outcomes. However, inducing a stronger immune response by using higher doses of LPS, such as those used by Wang et al., (2016), (+)-Naloxone only attenuates the TRIF pathway. Whether this phenomenon also occurs for (+)-Naltrexone remains to be determined. However, the collective results from chapters 3 and 5 largely support the conclusions by Wang et al., (2016): (+)-Naltrexone reduced the expression of TLR4, TRIF and IFNβ genes but did not alter the expression of MyD88 or IL-1β genes.

How and if (+)-Naltrexone results in a biased antagonism of the TRIF pathway still remains to be fully determined. *In silico* docking demonstrates (+)-Naltrexone (and (-)-Naltrexone) bind to the LPS binding pocket in MD2 interacting specifically with amino acids phenylalanine, isoleucine and valine at residues 76 and 147, 63 and 48 respectively (Hutchinson et al., 2010). It is unclear how this interaction translates to a potentially biased antagonism of TLR-TRIF and caution must be taken when interpreting computer simulations to model ligand binding as interactions from water molecules are largely ignored despite their importance.

The *in vivo* studies largely support the biased antagonistic nature of (+)-Naltrexone. However, there are conflicting studies regarding (+)-Naltrexone's mechanism of action. For example, (+)-Naltrexone significantly reduced cocaine-induced IL-1β signalling in the nucleus accumbens of rats potentially by blocking the docking of cocaine to MD2 (Northcutt et al., 2015); and reduced or had no effect on LPS-induced NFκB SEAP reported expression in HEK-Blue™-hTLR4 cells (Wang et al., 2016; Skolnick et al., 2014; Lewis et al., 2013; Stevens et al., 2013; Hutchinson et al., 2008). HEK-Blue™-hTLR4 cells are human embryonic kidney cells transfected with plasmids to over-express the genes for SEAP, TLR4 and co-receptors CD14 and MD2. The lack of

biased antagonism observed in HEK-Blue™-hTLR4 cells is likely due to the fact that embryonic kidney cells are not immunologically competent and may not express all the required downstream signalling proteins necessary for the detection of biased antagonism. For example, LPS does not induce NO production in HEK-Blue™-hTLR4 cells, a key indicator of TRIF-IRF3 activation (Wang et al., 2016). Further, it is unclear how much TLR4 and co-receptors are over produced in HEK cells limiting the applicability of these cells to assess a TLR4 signal (Ashwood et al. unpublished).

Skolnick et al., (2014) additionally claim that the lack of efficacy of (+)-Naltrexone in attenuating LPS-induced SEAP expression limits its translational applicability. This statement largely ignores the diverse non-immunological role of TLR4 in the central nervous system and does not consider that TLR4 activation has divergent signalling outcomes reflecting on the type of cell it is expressed in. For example, LPS or alcohol-induced TLR4 signalling modulates GABA and dopamine release by neurons (Harris et al., 2017; Aurelian et al., 2016; Bajo et al., 2014; Blednov et al., 2011) and induces NFκB, AP-1 and IRF3 leading to the upregulation of IL-1β, TNFα, IFNβ, COX-2 and iNOS in microglia and astrocytes (Fernandez-Lizarbe et al., 2013; Blanco et al., 2005). Therefore, it is likely that a LPS or alcohol-induced TLR4 signal is unique to each cell type. Consequently, when assessing TLR4 function it is important to include an array of cell types.

The *in vitro* characterising of (+)-Naltrexone is fundamentally necessary for ascertaining how this drug interacts with and attenuates TLR4. However, this system is inherently confounded as it negates how (+)-Naltrexone interacts with a complex environment such as the brain that can lead to different results (as alluded to above). For example, (+)-Naltrexone significantly reduced cocaine-induced IL-1β signalling in the nucleus accumbens of rats (Northcutt et al., 2015) and down-regulated the mRNA

expression of *Lepr*, *Rxfp1, Creb1*, *Grin* and *Md2* in the preceding chapters. This highlights that (+)-Naltrexone may indirectly modulate the expression of other inflammatory mediators and neurotransmitter systems by inhibiting the expression of NO, TNFα and IFNβ. Alternatively, the divergent signalling TLR4-TRIF outcomes in neurons may additionally underscore the effects of (+)-Naltrexone on neurotransmitter genes.

By exclusively using (+)-Naltrexone to probe the TLR4-TRIF pathway, the effect of MyD88 signaling in reward and anti-reward was not assessed in this thesis. Interestingly, there is some evidence to suggest the MyD88 pathway contributes to reward behavior, however, these effects are inconsistent (Blednov et al., 2017b). Owing to time constraints and feasibility of obtaining a selective pharmacological MyD88 inhibitor this study did not address the role of MyD88 in reward and anti-reward behaviors. Consequently, future studies should ascertain the relative contribution of MyD88 signaling in reward and anti-reward behavior.

Results from our study suggested that aspects of the MyD88 pathway are activated following our three different regimes of alcohol exposure. For example, acute alcohol exposure (study 1) increased *Il1b* and *Ccl2*, alcohol priming (study 2) increased *Ccl2* and chronic exposure of alcohol (study 3) increased *Tirap*, *Rela (*NFκB), *Il1b* and *Ccl2* in addition to the increased TRIF-IFNβ signalling we observed. CCL2, IL-1β and NFκB are all downstream of MyD88 (Akira & Takeda, 2004) suggesting this pathway is potentially involved in alcohol-induced neuroinflammation. However, IL-1β expression can be induced by non-TLR receptors including; P2X7 (an ATP receptor), RIG-1 and AIM2 receptors (Guo et al., 2015; Kanneganti, 2010) indicating other mechanisms may potentially underlie alcohol-induced neuroinflammation.

As (+)-Naltrexone did not modify behaviours associated with reward and anti-reward following chronic alcohol, it highlights the possibility that MyD88 signalling alone or together with TRIF signalling may be involved in these behaviours as $TLR4^{-/-}$ mice are protected against the behavioural effects of protracted withdrawal (Pascual et al., 2011).

8.4 Is an immune response towards alcohol the same as ethanol?

An important consideration when assessing the translational potential of this study is the use of ethanol as a surrogate marker for alcohol. Ethanol often comprises less than half the total volume of alcoholic beverages with the remaining 50 – 99% consisting of carbohydrates, alditols, n-chain alcohols, proteins, amino acid, amines, inorganic and organic anions, vitamins, phenolic acid, yeast products and sulphur compounds (Heymann & Ebeler, 2016; Piggott, 2011). Many of these compounds exhibit pro- and anti-inflammatory actions, collectively influencing the immune response induced by ethanol. For example, a comparison of the immunogenicity of ethanol, stout and pilsner (matched ethanol concentration) determined stout and pilsner caused less liver dysfunction (as inferred by serum alanine aminotransferase, oxidised lipids and hepatic lipid levels), reduced intestinal permeability and TLR4 inflammation markers including MyD88, iNOS, TNFα and IκBα, NFκB p65 expression compared to ethanol (Landmann et al., 2015; Kanuri et al., 2014) in the livers of mice. However, the effects of beer, wine and spirits on the human peripheral immune system are mixed with studies demonstrating both increases, decreases and no effect on the immune response (for example Romeo et al., 2007; Watzl et al., 2004; 2002).

No study has currently examined the net effects of these beverages on the immune response within the central nervous system. However, individual components of alcoholic beverages have been assessed for the capability to alter immune responses by microglia. For example, the flavonoid resveratrol is hypothesised to be a natural

TLR4 antagonist (Rahimifard et al., 2017) as studies using primary microglia have shown it supresses LPS-induced IL-1β, TNFα, NO and PGE production by inhibiting the phosphorylation of MAPK and IκBα and NFκB (Capiralla et al., 2011; Meng et al., 2008; Kim et al., 2007; Bi et al., 2005). In peripheral macrophages, resveratrol suppresses LPS-induced NO, IFNβ, TNFα, MyD88, IκK, TRAM, TRIF and TBK1, and reduced the translocation of IRF-3, AP-1, STAT-1 and NFκB following LPS, suggesting it attenuates both the MyD88 and TRIF pathways (Yang et al., 2014; Zong et al., 2012; Qureshi et al., 2012; Kim et al., 2011; Youn et al., 2005). It is important to note while these pre-clinical models have established an anti-inflammatory role for resveratrol it is unknown whether it has the same effect on humans or rodents when consumed in alcohol beverages. The oral absorption for resveratrol is approximately 75 per cent. However, the bioavailability in systemic circulation is less than one per cent and therefore whether resveratrol can enter the brain to exert anti-inflammatory effects remains to be determined (Walle, 2011). Therefore, while there are many compounds within alcoholic beverages that have the potential to be anti-inflammatory, the low levels and limited bioavailability of these compounds limit their efficacy. Further, long-term use of alcohol causes inflammation within the liver (Lieber, 1997) and brain (Crews et al., 2017) suggesting these compounds may simply slow the inflammatory actions of ethanol but do not prevent it.

Collectively, this highlights a fundamental problem inferring the actions of ethanol onto alcoholic beverages, it is much more inflammatory and can induce more profound addictive tendencies in rodents compared to the parent beverage. Consequently, future research should focus on how these two differ and how we can better reconcile the effects of ethanol to that of alcoholic beverages.

8.5 Inferring reward from the behavioural paradigms

Another important caveat when considering the translational potential of this study is the manner in which reward behaviour was inferred. Achieving consilience between a rodent's behaviour and molecular events, with that observed in humans is one of the greatest problems facing translational medical science, especially in complex areas like addiction research. Addiction is an entirely human phenomenon, and while some behavioural characteristics associated with the disorder can be successfully modelled, creating models with predictive, construct and face validity is difficult (Ripley & Stephens, 2012; Sanchis-Segura & Spanagel, 2006). Oral self-administration procedures such as two-bottle choice, demonstrate face and construct validity of human alcohol consumption. Like humans, this paradigm enables rodents to choose to drink alcohol (or not), and has been used to identify pharmacological targets which prevent excessive drinking, such as (-)-Naltrexone (Hendershot et al., 2016; Middaugh & Bandy, 2000; Phillips et al., 1997). Further, these methods are technically simple and can create reproducible results. However, alterations in the experimental design, the theory behind alcohol drinking and the interpretation of results often cloud the conclusions of this paradigm (Ripley & Stephens, 2012). For example, the concentration of alcohol influences the outcome as low or high concentrations are readily consumed or rejected owing to their sweet or aversive tasting properties respectively (Kiefer, 1995; Kiefer et al., 1995). Further, the fast metabolic rate in mice and the temporal pattern of consumption (rodents drink in bouts rather than all at once as observed in humans) often makes it difficult to reach physiologically relevant ethanol concentrations within the brain (Crabbe et al., 2011). Consequently, using low concentrations of alcohol in the two-bottle choice preference test may reflect different aspects of drinking but does not model one of the key features of human alcoholism; drinking to the point of intoxication. Thus, a range of concentrations must be used to assess factors influencing the consumption of alcohol at low (primarily a taste

phenomenon) and high (a balance between aversive taste, hedonic, and reward) concentrations.

Another important consideration are the variables used to infer the “wanting” and “liking” components of reward in two-bottle choice: water, alcohol and total fluid intake. However, the interpretation of the results is often convoluted. For example, consider two rodents, A and B. Rodent A typically has high levels of intake, whereas rodent B displays low levels of intake. Given rodent A’s higher intake, we would assume that A finds alcohol more “pleasurable” and therefore is more likely to consume it. However, is alcohol more “pleasurable” to rodent B because they reach the same rewarding effects at a much lower amount of alcohol (and consequently exhibit reduced intake)? This is impossible to tell using two bottle choice. Additionally, are the aversive effects of alcohol (such as the bitter taste) more prevalent in rodent B? Consequently, this technique is often considered a rough measure of “liking” and provides limited information regarding the motivational (“wanting”) component of alcohol reward because the effort to consume alcohol is minimal (Ripley & Stephens, 2012). Therefore, this technique should primarily be used to assess the “liking” component of reward behaviour and, techniques such as the operant self-administration model, may be more suitable to assess the “wanting” component of reward.

Conditioned place preference assesses the “wanting” component of drug-induced reward as well as memory (Bardo & Bevins, 2000). In brief, it measures the ability of a drug to cause preference for an environment in which the drug is paired compared to an environment in which the subject receives vehicle control. Conditioned place preference is relatively easy to perform and can generate replicable dose-response effects. And while conditioned place preference lacks face validity, it has demonstrated construct validity and has reinforced findings relating to the “rewarding” effects of

alcohol in the two-bottle choice tests (Huston et al., 2013; Bardo & Bevins, 2000). However, this procedure is sensitive to methodological variations for example, the "preference" for a chamber changes reflecting the duration of the test; differs across species; and the psychological underpinnings are not well understood (it involves learning, spatial discrimination, memory, reward) (Sanchis-Segura & Spanagel, 2006). Therefore, it is unclear how minor methodological variations may alter the psychological processes and consequently, the final outcome may not reflect reward behaviour (Sanchis-Segura & Spanagel, 2006). However, these two paradigms are among the most replicable and feasible used in addiction research. Experiments using more complex models such as operant self-administration should be performed in the future to validate and expand upon the results of this study.

A final caveat to consider when examining reward-like behaviour in rodents is the background strain of the rodents. Typically, studies investigating the rewarding (or anti-rewarding) properties of alcohol use alcohol-preferring mice such as the C57BL/6J strain. This strain has an inherit predisposition to exhibit exaggerated responses towards alcohol such as high alcohol intake (Yoneyama et al., 2008). When assessing the effects of an intervention, the behavioural response may be exaggerated or minimised owing to floor or ceiling effects owing to C57BL/6J's genetic predispositions, which in turn, mitigate or enhance the effect of intervention. Consequently, C57BL/6J mice may be used as a model designed to reflect humans who are genetically predisposed to developing alcohol dependence. By contrast, the studies presented in this thesis used Balb/c mice a strain of mice which does not exhibit preference or avoidance towards alcohol (Yoneyama et al., 2008). Consequently, the effects of an intervention may be subtler as they do not exhibit exaggerated behaviours towards alcohol.

8.6 The effects of TLR4 on reward

The role of the TLR4 pathway in reward behavior (as inferred by the results presented in this thesis) are complex and depends upon brain region examined, ontological period and duration of alcohol exposure. Therefore, to ensure clarity in the ideas presented, the discussion of the results will focus on each study individually rather than considering whether TLR4 is involved in all reward behaviours.

8.7 The effects of TLR4 on acute reward

8.7.1 Action of (+)-Naltrexone

There is a growing body of evidence implicating TLR4 in the acute rewarding effects of alcohol (Harris et al., 2017; Mayfield et al., 2016). However, the results are far from uniform with studies demonstrating TLR4 reduces or has no effect on reward behaviour in rodents (Harris et al., 2017). The differing conclusions are likely attributable to the differences in strain and the method of attenuating TLR4 and inferring reward behaviour. The results presented in this thesis suggest (+)-Naltrexone reduces both the “liking” and “wanting” components of alcohol reward, as inferred by a reduction in the amount of alcohol consumed and time spent in the conditioned environment during the two-bottle choice tests and conditioned place preference tests respectively. However, (+)-Naltrexone additionally reduced saccharin intake – an effect consistent with other TLR4 antagonists (Bajo et al., 2016). This suggests TLR4 is pivotal to the normal homeostatic functioning of the reward pathway as blockade of TLR4 reduces the ability to feel reward immediately following an innately likable solution.

The effect of TLR4 on reducing the “wanting” component of reward is potentially attributable to a downregulation of tyrosine hydroxylase (*Th*) mRNA – the key enzyme responsible for dopamine synthesis. This supports the observations demonstrating that TLR4 regulates the expression of TH in the VTA via PKC and CREB (Aurelian et

al., 2016). Further, (+)-Naltrexone and (+)-Naloxone, a chemically and functionally similar compound, reduced cocaine and morphine-induced dopamine release in the nucleus accumbens within 10 minutes of administration (Northcutt et al., 2015; Hutchinson et al., 2012). This suggests TLR4's effect on the dopamine system is not limited to gene regulation but also occurs at the level of protein/neurotransmitter release as well. Whether this effect occurs with alcohol is an important experiment which needs to be performed in the future.

Having a drug which blocks the rise in dopamine following acute alcohol exposure may be beneficial as it prevents the transfer of motivational significance towards alcohol (or any drug of abuse). This would prevent the increase in the "wanting" component of reward; a key feature assisting the transition from occasional drinking to dependence (Berridge & Robinson, 2016). This may also prove fruitful for other disorders which have a motivation or a learnt aspect to them such as phobias. Unfortunately, drugs which exclusively block dopamine signalling following long-term alcohol use have failed to prevent relapse or alcohol drinking in humans. Further these drugs have pronounced adverse side effects such as anhedonia and loss of enjoyment in individuals (Swift, 2010). Consequently, the timing of administration is crucial to prevent the transfer of motivational importance following alcohol use and may therefore be only beneficial for people at risk of developing, but do not currently have an alcohol use disorder.

It is unclear how (+)-Naltrexone, and other TLR4 antagonists modify the "liking" component of reward. Given the "liking" component is a rapid molecular event, it is presently unclear whether alcohol-induced neuroimmune signalling could occur on this time scale given that four doses of alcohol are required before neuroimmune protein changes are observed (Qin et al., 2008). Despite this, (+)-Naltrexone reduced

saccharin and alcohol intake after the second day of consumption in the 2 h limited access paradigms (post hoc analysis). Importantly, the intake in g/kg was significantly less than that of the oral gavages required to elicit a neuroimmune protein change by alcohol (as inferred by Qin et al., (2008)) and it is unclear whether or how basal neuroimmune signalling modifies a response from a non-drug of abuse like saccharin. This suggests that (+)-Naltrexone may be modifying the release of protein from glia or is directly modifying basal/or induced TLR4 signalling on neurons altering their function and subsequent neurotransmitter output. This in turn would modify the behavioural response to alcohol and saccharin.

Alternatively, Thomas et al. (in preparation) demonstrated *in silico* that (+)-Naltrexone binds to the μ opioid receptor (a key component of "liking") at high micromolar concentrations. Further the binding energy was similar to DAMGO, a synthetic high affinity μ opioid receptor agonist. This study additionally demonstrated (+)-Naltrexone at high concentrations can displace [H^3]Diprenorphine in HEK μ opioid receptor over-expression cell lines and rat brain membrane preparations. However, caution must be used when interpreting these findings as the concentrations of (+)-Naltrexone are much greater than those used *in vivo* and result in saturation causing non-specific binding.

The concentrations of (+)-Naltrexone used in the aforementioned studies are thought to be insufficient to antagonize μ opioid receptor. Interestingly, a metabolite of Naltrexone, 6-β-Naltexol, is a μ opioid receptor antagonist *in vitro* and *in vivo* (Akala et al., 2008; Rodgers et al., 1980; Dayton & Inturrisi, 1976; Malspeis et al., 1975). Importantly, peripheral injection of 6-β-Naltrexol significantly reduces alcohol and sucrose drinking in rodents. However, higher concentrations were required compared to Naltrexone (Stromberg et al., 2002). Given that Naltrexone has a half-life of

approximately one hour in rodents, it raises the possibility that some of the effects such as reduced alcohol drinking and saccharin drinking in the acute studies (where (+)-Naltrexone is administered up to 8 times over the course of the tests) may be attributable to 6-β-Naltexol and not to the parent compound. However, whether 6-β-Naltrexol is found within the brain at physiologically relevant concentrations and is in the correct stereochemistry to exert biological activity following administration of (+)-Naltrexone remains to be determined.

Given the effects on the "liking" component of reward, the translational applicability of (+)-Naltrexone is questionable. Under naïve circumstances, alcohol is consumed principally for its euphoric properties – an effect attributable to the "liking" component of reward. Using (+)-Naltrexone to attenuate this effect is unlikely to be well received for occasional drinkers (unlike the benefit of attenuating dopamine) as it blocks the desired pharmacological effects. However, it may serve a purpose for teenagers or people at risk for developing an alcohol use disorder. By blocking the "liking" component of reward, the very reason to drink is removed potentially preventing the desire to consume more.

8.7.2 Influence of circadian rhythm

This is the first study to highlight how the efficacy of a TLR4-TRIF antagonist on alcohol-induced reward is dependent on circadian rhythm. While it may seem obvious that (+)-Naltrexone's effect would be greatest during the phase in which rodents consume the most alcohol - as it is not limited by floor/ceiling effects owing to low levels of intake observed during the day; it does not explain why the preference ratio or conditioned place preference was reduced. In this thesis, it was postulated that the circadian fluctuation in genes pertaining to TLR4-TRIF pathway, thirst and the "liking"

and “wanting” components of reward underlie the altered efficacy of the drug. However, it is unclear whether and how this finding would impact human drinking behaviour.

Interestingly specific polymorphisms within clock-related proteins such as PER and BMAL1 are correlated with alcohol consumption, abuse and anxiety in humans (Perreau-Lenz & Spanagel, 2015 for review) and like rodents, chronic alcohol consumption in humans disrupts the rhythmic expression of CLOCK proteins (Huang et al., 2010). Similarly, dopamine concentrations fluctuate throughout the day in humans, as does the function and cellular distribution profile of the immune system (Korshunov et al., 2017; Boivin, 2003). However, unlike rodents which exhibit a greater preference and intake of alcohol corresponding to the phase in which dopamine transmission and immune signalling is highest, humans do not. Rather, the acute consumption of alcohol is constrained by environmental factors such as work, school or social commitments. Consequently, alcohol intake usually occurs when humans have free times which is typically in the evening or afternoon (heading towards the inactive phase); a time of enhanced inflammatory immune activity and metabolism (Marcheva et al., 2013; Lange et al., 2010).

This study adds to the growing field of chronotherapeutics: a field which considers circadian fluctuation in immune and neuronal behaviour as well as absorption, distribution and metabolism to determine the most effective time to administer a drug. Links between circadian rhythm, inflammation and disease severity have already been studied in the context of rheumatoid arthritis (RA). Studies have demonstrated that administration of glucocorticoids to coincide with peak nocturnal rise in IL-6 significantly reduces joint stiffness and pain compared to when the same dose is taken in the morning. It is additionally hypothesised that these rhythmic fluctuations in cytokines may also be influencing psychiatric disorders which are comorbid with RA,

such as depression (Buttgereit et al., 2015). The study in this thesis is the first to demonstrate that the efficacy of a TLR4 antagonist is dependent on time-of-day. Future studies are required to determine whether the efficacy of clinically approved drugs to treat alcohol dependence depend on the time-of-day.

8.8 The effects of TLR4 on alcohol priming

The results from chapter 3 highlighted that acute exposure to alcohol increases the expression of TLR4 and its immune mediators, and that attenuation of TLR4-TRIF pathway reduces reward behaviour. The proceeding chapter sought to determine whether the rise in immune mediators persists for several weeks post exposure and if they subsequently contribute to reward behaviours later in life. Adolescent mice were examined as the effects of alcohol can be particularly detrimental during this developmental period. Further, unlike adults, adolescents often have significant abstinent periods from alcohol.

Few studies have examined how TLR4 influences the molecular and behavioural consequences of alcohol-induced sensitisation during adolescence. The results presented in chapter 5 largely contrast those by Montesinos et al., (2016). Their study demonstrated adolescent alcohol exposure potentiated the “wanting” component of reward and anxiety behaviour but did not alter “liking” later in life as indicated by the 48 h two bottle choice, elevated plus maze and saccharin intake respectively. Importantly, the effects of adolescent alcohol exposure were nullified in TLR4$^{-/-}$ mice. In contrast, our study demonstrated that adolescent alcohol exposure potentiated both alcohol “wanting” and “liking” but it did not alter anxiety behaviour later in life. Further, attenuating TLR4 prevented alterations to the “liking” (inferred by drinking in the dark) but not “wanting” component of reward nor did it alter anxiety behaviour. The discrepancies between the two studies are likely attributable to the strain of rodents,

the behavioural paradigms, genetic vs pharmacological antagonism of TLR4 and perhaps most importantly, the adolescent model of alcohol exposure. This highlights the need for further studies in this area. The study by Montesinos et al., (2016) used high doses of alcohol, and alcohol was administered for twice as long compared to the current study. This would likely have engaged the neuroimmune system more and could potentially recruit other neurological substrates which are more resilient to lower doses of alcohol such as the amygdala. This in turn would sensitise adolescence to reward and anxiety behaviours later in life to a greater extent than our study.

By contrast, the model used in this study was designed to more closely resemble what occurs in humans. Most adolescents report consuming alcohol infrequently (NIAAA, 2017). The infrequent use may serve to limit the neuroimmune response towards alcohol thereby reducing the adverse effects associated with adolescent alcohol use – an effect mirrored in this study (no anxiety alterations were observed). Further, adolescent humans are more resilient towards the effects of alcohol than their rodent counterparts (Doremus-Fitzwater et al., 2015; Kane et al., 2013). While on average, most rodents who consume high levels of alcohol go on to develop behavioural deficits associated with alcohol use later in life, the same cannot be said for humans (McCambridge et al., 2011). Most adolescents who binge drink do not develop problems associated with alcohol use later in life. This is because neurodevelopment and alcohol dependence are multifactorial and are influenced by environmental and genetic processes. By contrast, laboratory mice which are genetically homogenous and reared in the same environment are likely to exhibit similar developmental and alcohol-related outcomes.

In preclinical settings chronic high levels of alcohol are commonly administered resulting in profound neurodevelopmental alterations (Ward et al., 2014). This is

particularly problematic because this manner of alcohol exposure in rodents can exaggerate the behavioural and molecular consequences of alcohol use and thus no longer reflect what is occurring in humans. The problem is furthered as neurobiological models of human adolescent alcohol use are largely based upon results from rodent studies. Therefore, this study aimed to achieve better consilience between rodents and human's alcohol exposure outcomes.

The results from this study suggest that (+)-Naltrexone failed to modify adolescent alcohol potentiated "wanting" behaviour later in life despite a reduction in tyrosine hydroxylase mRNA. The increased "wanting" for alcohol imparted by adolescent alcohol exposure may be attributable to the persistent increases in dopamine receptors and the downregulation of GABA receptor mRNA, indicating that the reward pathway is sensitised towards dopamine and lacks inhibitory control respectively. By contrast $TLR4^{-/-}$ mice are protected against potentiated cocaine-induced CPP and 48 h alcohol intake imparted by adolescent alcohol exposure. This raises the possibility that either or both the MyD88 and TRIF pathways are required for the sensitization of the "wanting" component of alcohol reward. This suggests TLR4-TRIF may not be involved in the sensitisation of alcohol "wanting" or is part of a larger system which collectively regulates this aspect of reward. It is important to note that, the genes assessed only represent a small part of all the processes which are potentially modulated by alcohol. Thus, there may be other mechanisms which underlie why the increased "wanting" but not "liking" component of alcohol reward remained elevated.

(+)-Naltrexone successfully reduced the adolescent alcohol potentiated "liking" component of reward as inferred by a reduction in alcohol intake during the drinking in the dark tests. These effects are unlikely due to the downregulation of *Th* and *Gabra2* mRNA. Rather the reduction in liking is likely related to the decreased expression of

Tlr4 or *Ifnb (*assuming the increases in mRNA translated to increases in protein). The mechanism behind how an IFNβ signal modulates the "liking" component of reward remains to be fully determined. However, associative evidence suggests IFNβ directly interacts with opioid and cannabinoid systems, key molecular mediators of "liking" (Downer et al., 2012; Lee et al., 2010; Wang et al., 2006; Jiang et al., 2000).

The persistent upregulation of inflammatory mediators following adolescent alcohol exposure likely results from epigenetic remodelling of TLR4-related gene activity. Given these mediators remained elevated into neurobiological maturity it reinforces the findings that alcohol exposure during adolescence can result in long-lasting consequences. However, it is unclear whether, or how much, alcohol is required in humans before this type of remodelling (which is typically observed in rodents) occurs – a topic for future experiments.

A key advantage of this study was the implementation of a pre- and post-treatment paradigm of (+)-Naltrexone. Pretreatment paradigms often have limited applicability in humans. However, they often provide insight into specific mechanisms in a preclinical setting. Despite this, few studies have evaluated how antagonists or agonists function once the pathology has commenced. Results from this study indicated there was no difference between the pre- and post-treatment paradigms regarding the behavioural or molecular endpoints (effect of order, $p > 0.05$). This indicates that attenuating TLR4 signalling post alcohol exposure can still be beneficial to reduce increased alcohol "liking" and inflammatory gene transcription later in life: increasing the therapeutic relevancy of (+)-Naltrexone. However, 30 minutes is a relatively short time frame post exposure. Thus, it is likely that TLR4 signaling events triggered by alcohol are ongoing. For example, *in vitro* studies have shown TLR4-MyD88 interactions are ongoing 30 min post alcohol exposure (Fernandez-Lizarbe et al., 2009). It would be interesting to

determine how far out from the initial exposure (+)-Naltrexone remains effective and whether adolescents would take a prophylactic medication given their risk-seeking nature.

A key limitation of the studies in chapters 3, 5 and 7 is that sex differences were not widely explored or powered intentionally for specific analysis. A brief statistical analysis indicated there was no effect of sex on any behavioral or molecular outcomes in chapter 5. However, this finding largely contrasts those in both humans and rodents. For example, females consume more alcohol at a faster rate, exhibit heightened “liking” and “wanting” following alcohol exposure, display greater motor and cognitive impairment, have more severe withdrawal symptoms and are more likely to relapse sporadically than males (Foster et al., 2015; Schulte et al., 2009; Walter et al., 2005; Wilsnack et al., 2000). Further, female adolescent mice appear more sensitive to the long-lasting consequences of drugs of abuse with greater risk of consuming alcohol later in life (Mateos-García et al., 2015).

Numerous factors have been attributed to sex differences including reproductive hormones, GABA, glutamate and endogenous opioid signalling. However, sex differences may also be attributable to alterations in TLR4-based neuroimmune activity. For example, female mice mount more robust immune responses than males, with increases in pro-inflammatory cytokines and glial activation following LPS (Doyle & Murphy, 2016). Further, in response to acute alcohol exposure, female mice exhibit more pronounced neuroinflammatory responses with greater elevations in glial reactivity, TLR4, IL-1β, TNFα, COX-2, iNOS and NFκB p65 expression in brain regions associated with reward (prefrontal cortex, VTA and nucleus accumbens) compared to males (Baxter-Potter et al., 2017; Pascual et al., 2016; Alfonso-Loeches et al., 2013).

However, whether these effects remain following chronic alcohol administration remains to be determined.

Few studies have examined the effect of sex on TLR4, alcohol reward and anti-reward behaviours. Blednov et al., (2017b) demonstrated female TLR2$^{-/-}$ mice drank less than female wildtype mice – an effect absent in male mice. Further MyD88$^{-/-}$ male mice drank less than wildtype whereas female MyD88$^{-/-}$ did not. However, these authors did not compare male vs female drinking, limiting the findings of their results. Future studies are therefore required to fully elucidate the role sex-of-animal plays in TLR4-induced reward behaviour.

8.9 TLR4 alcohol and chronic reward and anti-reward

Long-term alcohol use is associated with TLR4-dependent neuroplastic and epigenetic events, which assist in desensitising the reward pathway, recruiting the anti-reward pathway and from a neuroimmune perspective increase the likelihood of transcribing genes pertaining to the TLR4 pathway. The increase in TLR4 and its immune mediators further alter and augment reward and anti-reward behaviours respectively (in addition to the pre-existing neuronal changes which drive these behaviours). Thus, targeting TLR4 may alleviate some of the symptoms of acute withdrawal and lessen the rewarding components of alcohol prior to withdrawal.

Given that (+)-Naltrexone reduced the immediate and priming of immune mediators and this coincided with a reduction (although not always consistently) in reward-like behaviour (conditioned place preference and two-bottle choice), we sought to investigate whether (+)-Naltrexone could reduce the rise in immune mediators following long-term alcohol exposure and whether this influenced reward and anti-reward behaviours before and during withdrawal. Consistent with Harris et al., (2017),

pharmacologically targeting the TLR4-TRIF pathway failed to reduce the “liking” or “wanting” of alcohol as inferred by sucrose and alcohol two-bottle choice tests before and during withdrawal. Further, targeting the TLR-TRIF pathway did not modify sucrose preference or intake (measures of anhedonia) or elevated plus maze performance (measure of anxiety) during withdrawal despite a reduction in immune mediators in the amygdala and nucleus accumbens. This highlights that acute blockade of TLR4-TRIF cannot overcome existing neuroplastic and epigenetic events which confer reduced and enhanced reward and anti-reward behaviours respectively. Interestingly, global knockout of TLR4 reduces alcohol drinking, sucrose deficits, elevated plus maze and open field test performance (indicators of reward and anti-reward behaviour) during and before protracted withdrawal (two weeks post alcohol) (Pascual et al., 2011) suggesting either the MyD88 or both the MyD88 and TRIF pathways are required to modify reward and anti-reward behaviours following long-term alcohol use. In support of this, TNFα, a cytokine which requires activation of the TRIF and MyD88 pathway for transcription, can sensitise mice to alcohol withdrawal and blocking TNFα can reduce CRF release in the amygdala, a key contributor of anti-reward and reward behaviour (following long-term alcohol use) respectively (Knapp et al. 2011).

There are many reasons why (+)-Naltrexone failed to modify reward and anti-reward behaviour (alcohol and sucrose preference, and elevated plus maze and open field test respectively). For example, the limited half-life of (+)-Naltrexone may result in a transient short-lived decrease in immune mediators; additional pattern recognition receptors, alcohol metabolites and stress hormones (see below) may additionally contribute to reward and anti-reward behaviours. Collectively these processes likely underscore the continual elevation in immune mediators thus propagating reward and anti-reward behaviours.

8.9.1 Role of alcohol's metabolites

A fundamental tenant of this study is that an alcohol-induced immune response (via TLR4) alters neurotransmission, plasticity and in turn reward and anti-reward behaviours. However, we are beginning to appreciate the role of ethanol's metabolites in the immunological and neurobiological effects induced by alcohol consumption.

Following acute intake, ethanol is metabolised in the liver to form acetaldehyde, a short-lived highly reactive compound, and then to acetate (Zakhari, 2006; Jones, 1991). Following long-term alcohol intake, ethanol is metabolised in other organs, including the brain (Zakhari, 2006). In addition, long-term alcohol use results in non-oxidative metabolism of alcohol increasing the number of metabolites for example, ethyl glucuronide, ethyl sulphate, free fatty acid esters such as ethyl pyruvate and phosphatidylethanol (a phospholipid bound to ethanol). Unlike acetaldehyde and acetate, these metabolites are long lasting, often persist for days to weeks and can be formed within the brain (Maenhout et al., 2013; Calabrese et al., 2001; Lundqvist et al., 1994). Given these findings, it has been hypothesised that reward and anti-reward behaviours are partly attributable to the effects of ethanol's metabolites.

Interestingly, mice readily self-administer and exhibit conditioned place preference towards acetaldehyde (see Deng & Deitrich, 2008; Quertemont & Didone, 2006 for review). Further, this compound is a 1000 fold more potent at generating reward-like behaviour compared to its parent compound (ethanol) when self-administered into the VTA (Rodd-Henricks et al., 2002). Acetaldehyde can also induce aversive responses (as measured by conditioned taste aversion), anxiety, memory impairment and loss of righting reflex (Quertemont & Didone, 2006). Consequently, acetaldehyde is considered a contributor to the development of alcohol dependence and addiction.

Further, this metabolite can induce an immune response by increasing the expression of cytokines such as TNFα and CCL2; ROS production; and transcription factors including NFκB and MAPKs (Ceni et al., 2014; Redmond et al., 2009; Gomez-Quiroz, 2003). Acetaldehyde is further hypothesised to cause intestinal permeability by disrupting tight junctions in the colon (Basuroy et al., 2005; Rao, 1998) and can act synergistically with LPS to potentiate immune response (Gutierrez-Ruiz et al., 2001). Given acetaldehyde acts in concert with LPS, it highlights a potential role of TLR4 in mediating this response. Thus, it raises the possibility that acetaldehyde increases TLR4 and neuroimmune activation, propagating ethanol reward and anti-reward behaviours.

In addition, the long-lived minor metabolites such as ethyl-glucuronide directly activate TLR4 inducing NFκB activation (Lewis et al., 2013). This in turn results in exacerbated pain which can be reversed by (+)-Naloxone (Lewis et al., 2013). Given TLRs pivotal involvement in the reward behaviours, it represents another mechanism whereby alcohol can cause long-term TLR4 activation.

8.9.2 Role of additional pattern recognition receptors

The mechanism underlying alcohol-induced TLR4 signalling remains to be determined. Early experiments hypothesised alcohol activated TLR4 directly (Blanco et al., 2005). However, subsequent studies have largely disproved this notion; instead studies have suggested alcohol promotes the release of HMBG1 which subsequently binds to, and activates TLR4 expressed on neurons and glia (Crews et al., 2017). However, the mechanism underlying the release of HMGB1 remains to be determined (I speculate it is likely due to alterations in osmotic stress or production of reactive oxygen species induced by local metabolism of ethanol) (Tsung et al., 2007). HMGB1 interacts with multiple pattern recognition receptors including TLR2, TLR4, TLR5, TLR9, RAGE and

the chemokine receptor, CXCR4 (Das et al., 2016; Yang et al., 2015; Yu et al., 2006) with the degree of engagement dependent on the reduced status of HMGB1 (Yu et al. 2006). Further, HMGB1 release unlikely accounts for the acute effects of alcohol-induced TLR4 signalling as *in vitro* studies have shown that HGMB1 is released 24 h post alcohol exposure (Lawrimore & Crews, 2017).

Long-term alcohol use additionally increases intestinal permeability. This causes the translocation of PAMPs from the colon into the blood and liver, inducing TLR activation and the release of inflammatory mediators primarily from macrophages. These mediators subsequently cross the blood brain barrier and are hypothesised to induce a neuroimmune response (Cui et al., 2014; Mayfield et al., 2013). Consequently, the neuroimmune response towards alcohol is complex and is unlikely to be solely mediated by TLR4 which may explain why acute attenuation of TLR4 did not alter reward or anti-reward behaviour following long-term alcohol use. In support of this notion, alcohol increases the expression of multiple parallel neuroimmune pathways. For example, alcohol significantly up-regulates the expression of TLR2 on microglia and TLR3, RAGE and TLR7 on neurons (Coleman et al., 2017; Lawrimore & Crews, 2017; Vetreno et al., 2013). Furthermore, the expression of TLR3 and RAGE also correlated with lifetime consumption of alcohol in humans (Vetreno et al., 2013). Taken together with the increase in expression of TLR4, this further highlights additional TLRs as key regulators of alcohol intake (Vetreno et al., 2013). However, few studies have considered the behavioural ramifications of the engagement of other TLRs in regards to alcohol's pharmacodynamics. $TLR2^{-/-}$ mice are protected against alcohol-induced cytokine and chemokine increases (Pascual et al., 2015) motor impairment and sedation (Blednov et al., 2017a; Corrigan et al., 2015). Further, $TLR2^{-/-}$ but not $MyD88^{-/-}$ or $TLR4^{-/-}$ mice consume significantly less alcohol but more saccharin compared to wildtype (C57BL/6J) mice across a range of drinking paradigms (Blednov et al., 2017b)

indicating that TLR2 modifies the aversive component or taste alcohol, as the “liking” component of reward was increased. Given that TLR2 exclusively signals through MyD88 it is unclear why $TLR2^{-/-}$, but not $MyD88^{-/-}$ mice, do not display altered drinking behaviour. If TLR2 is contributing to the reward and anti-reward behaviours following long-term alcohol use it, it may provide an additional reason as to why (+)-Naltrexone, a TLR4-specific antagonist, failed to modify these behaviours before, and during withdrawal. Therefore, future experiments should examine whether and how other TLRs contributes to reward and anti-reward behaviours.

8.9.3 Role of stress hormones

A key driver of anti-reward behaviours (anxiety and anhedonia) are stress hormones and neuropeptides such as CRF, ACTH, NPY and dynorphin. These are initially upregulated to limit the effects of alcohol on the CNS (opponent B) but remain elevated even in the absence of alcohol creating stress-like behaviours including anxiety and depression. Importantly, these hormones and neuropeptides interact with TLR4 and the neuroimmune system to exacerbate withdrawal symptoms. For example, TNFα and CCL2, cytokines produced by alcohol-induced TLR4 activation, sensitise mice to alcohol withdrawal and exacerbate symptoms of anxiety and depression (Harper et al., 2016; 2014; Ming et al., 2013). It is presently unclear whether and how the bidirectional relationship between stress and TLR4 augment withdrawal behaviours. Preliminary evidence demonstrates CRF augments the release of cytokines following TLR4 stimulation (Hu et al., 2016), and CRFR1A antagonism reduces alcohol-induced TLR4 and cytokine expression during withdrawal (Knapp et al., 2016; June et al., 2015; Whitman et al., 2013). Collectively these results suggest that in addition to its role in stress and anxiety during withdrawal from alcohol, CRF increases the production of TLR4-related cytokines and chemokines which further augment withdrawal-like behaviours (anxiety and relapse) in mice (Knapp et al., 2016; June et al., 2015).

8.10 Translational applicability

Modelling alcohol dependence and the consequential behavioural and molecular alterations in rodents is difficult (Ripley & Stephens, 2012). This problem is further confounded as there is no universally accepted theory of addiction nor is there likely to be a single theory which can adequately account for such a diverse, complex and multifactorial disorder. Further, we are only beginning to appreciate that like depression and autism, alcohol dependence is a spectrum disorder with different population subtypes more susceptible to specific triggers and treatments. While rodent models can model particular aspects of the disorder, the results obtained in these studies are subject to interpretation and may be modelling an entirely different phenomena and biological process than what is observed in the clinic. This may be a key reason why so many drugs have failed in clinical trials.

Preclinical research has demonstrated the importance of TLR4 and the neuroimmune system in models of alcohol dependence in rodents. It is unclear whether this system plays a role in the pathogenesis of alcohol dependence in humans. Post-mortem studies demonstrate increased glial reactivity in the prefrontal cortex, entorhinal cortex and hippocampus in alcohol dependent subjects compared to healthy controls (He & Crews, 2008). Alcohol dependent subjects exhibit increased expression of TLR2, 3, 4, RAGE and HMGB1 in the orbitofrontal cortex compared to controls; an effect which correlates with the estimated life-time consumption of alcohol (Vetreno et al., 2013). Furthermore, microarray data identified immune genes are differentially expressed in the brains of alcohol dependents compared to controls (Ponomarev et al., 2012; Lewohl et al., 2011; Jianwen Liu et al., 2005). Importantly, there are commonalities between the expression of neuroimmune-related genes in the brains of humans and mice suggesting similar pathways are activated between the two species following long-term alcohol use (Tabakoff et al., 2008; Mulligan et al., 2006). Of increasing

interest are pathways relating to NFκB (Ökvist et al., 2007). Okvist et al., (2007) demonstrated the expression of NFκB p65-p50 heterodimer and p50-p50 homodimers were increased in the brains of alcohol dependents compared to controls. Further, the DNA binding of NFκB was increased and 479 NFκB-dependent genes were differentially regulated between alcohol dependents and control individuals highlighting the potential importance of this transcription factor in alcohol addiction (Liu et al., 2009; Ökvist et al., 2007). However, no study has indicated whether neuroimmune signaling occurs within the human brain and whether this can modify reward or anti-reward behavior.

Alcohol dependent subjects additionally exhibit increased circulating LPS, a key mechanism which is thought to underlie neuroimmune activation. Alcohol renders the lumen of the intestines “leaky” enabling the translocation of microbial products such as LPS into systemic circulation. Binge drinkers and non-cirrhotic alcohol dependence have increased circulating LPS compared to individuals who drink in moderation (Leclercq et al., 2014; 2012; Ward et al., 2014) a finding mirrored *in vivo* (Mandrekar & Szabo, 2009). Furthermore, the immune system of binge drinkers and alcohol dependents were sensitized, exhibiting increases in cytokines and MAPKs. The rise in immune mediators was transient and resolved three weeks after withdrawal. During the withdrawal processes however, the expression of circulating cytokines correlated with alcohol consumption and alcohol craving scores (Leclercq et al., 2014; 2012). Interestingly, the anti-inflammatory cytokine IL-10 was inversely correlated with measures of anxiety, depression and craving three weeks post exposure, indicating a potent role of IL-10 in modulating the psychological responses about alcohol – an effect reinforced by *in vivo* observations (Marshall et al., 2016). This reinforces the hypothesis that cytokines cross the blood brain barrier and affect brain function pertinent to alcohol reward and anti-reward.

These caveats have not detracted researchers trialing a glial attenuator to treat alcohol dependence. The phase 1 clinical trial of Ibudilast demonstrated that the drug is safe and with few adverse effects. Initial experiments determined there was no effect of Ibudilast on the levels of craving, stimulation, sedation, positive mood, “liking” and “wanting” behaviour. However, Ibudilast decreased aspects of negative mood following alcohol exposure and was associated with mood improvements and reduced measures of stress and alcohol cue exposure and basal levels of craving. This effect was more pronounced in individuals with higher depressive symptoms but not higher alcohol use (Ray et al., 2017). Even though this study was not designed to address efficacy, Ibudilast attenuated measures mainly associated with relapse but not the rewarding properties of alcohol. This suggest that the neuroimmune activation in humans plays a much greater role in influencing withdrawal and craving than it does in reward. This idea is furthered by examining the efficacy of Ibudilast in opioid withdrawal in opioid addicts. Ibudilast reduced the withdrawal symptoms of anxiety, perspiration, restlessness and stomach cramps compared to the placebo control group (Cooper et al., 2015).

While Ibudilast has shown promise in its ability to mitigate aspects of withdrawal, it is unlikely (+)-Naltrexone will be used for alcohol dependence. (+)-Naltrexone was unable to modify aspects of reward or anti-reward behavior. Furthermore, given it downregulates tyrosine hydroxylase mRNA, it may worsen symptoms of anhedonia which are characterised by dopamine deficiency – thus propagating relapse.

8.11 Is TLR4 a good therapeutic target for the treatment of addiction and dependence?

From a pharmacological perspective, TLR4 is an attractive therapeutic target. This is due, in large part to the functional redundancies of Toll-like receptors. Each TLR

recognises specific molecular epitopes present on bacteria and viruses (Takeuchi & Akira, 2010). However, each bacteria and virus has thousands of epitopes each of which are recognised by various TLRs and other pattern recognition receptors. Consequently, attenuating one TLR may reduce the ability to detect specific parts of bacteria, yet additional TLRs can compensate for the loss, thus limiting the immunosuppressive effects of TLR antagonists (Bachtell et al., 2015; Akira & Takeda, 2004). This is particularly pertinent for individuals who consume alcohol, as this drug causes immunosuppression and immune dysfunction in the periphery. Unfortunately, antagonising TLR4 within the central nervous system is problematic. Traditional high affinity and specific TLR4 antagonists such as LPS:RS do not readily cross the blood brain barrier (Banks & Robinson, 2010). Consequently, lower affinity TLR4 antagonists such as (+)-Naltrexone and T543216 are used. These compounds are further limited by their short-half lives and potential off-target side effects thus limiting their efficacy (Wang et al., 2016; Chavez et al., 2011). A new longer lasting, high affinity TLR4 antagonist is therefore required.

(+)-Naltrexone attenuates the “liking” and “wanting” components of reward following acute alcohol use but has limited efficacy following long-term alcohol use. Therefore, (+)-Naltrexone may prove fruitful for preventing the transition from occasional use to dependence, or mitigate some of the detrimental effects of adolescent alcohol use. However, given this drug downregulates *Th* and *Gabra2* mRNA, it is unlikely to be beneficial following long-term exposure as these processes assist in creating anhedonia and compulsivity respectively (Wise, 2004). Future studies should determine whether higher doses or a longer duration of (+)-Naltrexone treatment is effective at reversing the increase in immune mediators and drinking behaviour following long-term alcohol use.

However, viewing addiction and dependence as a purely molecular disease is restrictive. Addiction and dependence arise from a plethora of reasons including family history, genetics, behavioural traits and socio-economic states and psychological reasons such as; impulsivity, detrimental learned coping mechanisms, low self-esteem, depression, stress, resilience and developmental maturity (for example Velez et al., 2017; Verdejo-Garcia et al., 2017; Yap et al., 2017; Hägele et al., 2014; Merrill & Read, 2010). Further, one's environment plays a pivotal role in the progression of alcohol addiction and dependence. For example, if an individual's motivator to consume alcohol is due to stress from work or home-life using a TLR4 antagonist or any molecular antagonist is unlikely to treat the underlying cause. Thus, any pharmacological approach should occur in conjunction with psychological counselling.

8.12 Conclusion

The studies carried out in this thesis aimed to better determine which TLR4 pathway was elevated following various alcohol exposure paradigms. Acute and chronic alcohol exposure increased all aspects of the TLR4 pathway including co-receptors, the MyD88 and TRIF pathway and endogenous agonists. In contrast, acute exposure followed by a period of deprivation resulted in a persistent increase in *Tlr4*, *Trif* and *Ifnb* suggesting that only the TRIF but not MyD88 pathway remains in a sensitised state.

This thesis additionally sought to address whether acutely attenuating the TLR-TRIF pathway via (+)-Naltrexone would successfully reduce the "liking" and "wanting" of reward generated by acute alcohol intake; the reward-priming effect of adolescent alcohol use; and the reward and anti-reward behaviours following long-term alcohol use. The results suggest (+)-Naltrexone reduced "liking" and "wanting" components of reward following acute use. However, (+)-Naltrexone had limited efficacy reversing the

reward sensitising effects of adolescent alcohol use and had no effect on anti-reward and the “liking” and “wanting” components of reward following long-term use. Consequently, this drug may be beneficial for people at risk of developing alcohol dependence by blocking the transition from impulsive to compulsive behaviour, but it is unlikely to have any therapeutic benefit for treating alcohol dependence.

8.13 References

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Appendix: Pharmacological characterisation of the action of alcohol and (+)-Naloxone in BV2 cells

9.1 Introduction

Neuroimmune activation has recently been implicated as a key mechanism underlying drugs of abuse-related behaviours including cognitive dysfunction, pain, anxiety, depression and addiction (Crews & Vetreno, 2015; Cui et al., 2014; Hutchinson et al., 2011). Particular emphasis has been placed on microglia as crucial mediators underlying drug-related behaviours (Cadet & Bisagno, 2014; Cui et al., 2014; Kovács, 2012). Microglia are the primary immune cells within the central nervous system and in response to drugs of abuse such as cocaine and opioids, they become activated and shift their phenotype to one more in line with an immune response (see Lacagnina et al., 2016; Coller & Hutchinson, 2012, for review). This typically manifests as elevations in cytokines, reactive oxygen species, proteases and immune-related transcription factors (for example, Liao et al., 2016; Wang et al., 2012). Crucially, drug of abuse-induced immune responses is lower in magnitude when compared to those induced by traditional immunogens such as the bacterial cell wall component, lipopolysaccharide (LPS) (Wang et al., 2012). The comparatively reduced immune response occurs within discreet brain regions (typically associated with addiction and depression) and are therefore thought to function more akin to neurotransmitters than their traditional inflammatory role (see Lacagnina et al., 2016 for review). This finding is further supported as attenuating cytokines, immune-related transcription factors or microglia alter neuronal signalling and in turn adverse behaviours induced by drugs of abuse such as addiction (Marshall et al., 2016a; 2016b; Bell et al., 2015; Beardsley et al., 2010; Bland et al., 2009; Hutchinson et al., 2009). Consequently, identifying the mechanism which underlies drug of abuse-induced microglial activation is of importance.

Crucially, the mechanism underlying drug-induced microglial activation is beginning to be elucidated. Both opioids and cocaine are thought to bind to Toll-like receptor 4 (TLR4), a pattern recognition receptor, originally characterised as the receptor detecting LPS (Northcutt et al., 2015; Hutchinson et al., 2010). TLR4 is broadly expressed throughout the central nervous system however, the highest level of expression is on microglia (Bsibsi et al., 2002). Binding to this receptor either on microglia or other neuroimmune cells results in the activation of two divergent signalling pathways; MyD88 and/or TRIF (Akira & Takeda, 2004). Activation of the MyD88 pathway leads to the activation of early phase NFκB and the upregulation of proinflammatory cytokines such as IL-1β and TNFα. Activation of the TRIF pathway leads to late phase NFκB and IRF3 activation, and the upregulation of type one interferons and anti-inflammatory cytokines (Akira & Takeda, 2004). While other neuroimmune cells additionally express TLR4 it is unclear whether they possess the necessary co-receptors and signalling pathways to induce a prototypical immune response. Hence the activation of TLR4 on these cells may result in a different outcome when compared to a microglial TLR4 response (Okun et al., 2011).

Both opioids and cocaine are thought to bind to TLR4 activating the MyD88 and TRIF pathways (Northcutt et al., 2015; El-Hage et al., 2011; Hutchinson et al., 2010). However, the TLR4 response towards other drugs of abuse, such as alcohol is ambiguous with *in vitro*, *ex vivo* and *in vivo* studies reporting conflicting results. For example, studies have shown that alcohol activates both MyD88 and TRIF pathways; potentiates but does not initiate a TLR4 signal; or attenuates a TLR4 response (Lawrimore & Crews, 2017; Marshall et al., 2013; Goral et al., 2011; Fernandez-Lizarbe et al., 2009; Qin et al., 2008; Lee et al., 2004).

Consequently, an aim of this study was to determine whether alcohol induces or modulates an immune response *in vitro* with microglia-like cells (BV2 cells). Given TLR4's pivotal role in the immune activating actions of opioids and cocaine, this study further sought to examine how attenuating TLR4 may influence the immune response elicited by alcohol. To address this aim, both traditional and novel TLR4 antagonists were utilised. Although traditional TLR4 antagonists such as LPS:RS are potent TLR4 modulators, their poor brain penetrance limits their translatable efficacy (Banks & Robinson, 2010). By contrast, (+)-Naloxone, an enantiomer of (-)-Naloxone has high brain penetrance and therefore represents a more translatable antagonist (Wang et al., 2016; Selfridge et al., 2015). Interestingly, recent research has demonstrated that (+)-Naloxone is a biased TLR4 antagonist; selectively inhibiting the TLR4-TRIF pathway (Wang et al., 2016). Whether LPS:RS exhibits biased antagonism remains to be fully determined.

9.2 Methods

9.2.1 Cell culture

BV2 microglia-like cells (Blasi et al., 1990) were maintained in Dublecco's Modified Eagle Media (DMEM) (Sigma-Aldrich, NSW, Australia) supplemented with 10 per cent (v/v) Fetal Bovine Serum (Life Technologies, CA, USA), 2mM L-glutamine (Sigma-Aldrich), 50U/mL Penicillin + 50ug/mL Streptomycin (Sigma-Aldrich) and 100ug/mL Normocin (Invivogen, CA, USA). Cells were grown in a humidified incubator (5 per cent CO_2/ 95 per cent air) at 37^{o}C.

BV2 cells were seeded at differing densities reflecting the molecular analysis endpoint. For cell viability, immunocytochemistry, protein and mRNA isolation experiments, cells were seeded at 1x10^{4}cells/well in 96 well plates, 2x10^4 cells/well in 24 well plates and 3x10^5 cells/well in 6 well plates respectively.

9.2.2 Drugs

(+)-Naloxone, a pharmacological TLR4 antagonist was synthesised and supplied by Dr Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute of Alcohol Abuse and Alcoholism, MD, USA). Ethanol (99.5%) (herein referred to as alcohol) was purchased from Chemsupply (Gliman, SA, Australia). Ultrapure Lipopolysaccharide and Lipopolysaccharide from *Rhodobacter sphaeroides* (LPS:RS) were purchased from Invivogen. All drug stocks were made in phosphate buffered saline (PBS) and working solutions were diluted in serum free media (DMEM + L-glutamine).

9.2.3 Experimental Design

9.2.3.1 Immunological characterisation of alcohol

The first series of experiments aimed to determine whether alcohol can induce an immune response in BV2 cells. BV2 cells were incubated with 100mM of alcohol for 0.5, 2, 4 or 24 h reflecting the desired molecular analysis (figure 1a). LPS (100ng/mL), a known TLR4 agonist, and volume matched PBS (vehicle) were included as positive and negative controls respectively. The concentration of alcohol selected for this study was based upon previous findings demonstrating that this concentration successfully upregulates proteins and mRNA belonging to the TLR4 pathways (Lawrimore & Crews, 2017; Fernandez-Lizarbe et al., 2009). Similarly, the duration of alcohol exposure was based upon previous studies indicating TLR4-related cytokines and transcription factors were upregulated approximately 4 and 24 h post exposure (Lawrimore & Crews, 2017; Fernandez-Lizarbe et al., 2009). The concentration of LPS was selected based upon a dose response (supplementary figure s1 and s2) demonstrating moderate expression of cytokines with comparatively low amounts of cell death.

A second series of experiments aimed to determine whether alcohol modulates a TLR4 response in BV2 cells. BV2 cells received a pretreatment of alcohol (100mM) or vehicle (volume-matched PBS) for 0.5 h, after which, LPS (100ng/mL) or vehicle (volume-matched PBS) was added to the wells. Cells were incubated in the pretreatment + treatment (co-treatment) for a further 24 h (figure 1b).

9.2.3.2 Characterisation of (+)-Naloxone's pharmacology

To ascertain how (+)-Naloxone modulates TLR4 signalling BV2 cells were pretreated with (+)-Naloxone (100 – 800 uM), LPS:RS (200ng/mL) or vehicle (volume-matched PBS) for 0.5 h after which LPS (100ng/mL) or vehicle (volume-matched PBS) was added to the wells. Cells were incubated in the co-treatment for a further 0.5, 2, 4 or 24 h depending on the experiment (figure 1c).

9.2.4 Protein isolation and western blotting

Following completion of the drug treatments, the supernatant was collected and spun down (500g for 10min at 4°C) to remove any suspended cells. The supernatant was subsequently aliquoted into separate tubes without disturbing the resulting pellet. The supernatant was stored at -80°C.

After removal of the supernatant, adherent cells were washed 3 times with ice cold PBS. Cells were subsequently incubated with 100uL of RIPA buffer on ice for 10 min. Cell lysates were then harvested, placed into 1.5mL tubes, and mixed using a rotatory mixer for 1 h at 4°C. Cell lysates were then centrifuged for 10 min at 20,000g (4°C) to remove cellular debris. The supernatant was transferred to a new tube and protein concentration was quantified.

Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, MA, USA) as per manufacturer instructions. Protein samples were normalised to 1.25ug/uL by diluting sample in Laemmli and RIPA buffer. Samples were then stored at -80°C.

Twenty-five micrograms of total protein (cell lysates) and 60uL of supernatant were resolved onto 4 – 12 or 12 per cent gradient bis-tris gels (Bolt™, Thermo Fisher) for 5 min at 180 V followed for a further 38 min at 200 V. Samples were subsequently transferred to a nitrocellulose membrane (BioRad, CA, USA) at 20 V for 1 h. After the transfer was complete, the membrane was stained for protein using Ponceau S (Sigma), washed and blocked with 5 per cent skim milk in Tris-buffered saline for 2 h at room temperature. The membrane was then stained with the following primary antibodies overnight at 4°C; Actin (Sigma-Aldrich A2066, 1: 2000), CCL5 (ab10394, Abcam, Cambridge, UK, 1:250), IRF3 (ab68481 Abcam, 1:1000), IFNβ (ab85803, Abcam, 1:1000) and NFκB p65 subunit (ab16502, Abcam,1:500). The following day, the membrane was washed 3 times with Tris-buffered saline + 0.05% Tween20 (Sigma) and then incubated with donkey anti-rabbit and goat anti-rat antibodies containing 700 or 800nm fluorophores (925-68073 and 925-32219, Li-Cor, NE, USA, 1:10,000) for 1 h at room temperature. The membranes were developed using an Odyssey Imaging System (Li-Cor).

9.2.5 Enzyme-linked immunosorbent assay (ELISA)

The expression of IL-1β and TNFα from cell supernatant and lysates were analysed using Mouse IL-1β and Mouse TNFα ELISA MAX™ kits (BioLegends San Diego, CA, USA) as per manufacturer instructions. Samples were diluted in a ratio of 1:10 (sample:assay diluent) for the TNFα ELISA. Absorbance was measured at 450nm with 540nm reference using Synergy MX plate reader (Biotek, VT, USA).

9.2.6 mRNA isolation and PCR

RNA was isolated using Maxwell® 16 LEV simply RNA Tissue Kit (Promega, Madison, WI, USA) as per manufacturer instructions. RNA was quantified using spectrophotometric analysis, with the quality of RNA verified by the OD260/280 ratio. 900 ng of RNA was reversed transcribed into cDNA using iScript^{TM} cDNA reverse transcription kit (BioRad, Hercules, CA, USA) as per manufacturer instructions.

Gene expression was assessed using iTaq^{TM} Universal SYBR® Green Supermix (as per manufacturer instructions). Real time PCR was performed using the CFX96 Touch^{TM} Real-Time PCR Detection System (BioRad). All primers were synthesised by Integrated DNA Technologies Pte. Ltd. (Baulkham Hills, NSW, Australia) with their sequences outlined in the supplementary materials (Table 1).

The relative difference in expression level of each of the genes of interest were normalised to the CT of GAPDH for both the test and control sample. The ΔCT of the test sample was normalised to the ΔCT a control sample (equal amount of cDNA from all samples), and then expressed as a ratio ($2^{-\Delta\Delta CT}$). This determined the relative fold change in expression.

9.2.7 Immunocytochemistry

9.2.7.1 Immunocytochemistry protocol

The translocation of NFκB p65 subunit and IRF3 was quantified using immunocytochemistry. In brief, BV2 cells were plated onto poly-D-lysine coated cover slips. After 24 h, cells underwent their respective treatments and were subsequently stained with Wheat Germ Agglutinin 633 (Molecular Probes, OR, USA) for 10 min at 37°C and fixed using 4 per cent paraformaldehyde + 5 per cent sucrose for 10 min at room temperature. Cover slips were washed 3 times with PBS, blocked with 5 per cent

skim milk for 10 min and stained for NFκB p65 subunit (ab16502, Abcam,1:500) or IRF3 (ab68481, Abcam, 1:200) overnight at 4°C. Secondary antibodies (donkey anti-rabbit IgG 488, Invivogen, 1:1000) and nuclear stain DAPI (Invivogen 1:10000) were then applied to the coverslips for 1 h at room temperature. Coverslips were then washed 3 times in PBS and inverted onto microscope slides for imaging.

9.2.7.2 Quantification of translocation and colocalisation

9.2.7.2.1 Nucleus: total cell ratio

Using the WGA channel, 10 cells were selected and a line drawn along the longest diameter of the cell. Using the RGB profiler plugin in Fiji (Image J) (Schindelin et al., 2012), the edges of the nucleus and cytoplasm were delineated. The mean grey intensity value was then calculated for NFκB p65 or IRF3 channels within the nucleus (between the two DAPI peaks) and within the cytoplasm (between the WGA peaks – nucleus). To determine the ratio of nucleus to total cell intensity, the nucleus intensity was divided by the total mean grey intensity (nucleus + cytoplasmic). This generated a ratio where by $x > 0.5$ indicates the greatest intensity resides in the nucleus; $x < 0.5$ indicates the greatest intensity resides in the cytoplasm; and $x = 0.5$ indicates equal intensity in the nucleus and cytoplasm.

9.2.7.2.2 Pearson's correlation coefficient and Spearman's Rank Value

To verify the results obtained using RGB profiler and pixel intensity, Pearson's correlation coefficient and Spearman's Rank Value were performed as per the coloc2 plugin in Fiji. In brief, the correlations assess whether pixels from two separate channels (for example 405 (DAPI) and 488nm (NFκB p65 subunit and IRF3) channels) overlap in the same space relative to a set of randomised pixels.

9.2.8 Cell viability assays

9.2.8.1 Neutral red

The neutral red assay is a quantitative measurement of cell cytotoxicity *in vitro* and was performed as outlined by (Repetto et al., 2008). In brief, cells were incubated in neutral red medium (40ug/mL) for 2 h at 37^{o}C (5 per cent CO_2/ 95 per cent air), washed with PBS and incubated with the neutral red destain solution with gentle agitation. After 10 min, the plate was measured at 540nm using the Synergy MX plate reader.

The neutral red assay is based on the premise that living cells will retain the neutral red solution and therefore will produce higher absorption values at 540nm. By contrast, dead/dying cells do not retain the neutral red solution and will therefore exhibit lower absorption values.

9.2.8.2 Lactate dehydrogenase

The lactate dehydrogenase assay measures the cytosolic release of lactate dehydrogenase into the supernatant and is used to infer cellular injury (Danpure, 1984). It was performed as per manufacturer instructions (Piece LDH Cytotoxicty Assay Kit, Thermo Scientific). Absorbance was measured at 490 and 680nm using the Synergy MX plate reader. LDH activity was determined by subtracting the 680nm from the 490nm absorbance value. A low absorption value indicates the concentration of lactate dehydrogenase in the supernatant is small suggesting low amounts of cellular injury. Higher absorption values indicate greater cellular injury as more lactate dehydrogenase is present in the supernatant.

9.2.9 Alcohol concentration assay

The concentration of alcohol in the supernatant was measured using a commercial kit (ADH-NAD Reagent Multiple Test Vial; Sigma-Aldrich) and performed as per the

manufacturer instructions. In brief, it estimates alcohol-induced reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH in the presence of alcohol dehydrogenase. The reaction is observed by recording the absorbance of 340 nM from the solution. High and low levels of absorption indicate higher and lower amounts of alcohol in the supernatant respectively.

9.2.10 Statistical analysis

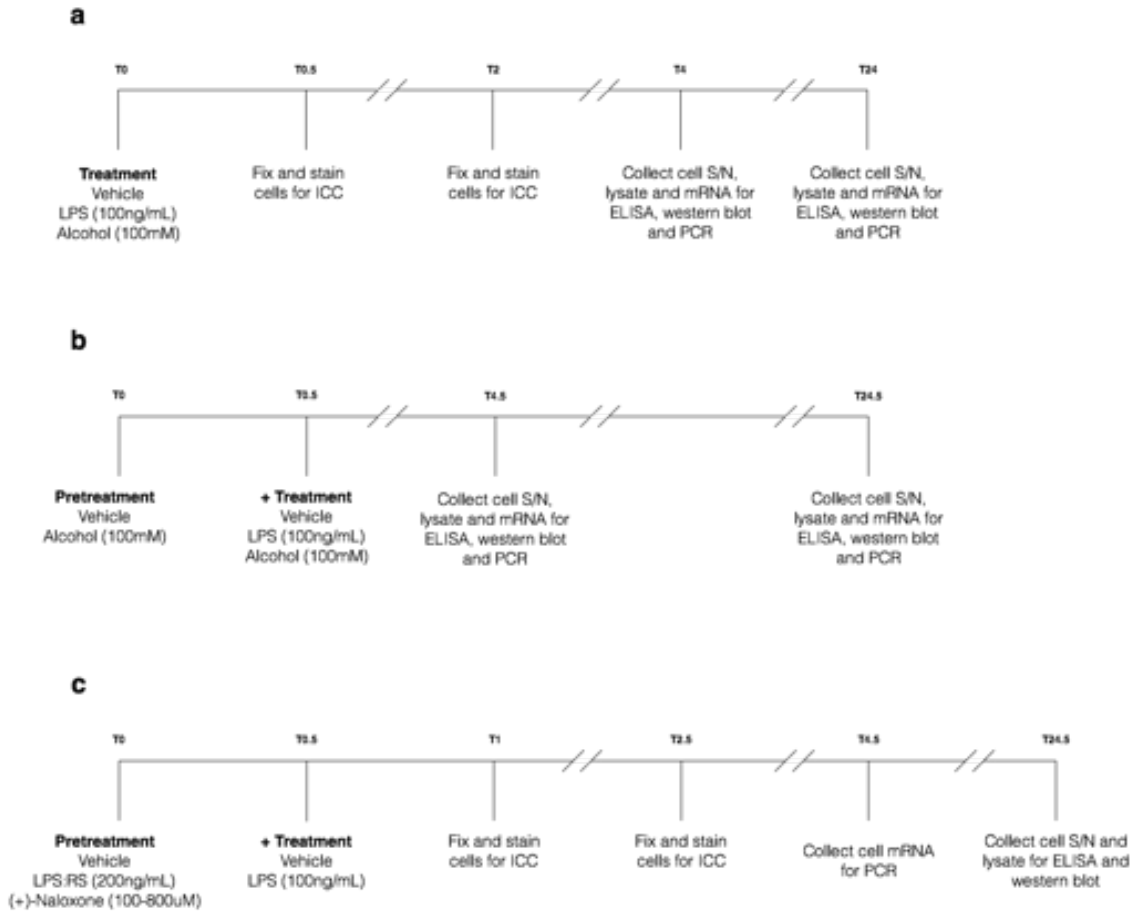
Experiments 1 and 2: immunomodulatory effects of alcohol

ELISA, western blot, immunocytochemistry, neutral red and lactate dehydrogenase assays were analysed using a two-way ANOVA with Tukey post-hoc (time x treatment or pretreatment x treatment). qPCR was analysed using a two-way ANOVA with Bonferroni post-hoc (time x treatment or pretreatment x treatment).

Experiments 3 and 4: characterisation of (+)-Naloxone

ELISA, western blot, immunocytochemistry, neutral red and lactate dehydrogenase assayswere analysed using a two-way ANOVA with Tukey post-hoc (concentration x treatment or pretreatment x treatment). qPCR was analysed using a two-way ANOVA with Bonferroni post-hoc (pretreatment x treatment).

All summary values presented as mean ± standard error of mean (SEM). *p*-values ≤ 0.05 were considered statistically significant.



ICC; immunocytochemistry; LPS, lipopolysaccharide; mRNA, messenger RNA; S/N supernatant.

Figure 1 Experimental timeline. To determine whether alcohol induces an immune response which parallels a TLR4 response in BV2 microglia-like cells, cells were incubated with alcohol, LPS or vehicle. Cell lysates, mRNA and supernatant were then collected for ICC (T0.5 and T2), gene expression analysis (PCR) (T4) and protein analysis (ELISA and western blots, T4 and T24) (a). To determine whether alcohol modifies a TLR4 response, cells were pretreated with alcohol or vehicle for 0.5 h and then stimulated using LPS or vehicle for 4 or 24 h. The treatments of LPS or vehicle occurred in the presence of the pretreatments (alcohol or vehicle). Cell lysates, mRNA and supernatant were then collected to determine whether the alcohol alters the expression of cytokines and transcription factors using ELISAs and western blots (b). To characterise the pharmacology of (+)-Naloxone, cells were pretreated with either

vehicle, LPS:RS or various concentrations of (+)-Naloxone (100 – 800uM). After 0.5 h cells were stimulated with LPS or vehicle in the presence of the pretreatment. Cells were collected for ICC, gene expression analysis (PCR) and protein analysis (ELISA and western blots) (c).

9.3 Results

9.3.1 Experiment 1: Does alcohol induce a TLR4 response in BV2 cells?

To determine whether alcohol induces an immune response which is similar to (albeit lower in magnitude) LPS, a traditional TLR4 agonist, BV2 cells were incubated with 100mM of alcohol. After 0.5 – 24 h, proteins and genes pertaining to the TLR4 signalling pathways were assessed. The expression of IL-1β, TNFα, IFNβ and CCL5 in cell supernatant and lysates were selected as they represent prototypical end-products of the MyD88 (IL-1β and TNFα) and the TRIF (IFNβ and CCL5) pathways. This would allow us to determine whether biased agonism/antagonism was present with the compounds being tested. However, the supernatant expression of IFNβ, and the supernatant and lysate expression of CCL5 was not detected following any drug treatment using western blots.

A two-way ANOVA determined the supernatant expression of TNFα and the lysate expression of IL-1β, IFNβ and TNFα were significantly influenced by time (4 h vs 24 h) (figure 2) ($p < 0.05$ for all cytokines, see supplementary material for precise statistical information). Post hoc analysis determined the greatest expression of these cytokines occurred at 24 h. By contrast the expression of IL-1β in the supernatant was not altered by time (effect of time, $F_{(1, 5)} = 0.49$, $p = 0.51$) with similar expression at 4 and 24 h.

There was a significant effect of treatment (vehicle vs LPS vs alcohol) on the expression of IL-1β and TNFα from cell supernatant and lysates ($p < 0.0001$ for all cytokines, see supplementary material for precise statistical information). Tukey post hoc analysis determined LPS significantly potentiated IL-1β and TNFα expression compared to vehicle and alcohol at 4 and 24 h. Crucially, there was no post hoc difference between alcohol and vehicle at either time point indicating a similar level of expression between these treatments. By contrast, the lysate expression of IFNβ was

not significantly influenced by treatment (effect of treatment, $F_{(2, 10)} = 1.17$, p = 0.36). No post hoc differences were found between the groups. Collectively, the results suggest LPS but not alcohol increases the expression of proteins pertaining to the MyD88 but not TRIF pathway following 4 and 24 h of exposure.

To infer whether these drugs modulate the transcription of the assessed proteins, qPCR was performed on *Il1b*, *Tnfa*, *Ifnb* and *Ccl5* genes (figure 3). Unlike protein expression, a two-way ANOVA determined there was no effect of time on the expression of *Il1b*, *Tnfa*, *Ifnb* and *Ccl5* ($p > 0.05$, see supplementary material for precise statistical information). There was however, a significant effect of treatment for the expression of *Il1b* (effect of treatment, $F_{(2, 6)} = 7.86$, $p = 0.041$), *Tnfa* (effect of treatment, $F_{(2, 6)} = 10.48$, $p = 0.026$) *and Ifnb* (effect of treatment, $F_{(2, 6)} = 29.94$, $p = 0.0039$) but not *Ccl5* mRNA (effect of treatment, $F_{(2, 6)} = 0.19$, $p = 0.83$). Bonferroni post hoc analysis determined LPS significantly potentiated the expression of *Il1b*, *Tnfa* and *Ifnb* mRNA compared to vehicle and alcohol at 4 h, however, by 24 h the difference was mitigated. There was no difference between vehicle and alcohol across any gene or time point. Collectively, the results suggest LPS but not alcohol increases the expression of mRNA pertaining to the MyD88, and some aspects of the TRIF pathways following 4 h of exposure.

To determine whether these compounds alter the expression of transcription factors which may underlie the differences in gene expression, western blots were performed on NFκB p65 subunit and IRF3 (figure 4). A two-way ANOVA determined time significantly influenced the expression of p65 (effect of time, $F_{(1, 4)} = 5.04$, $p = 0.088$) and IRF3 (effect of time, $F_{(1, 4)} = 17.05$, $p = 0.015$). However, only the expression of p65 in vehicle treated cells differed between time points (Tukey post hoc). Further, there was a significant effect of treatment for p65 (effect of treatment, $F_{(2, 8)} = 12.78$, p

= 0.032) but not IRF3 expression (effect of treatment, $F_{(2, 8)}$ = 1.17, p = 0.36). There was a significant increase in p65 expression at 24 h following LPS treatment compared to vehicle and alcohol (post hoc). There was no difference in p65 or IRF3 expression between vehicle and alcohol across any time point.

To determine whether these compounds alter the potential activity of NFκB p65 and IRF3, immunocytochemistry was performed to see if these transcription factors translocate to the nucleus after 0.5 and 2h respectively (figure 5). A two-way ANOVA evaluating the staining intensity of the nucleus (or cytoplasm) to the total cell determined a significant effect of cellular compartment (nucleus vs cytoplasm) on the expression of NFκB p65 (effect of compartment, $F_{(1, 4)}$ = 6.38, p = 0.045) and IRF3 (effect of compartment, $F_{(1, 4)}$ = 54.67, p = 0.0018). Tukey post hoc analysis determined there was significantly greater expression of p65 and IRF3 in the cytoplasm in vehicle and alcohol treated cells. By contrast, a treatment of LPS caused a greater expression of p65 and IRF3 in the nucleus compared to the cytoplasm. There was no significant effect of treatment for p65 (effect of treatment, $F_{(2, 8)}$ = 4, p = 0.062) and IRF3 (effect of treatment, $F_{(2, 8)}$ = 4.1, p = 0.063). Compared to alcohol and vehicle however, LPS significantly increased the nuclear, and decreased the cytoplasmic expression of p65 and IRF3 (Tukey post hoc). There was no difference between vehicle and alcohol for either p65 or IRF3. Importantly, these results were verified using traditional colocalisation analysis techniques (supplementary figure s4).

Lastly, to determine whether these compounds induce cytotoxicity or injury, neutral red and lactate dehydrogenase assays were performed (figure 6). A two-way ANOVA found a significant effect of time for both neutral red (effect of time, $F_{(1, 8)}$ = 68.47, p < 0.0001) and lactate dehydrogenase assays (effect of time, $F_{(1, 8)}$ = 46.59, p = 0.0064). However, there was no post hoc differences between 4 and 24 h for any treatment for

either assay. There was a significant effect of treatment in the neutral red (effect of treatment, $F_{(2, 6)} = 68.26$, $p < 0.0001$) and lactate dehydrogenase assays (effect of treatment, $F_{(2, 6)} = 89.05$, $p < 0.0001$). Post hoc analysis determined significant differences in absorption in the lactate dehydrogenase and neutral red assays between the treatments. LPS significantly reduced absorption relative to vehicle in the neutral red assay. LPS additionally increased absorption compared to vehicle and alcohol suggesting this compound increases the release of lactate dehydrogenase into the supernatant thereby inferring an increase in cellular stress/injury. There was no difference between vehicle and alcohol in either assay indicating both compounds do not induce large amounts of cytotoxicity or cellular injury.

9.3.2 Experiment 2: Does alcohol alter a TLR4 response in BV2 cells?

Collectively, the results suggest that alcohol is unable to increase the expression or release of MyD88 and TRIF mediated cytokines, nor induce the activation of NFκB p65 or IRF3. This suggests that alcohol is unable to directly induce an inflammatory response, a finding which contrasts previous studies (Lawrimore & Crews, 2017; Fernandez-Lizarbe et al., 2009; Blanco et al., 2005). However, additional studies have suggested alcohol modulates rather than induces an immune response (for example, (Goral et al., 2011; Lee et al., 2004). To verify these findings, BV2 cells were incubated with alcohol and endogenous TLR4 agonist, HMGB1 (ab18650) to reflect the current hypothesis of alcohol-induced neuroimmune activity (Crews et al., 2017). However, despite ab18650 being purportedly “active”, initial experiments determined this product was unable to induce an IL-1β response from BV2 cells (supplementary figure s5) suggesting an “inactive” isoform (Yu et al., 2006). Consequently, cells were pretreated for 0.5 h with alcohol and stimulated for 24 h with LPS. It must be kept in mind however, that while both LPS and HMGB1 are TLR4 agonists, the mechanism of activation and

resulting outcomes may differ between the two. Therefore, caution must be used when extrapolating the findings of LPS to alcohol-induced neuroimmune activity.

A two-way ANOVA determined the supernatant and lysate expression of TNFα and IL-1β were significantly influenced by treatment (LPS vs vehicle (herein termed media)) (figure 7) ($p < 0.0001$ for all cytokines, see supplementary for precise statistical information). Tukey post hoc calculated LPS significantly potentiated the expression TNFα in both cell supernatant and lysates compared to media across both pretreament groups. By contrast, IL-1β expression in the supernatant and lysates differed only in vehicle pretreated cells, with LPS inducing a greater expression relative to media. There was no main effect of treatment on IFNβ expression (effect of treatment, $F_{(2, 10)} = 3.18$, $p = 0.15$) nor any post hoc differences. Again, LPS potentiated the expression of proteins pertaining to the MyD88 but not TRIF pathway.

A two-way ANOVA determined a significant effect of pretreatment (alcohol vs vehicle) on the supernatant and lysate expression of TNFα and IL-1β ($p < 0.0001$ for all cytokines, see supplementary for precise statistical information). An alcohol pretreatment significantly reduced LPS-induced increases in supernatant and lysate TNFα and IL-1β compared to vehicle (post hoc). However, IFNβ expression was not modified by pretreament (effect of pretreatment, $F_{(1, 5)} = 4.03$, $p = 0.12$) nor were there any post hoc differences between pretreatment groups. Collectively, the results suggested pretreating BV2 cells with alcohol significantly reduced LPS-induced expression of MyD88-dependent cytokines (IL-1β and TNFα).

To determine whether the immunomodulatory capabilities of alcohol were due to alterations in gene transcription, qPCR was performed (figure 8). A two-way ANOVA determined there was a significant effect of treatment on the expression of *Il1b* (effect

of treatment, $F_{(1, 3)}$ = 13.71, p = 0.034), *Tnfa* (effect of treatment, $F_{(1, 3)}$ = 17.38, p = 0.025) and *Ifnb* (effect of treatment, $F_{(1, 3)}$ = 8.56, p = 0.041) but not *Ccl5* (effect of treatment, $F_{(1, 3)}$ = 0.017, p = 0.90). Bonferroni post hoc analysis determined LPS significantly increased the expression of *Il1b*, *Tnfa* and *Ifnb* relative to media in vehicle pretreated cells. There was no post hoc differences for *Ccl5* mRNA. There was an additional main effect of pretreatment for *Il1b* (effect of pretreatment, $F_{(1, 3)}$ = 13.87, p = 0.034), *Tnfa* (effect of pretreatment, $F_{(1, 3)}$ = 22.39, p =0.018), *Ifnb* (effect of pretreatment, $F_{(1, 3)}$ = 73.93, p = 0.033) and *Ccl5* mRNA expression (effect of pretreatment, $F_{(1, 3)}$ = 29.34, p =0.012). Pretreating the cells with alcohol significantly reduced LPS-induced expression of *Il1b*, *Tnfa*, *Ifnb* and *Ccl5* mRNA compared to vehicle (Bonferroni post hoc).

To determine whether pretreating BV2 cells with alcohol alters the expression of transcription factors NFκB p65 and IRF3 western blot were performed (figure 9). A two-way ANOVA determined the expression of p65 and IRF3 were significantly influenced by treatment (effect of treatment, $F_{(2, 10)}$ = 4.73, p = 0.0095 and $F_{(2, 10)}$ = 13.47, p = 0.035 respectively). However, no post hoc differences were identified. Further, the expression of p65 and IRF3 were not influenced by pretreatment (effect of pretreatment, $F_{(1, 5)}$ = 0.96, p = 0.38 and $F_{(1, 5)}$ = 2.54, p = 0.21 respectively), with post hoc analysis determining there were no significant differences between any of the groups.

Neutral red and lactate dehydrogenase assays were performed to determine whether pretreating BV2 cells with alcohol reduces cell cytotoxicity and injury (figure 10). Two-way ANOVAs determined a significant effect of treatment in the neutral red (effect of treatment, $F_{(2, 10)}$ = 16.14, p = 0.056) and lactate dehydrogenase assays (effect of treatment, $F_{(2, 10)}$ = 415.9, p < 0.0001). Tukey post hoc analysis determined LPS

significantly reduced absorbance in the neutral red assay in BV2 cells pretreated with vehicle; indicating greater cell cytotoxicity. Similarly, post hoc analysis determined LPS increased absorbance in the lactate dehydrogenase assay compared to media across both treatment groups (indicating increased cellular injury). Additionally, there was a main effect of pretreatment for the lactate dehydrogenase (effect of pretreatment, $F_{(1, 5)} = 21.90$, $p = 0.0054$) but not neutral red assays (effect of pretreatment, $F_{(1, 5)} = 0.44$, $p = 0.57$). Pretreating cells with alcohol significantly reduced absorbance in the lactate dehydrogenase assay compared to vehicle suggesting a reduction in cellular injury.

Collectively, the results suggest pretreating cells with alcohol significantly supressed LPS-induced increases in both MyD88 and TRIF-dependent cytokines on a mRNA level and MyD88-dependent cytokines on a protein level. Further, alcohol did not alter the expression of transcription factors nor cytotoxicity. However, alcohol significantly reduced LPS-induced lactate dehydrogenase expression in the supernatant indicating a potential reduction in cellular injury.

9.3.3 Figures

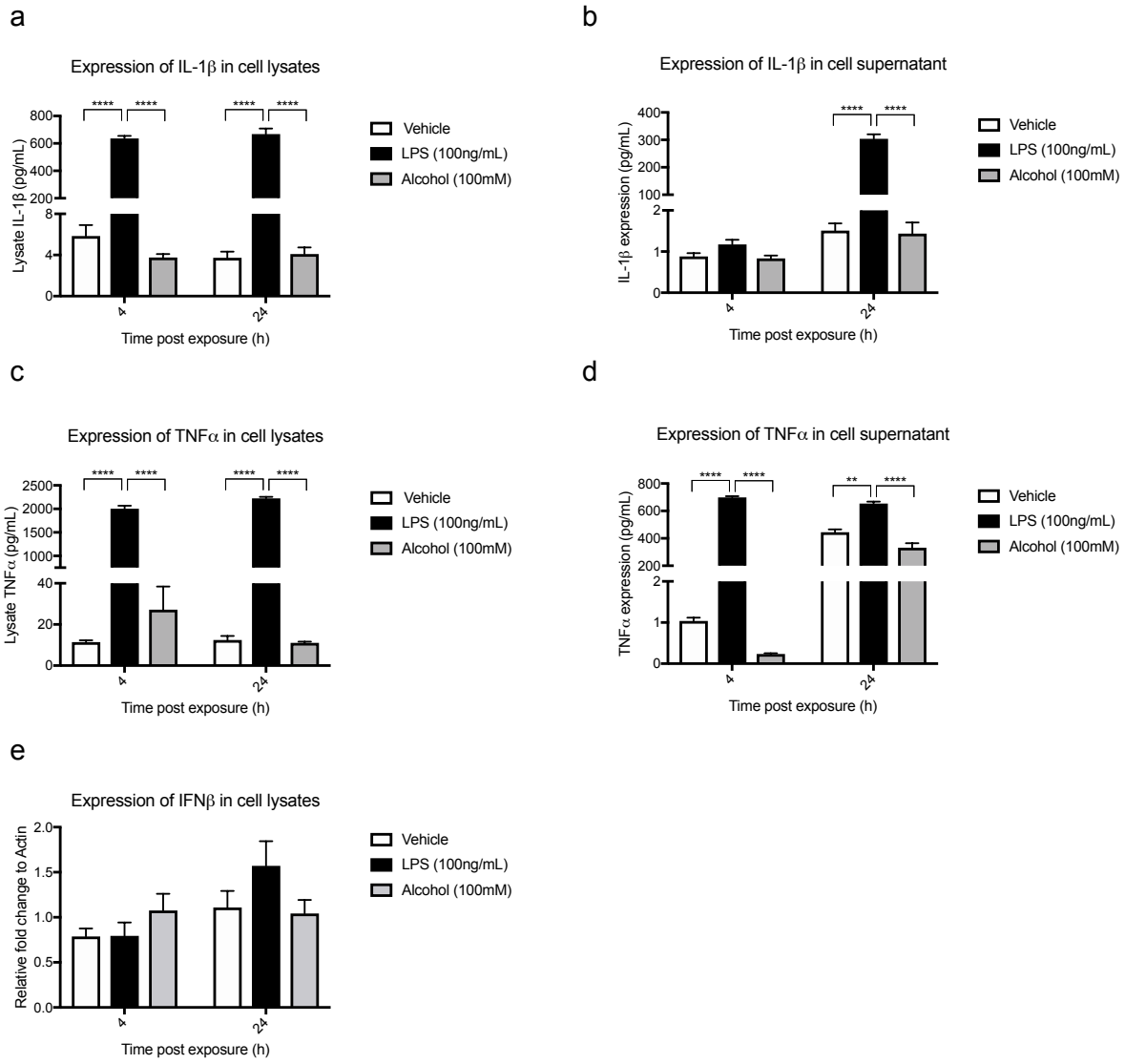


Figure 2 IL-1β (a – b), TNFα (c – d) and IFNβ (e) expression following 4 or 24 h of vehicle, LPS (100ng/mL) or alcohol (100mM). LPS significantly increased IL-1β and TNFα from cell lysates and supernatant. However, neither LPS nor alcohol increased IFNβ expression compared to vehicle. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

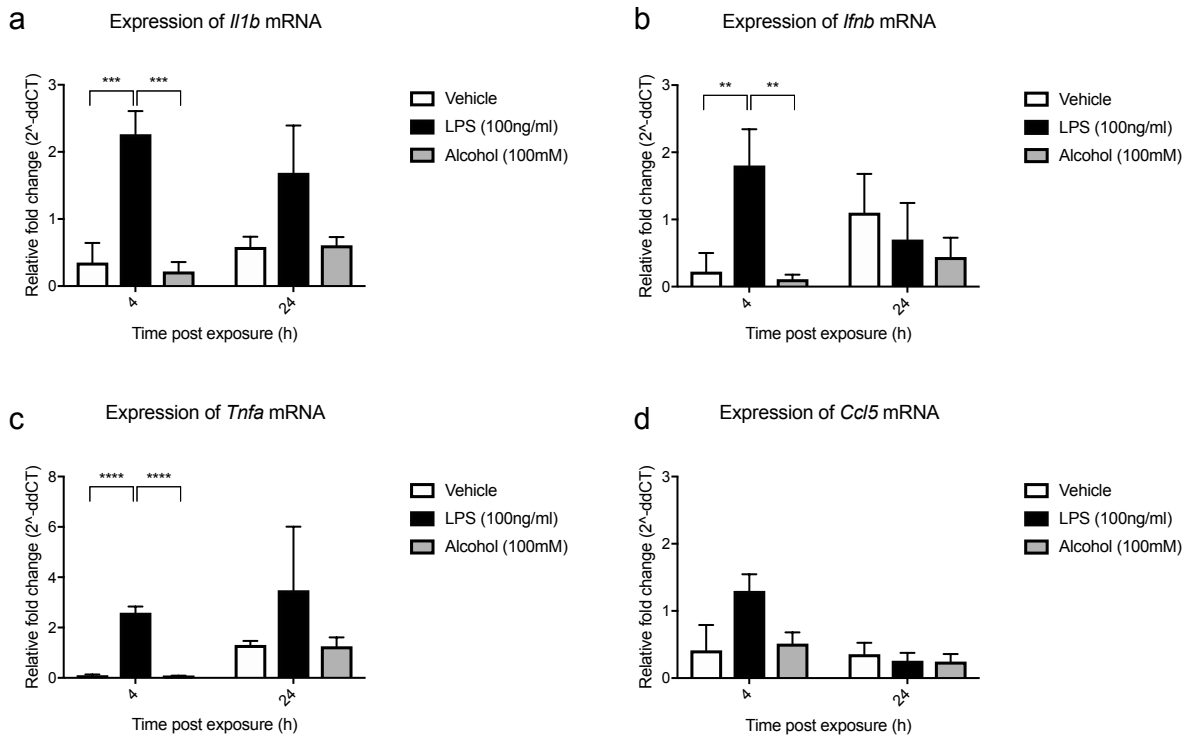


Figure 3 *Il1b* (a), *Ifnb* (b), *Tnfa* (c) and *Ccl5* (d) mRNA expression following 4 or 24 h of vehicle, LPS (100ng/mL) or alcohol (100mM). 4 h of LPS potentiates the expression of *Il1b*, *Ifnb* and *Tnfa* mRNA compared to vehicle and alcohol in BV2 cells. Neither alcohol or vehicle altered the expression of *Il1b, Ifnb*, *Ccl5* and *Tnfa* mRNA following 24 h of stimulation relative to vehicle. All data was analysed using a two-way ANOVA with Bonferroni post-hoc. Summary values represented as mean±SEM; n=3; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

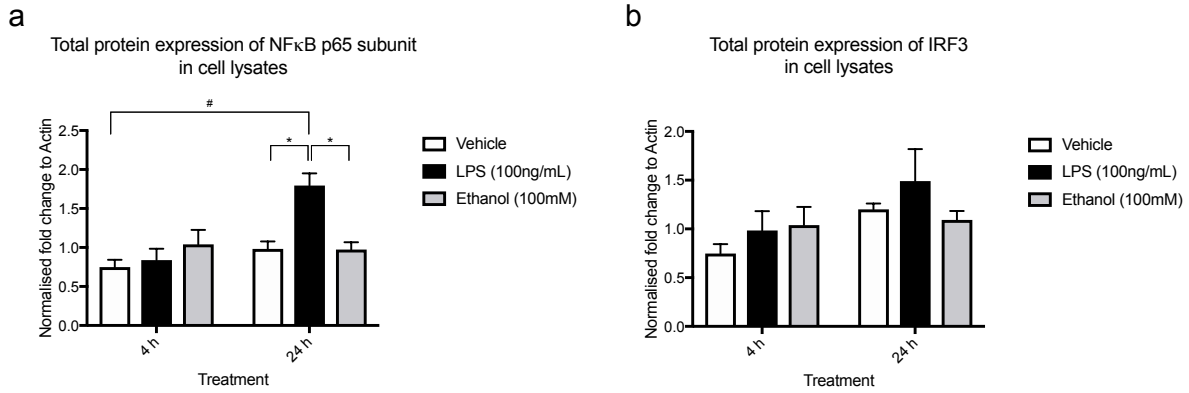


Figure 4 The expression of NFκB p65 subunit (a) and IRF3 (b) following 4 or 24 h of vehicle, LPS (100ng/mL) or alcohol (100mM). LPS, alcohol or vehicle had no effect on the expression of NFKB p65 or IRF3 following 4 h of stimulation. However, 24 h of LPS increased the expression of NFκB p65 compared to alcohol and vehicle-treated cells. Neither drug altered IRF3 expression at 24 h. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6; *$p < 0.05$ (between treatments); #$p < 0.05$ (between time).

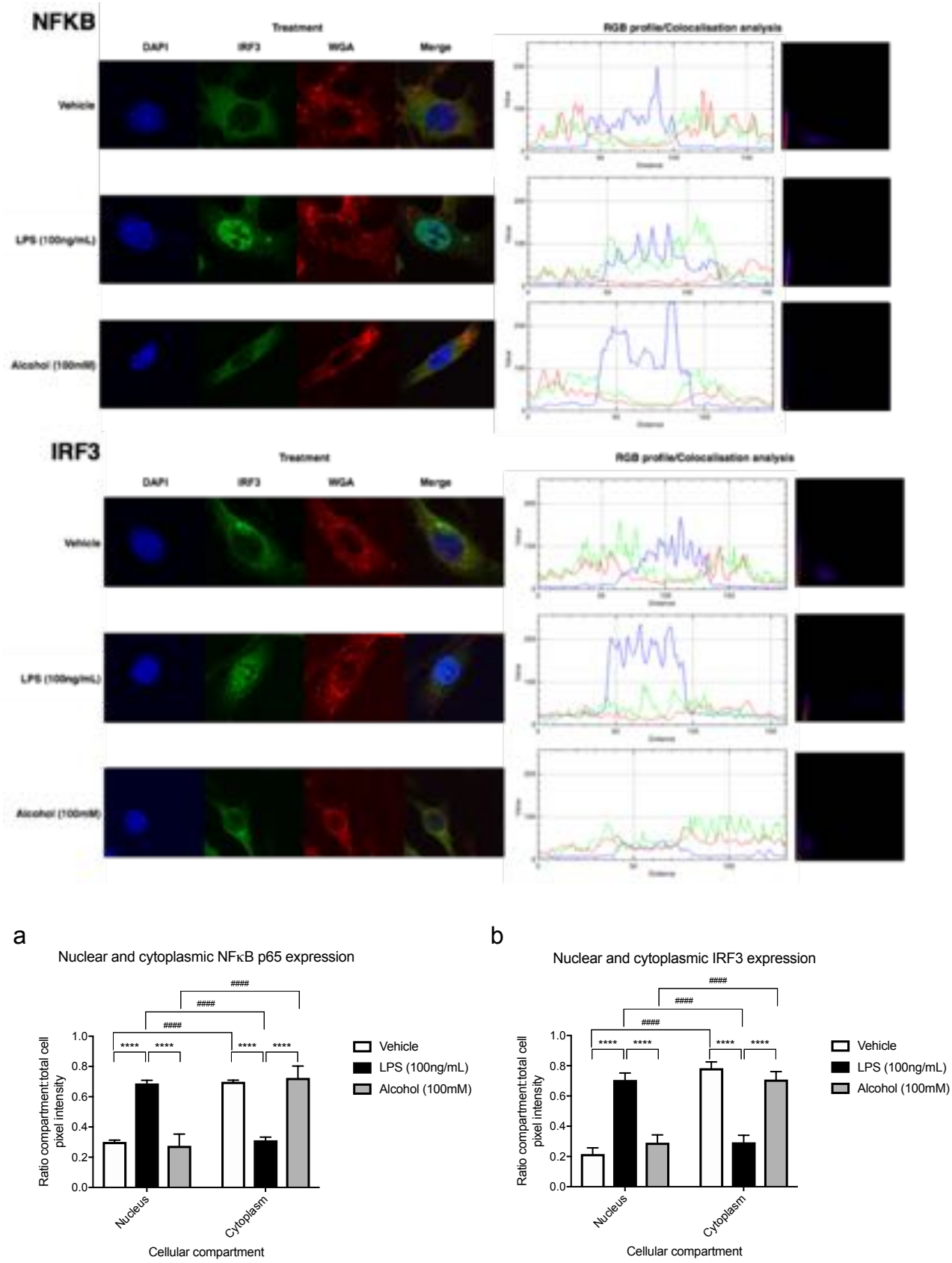


Figure 5 Translocation of NFκB p65 subunit (a) and IRF3 (b) at 0.5 and 2 h respectively following stimulation with vehicle, LPS (100ng/mL) or alcohol (100mM). LPS significantly increased the ratio of nuclear to cytoplasmic expression of

NFκB p65 and IRF3 compared to vehicle and alcohol treated cells. There was no difference between alcohol and vehicle treatments. The representative images of a and b and the corresponding RGB profile plot and colocalisation output from Fiji are shown above. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=4; ****p < 0.0001 (between treatments); ####p < 0.0001 (between cellular compartments).

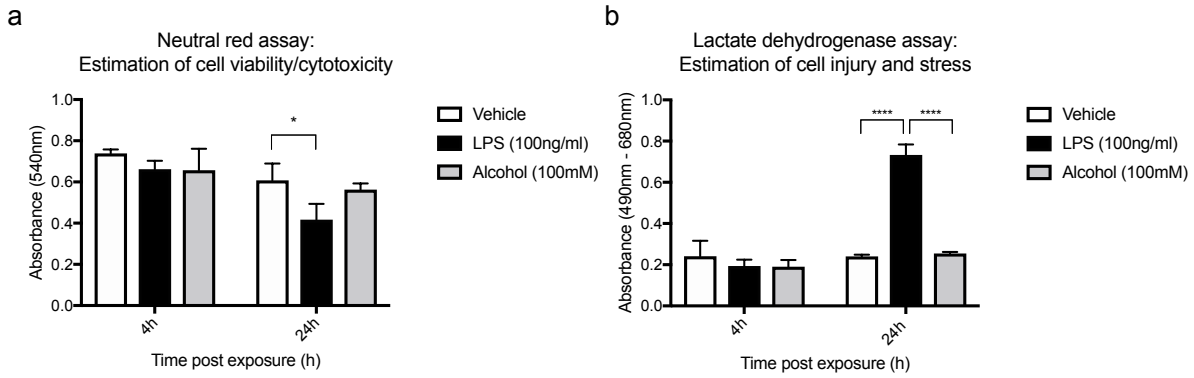


Figure 6 Estimation of cell cytotoxicity and injury following 24 h of vehicle, LPS (100ng/mL) or alcohol (100mM). Neither LPS or alcohol reduced the absorbance in the neutral red assay at 4 h indicating similar levels of cellular viability. However, LPS significantly reduced the absorbance at 24 h compared to vehicle-treated cells. This suggests LPS-induced cytotoxicity. (a). Similarly, LPS, alcohol or vehicle did not differ in regards to lactate dehydrogenase release at 4 h. However, LPS increases the release of lactate dehydrogenase into the supernatant following 24 h of stimulation compared to vehicle and alcohol treated cells (b). All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=4-8; ****$p < 0.0001$.

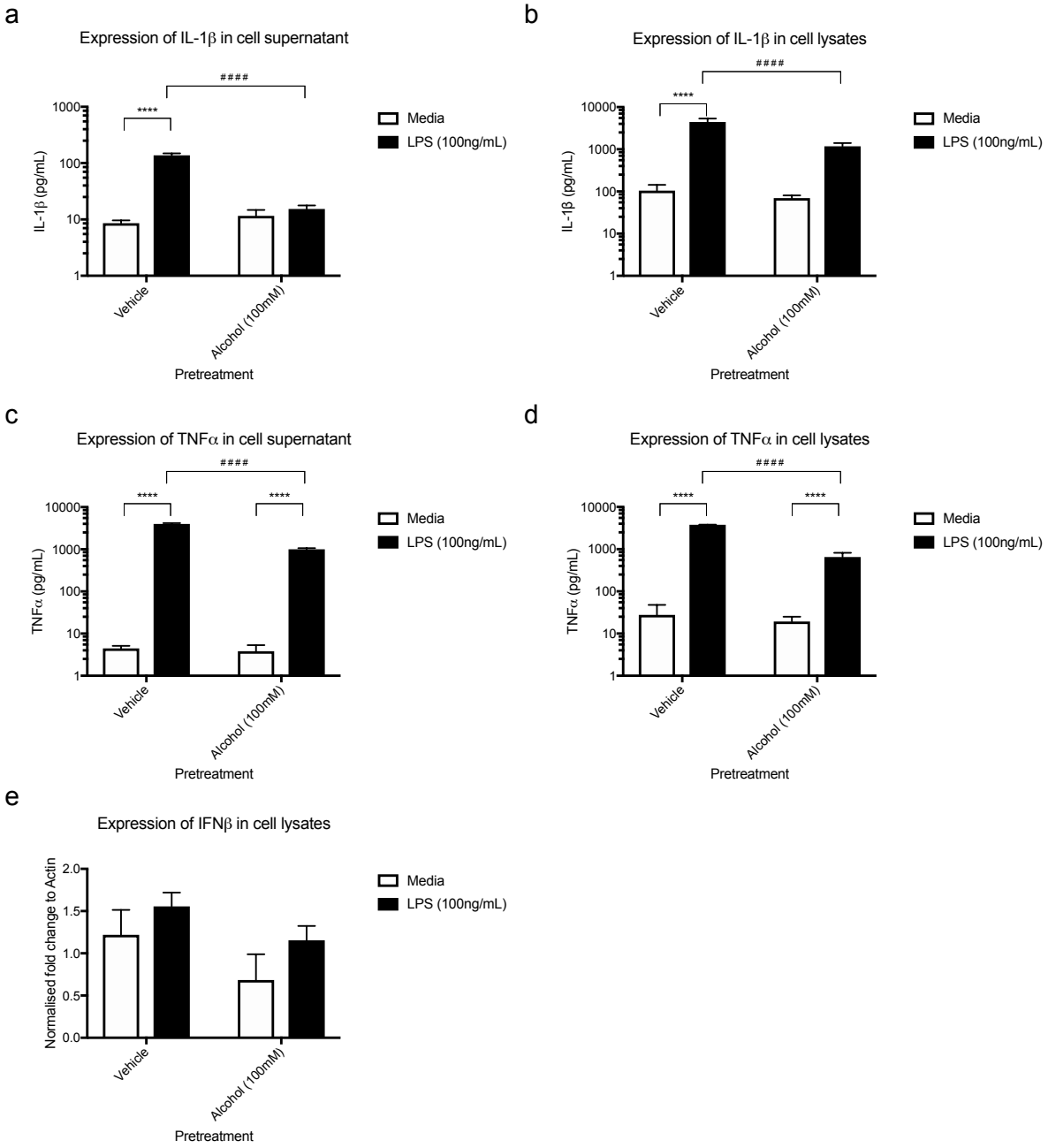


Figure 7 Alcohol pretreatment reduced LPS-induced IL-1β (a, b), TNFα (c, d) but not IFNβ (e) expression from BV2 supernatant and lysates. Pretreating with 100mM of alcohol significantly reduced LPS-induced IL-1β and TNFα expression in cell supernatant and lysates compared to vehicle pretreatment. However, neither alcohol nor LPS altered IFNβ expression in lysates. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6; ****$p < 0.0001$ (between treatments); #### $p < 0.0001$ (between pretreatments).

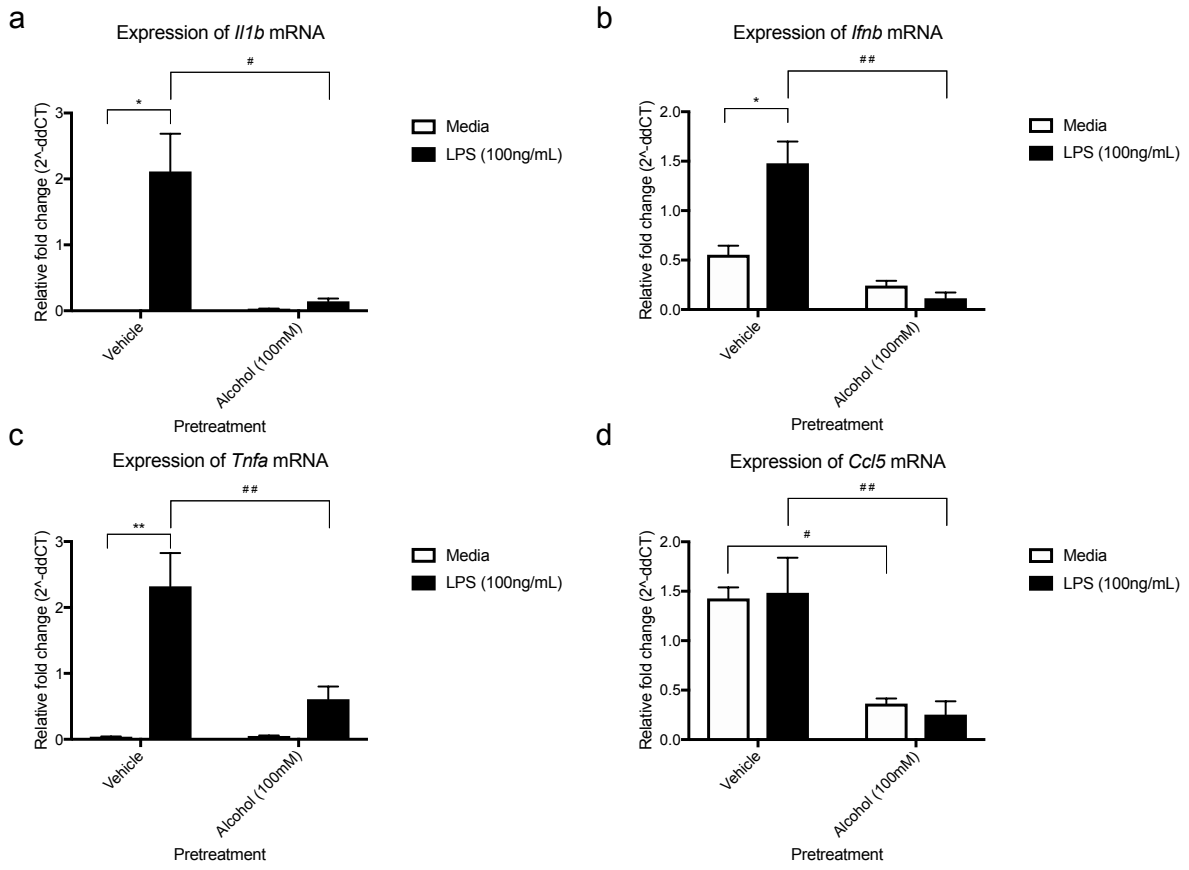


Figure 8 Alcohol reduced LPS-induced mRNA expression of *Il1b* (a), *Ifnb* (b), and *Tnfa* (c) but not *Ccl5* (d). LPS potentiated the expression of all 4 cytokine genes relative to media control. This effect was significantly supressed in cells pretreated with alcohol which demonstrated a reduction in LPS-induced *Il1b*, *Ifnb Tnfa*, and *Ccl5* mRNA expression. Summary values represented as mean±SEM; n=4; **p* < 0.05; ***p* < 0.01 (between treatments); #*p* < 0.05; ##*p* < 0.01 (between pretreatments).

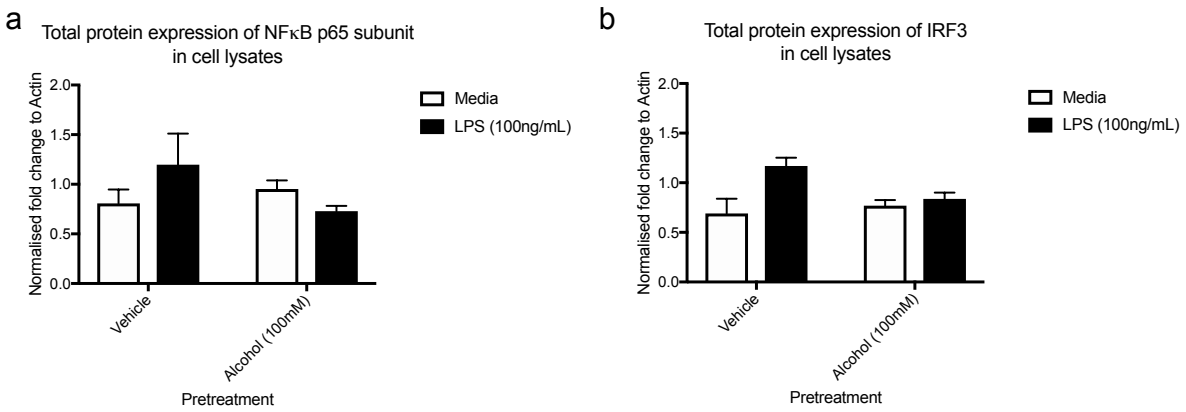


Figure 9 Alcohol pretreatment nor LPS altered the total expression NFκB p65 subunit (a) and IRF3 (b) from BV2 cell lysates. All data was analysed using a two-way ANOVA with Tukey HSD post-hoc. Summary values represented as mean±SEM; n=6.

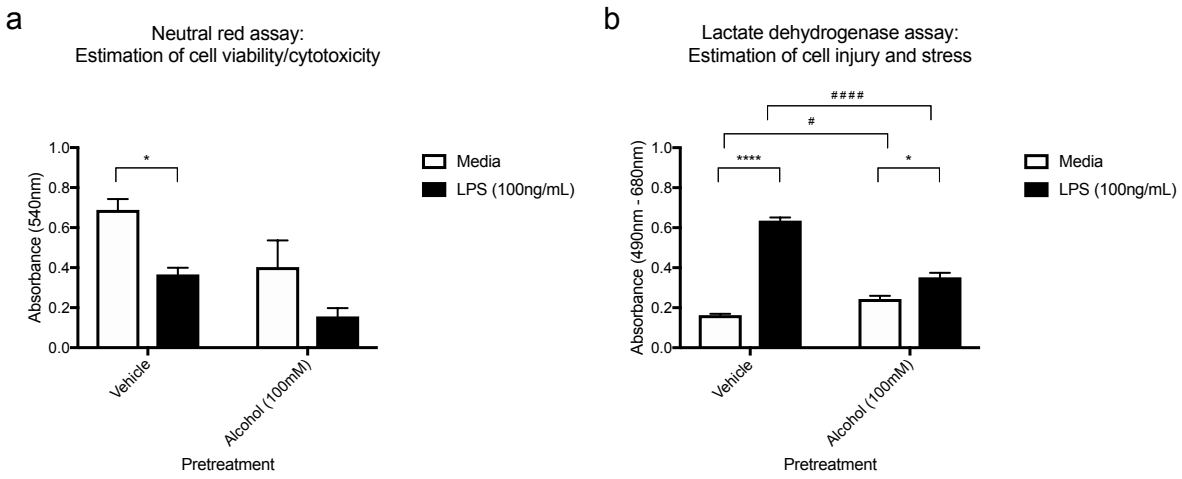


Figure 10 Alcohol had no effect on LPS-induced cell cytotoxicity (a) but reduced cellular injury (b). Incubating cells with alcohol has no effect on absorbance following stimulation with LPS compared to vehicle pretreated cells in the neutral red assay (a). However, pretreating cells with alcohol significantly reduced absorbance indicating decreased lactate dehydrogenases expression in the supernatant (b). All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6; ***p* <0.01, ****p* < 0.001 (between treatments); # *p* < 0.05, ###*p* < 0.001 (between pretreatments).

9.4 Interim discussion

Using multiple molecular analysis techniques, the results presented above indicate alcohol is unable to elicit an immune response from BV2 cells. Alcohol did not alter the expression of prototypical MyD88 and TRIF-dependent cytokines on a protein or mRNA level nor did it alter the expression of downstream transcription factors or their ability to translocate to the nucleus. These findings contradict existing studies (Lawrimore & Crews, 2017; Fernandez-Lizarbe et al., 2013; 2009; Blanco et al., 2005) that demonstrate alcohol significantly increases cytokines, immune-related signalling pathway proteins and transcription factors – a finding indicative of TLR4 activation. However, alcohol modulated a LPS-induced immune response. Pretreating cells with alcohol reduced the LPS-induced increases in MyD88 and TRIF cytokines. but did not alter the total expression of NFκB p65 or IRF3 suggesting the response may be attributable to altered translocation rather than total expression level. Further, pretreating cells significantly reduced markers of cellular injury but not overall cytotoxicity. Collectively the results suggest alcohol acts as an immunomodulator rather than a immunostimulatant.

The differing results observed between Lawrimore & Crews (2017), Fernandez-Lizarbe et al., (2009) and the current study are potentially attributable to duration and method of alcohol exposure, cell type and time points assessed. The present study used microglial-like BV2 cells. This cell line was designed as a substitute for primary microglia (Blasi et al., 1990). However, there is conjecture whether BV2 are an accurate model and substitute for primary microglia with studies demonstrating that the immune response towards LPS differs (Henn et al., 2009). Compared to primary microglia, BV2 often exhibit a less pronounced upregulation of genes and proteins following stimulation with LPS (Henn et al., 2009; Horvath et al., 2008), and may not possess specific transcription factors (for example, IRF5 and STAT1) and epigenetic

regulators (for example, NSD3) (Das et al., 2016). Consequently, immune features present in primary microglia may not be present in BV2 cells. Therefore, the lack of an alcohol-induced immune response in the present study may be due to the different cells types used by Fernandez-Lizarbe et al., (2009) (primary microglia) and the relative inability of BV2 cells to respond to weak immunogens.

Another important consideration is the time point at which protein and mRNA was assessed. The present study evaluated cytokine expression at 4 and 24 h. These time points were designed to partly align with those by Lawrimore & Crews, (2017). They demonstrated *Il1b* and *Tnfa* mRNA was significantly upregulated 24 h after alcohol exposure: a finding that contrasts those presented in this study. However, studies have additionally demonstrated that the response to alcohol occurs rapidly in primary microglia with increases in cytokines and signalling pathways proteins occurring 1 – 3 h post exposure (Lawrimore & Crews, 2017; Fernandez-Lizarbe et al., 2013; Blanco et al., 2005). Importantly, the immune response in BV2 cells not only differs in magnitude but also the activation time compared to primary microglia with the onset typically earlier and lower in magnitude (Henn et al., 2009). Consequently, the present study may be missing the peaks of an alcohol response.

The differing immune response towards alcohol may additionally be due to the method of alcohol exposure. The present study added alcohol to serum free DMEM and treated cells were placed in a humidified incubator. Alcohol evaporated relatively quickly with no detectable alcohol present in the media 12 h post exposure (see supplementary figure s3). By contrast, Lawrimore & Crews (2017) used a chamber to maintain alcohol saturation - limiting evaporation. Consequently, the concentration of alcohol did not differ across time and may therefore represent a more chronic treatment model. This is an important experimental consideration as it is hypothesised that chronic alcohol

increases cellular stress inducing the release of HMGB1, an endogenous danger signal. HMGB1 subsequently acts on TLR4 to induce an immune response from microglial cells (Crews et al., 2017; 2004). Given our study did not find an effect of alcohol on lactate dehydrogenase expression or supernatant HMGB1 expression (data not shown) it is likely the shorter exposure time was insufficient to induce cellular stress. By contrast, the chronic saturation model used by Lawrimore & Crews, (2017) demonstrated a significant increase in supernatant HMGB1 indicating this model may be more suited to explore the immune-inducing potential of alcohol.

This study additionally reinforced the observation regarding the immunosuppressive nature of acute alcohol on the immune system (Szabo & Mandrekar, 2009; MacGregor, 1986). Pretreating BV2 cells for 0.5 h with alcohol significantly reduced LPS-induced increases in *Il1b*, *Tnfa*, *Ifnb* and *Ccl5* compared to vehicle control. Importantly, the expression of NFκB and IRF3 remained unchanged suggesting the immune response is likely attributable to reduced translocation or alterations to the TLR4 signalling pathway. However, further experiments are required to confirm this.

Importantly, the mechanism underlying the effects of alcohol-induced TLR4 tolerance are being increasingly researched. For example, studies have shown acute alcohol exposure increases the expression of negative regulators of NFκB; anti-inflammatory cytokines; and decreases the expression of kinases and the formation of lipid rafts on the cell surface thereby preventing the interaction between TLR4 and its co-receptors (Goral et al., 2011; Mandrekar et al., 2009; 2008; 2007; Dai & Pruett, 2006; Dolganiuc et al., 2006; Pruett et al., 2004). These studies have focused on the MyD88 pathway. Our study is the first to demonstrate the effects of alcohol-induced tolerance also carry over to the TRIF pathway. Alcohol significantly reduced the expression *Ifnb* and *Ccl5* mRNA following LPS exposure suggesting that IRF3, STAT or late phase NFκB may

be attenuated. Whether this response is a consequence of the reduced TLR4-co-receptor interaction which also leads to MyD88 attenuation following acute alcohol, or whether there is a specific mechanism inducing TRIF-tolerance remains to be determined.

The finding that alcohol can induce tolerance but not elicit an immune response despite alcohol rapidly evaporating from the wells is interesting. Previous studies have shown that the acute immunosuppressive of alcohol occur quickly and is long-lasting (for example, Dolganiuc et al., 2006). Only a short pretreatment is required to elicit a long effect thus, the evaporation of alcohol observed above is unlikely to influence this response. By contrast, longer exposure times are potentially required to exert stress and the release of HMGB1 from BV2 cells. Consequently, this paradigm is more sensitive to the effects of evaporation.

Collectively, the results presented in this study demonstrate that acute alcohol exposure was unable to induce an immune response which parallels a response to prototypical TLR4 agonist, LPS. However, alcohol exhibited potent immunomodulatory abilities as it reduced the cytokine response following LPS. Importantly, this study demonstrates that alcohol suppresses aspects of both the MyD88 and TRIF-dependent pathways indicating alcohol severely hampers an ability to mount an immune response. However, the duration of suppression and the precise mechanism underlying TLR-TRIF based suppression remains to be fully elucidated.

9.5 Results

9.5.1 Pharmacological characterisation of (+)-Naloxone

Chapters 3, 5 and 7 demonstrated that acute and chronic alcohol exposure significantly upregulated cytokines and signalling proteins pertaining to the MyD88 and TRIF pathways. Furthermore, attenuating TLR4 mitigated the rise in immune gene expression following acute but not chronic alcohol exposure. Collectively, these experiments indicated alcohol potentiates TLR4 signalling *in vivo*. However, the preceding *in vitro* experiments were unable to replicate the immune-inducing ability of alcohol. Consequently, (+)-Naltrexone's mechanism of action in the context of an alcohol-induced immune response could not be explored *in vitro*.

Given alcohol could not elicit an immune response *in vitro*, the decision was made to explore and clarify how TLR4 antagonists function in the context of an immune response induced by LPS in BV2 cells. While LPS and alcohol differ in their potencies, half-lives and the mechanism underlying TLR4 activation (direct vs indirect), this model can still provide us with useful information discerning how TLR4 antagonists modulate an inflammatory response in microglia-like cells.

The availability of (+)-Naltrexone was extremely limited throughout my candidature. Therefore, the proceeding studies used a chemically similar compound; (+)-Naloxone, to antagonise TLR4. (+)-Naloxone is an enantiomer of a μ opioid receptor antagonist (-)-Naloxone. Importantly, (+)-Naloxone and (+)-Naltrexone are hypothesised to have the same binding site, binding motifs (Hutchinson et al., 2010), and affinity for TLR4 (Wang et al., 2016). Further, these drugs have almost identical structure and stereoisomerism of individual molecules and selectively antagonise the TLR4-TRIF pathway *in vitro* (Wang et al., 2016). While this approach can provide some insight into how (+)-isomers function at TLR4, caution must be used when interpreting and

inferring the actions of (+)-Naloxone to (+)-Naltrexone as the compounds differ in regards to half-life and the presence/absence of specific function groups (C=C bond and methyl group). Consequently, this study aimed to characterise the pharmacology of (+)-Naloxone in the context of an LPS-induced immune response by microglia-like BV2 cells.

The concentrations of (+)-Naloxone used in the following experiments ranged from 100 – 800uM and were based upon a previous study demonstrating these concentrations successfully attenuated a LPS-induced TLR4-TRIF response (Wang et al., 2016).

The first series of experiments were designed to assess (+)-Naloxone influence on cytotoxicity and cellular injury. Cells were pretreated with (+)-Naloxone (100 – 800uM) or vehicle* (volume-matched PBS, figures 11 – 13 x = 0) for 0.5 h. After which LPS (100ng/mL) or vehicle (volume-matched PBS) was added to the wells. Cells incubated in the co-treatment for a further 24 h and then underwent the neutral red and lactate dehydrogenase assays to determine whether (+)-Naloxone and/or LPS influenced cell death or injury.

A two-way ANOVA determined a main effect of treatment (LPS vs vehicle) on absorption in the neutral red and lactate dehydrogenase assays (effect of treatment, $F_{(1, 11)} = 578.3$, $p < 0.0001$ and $F_{(1, 11)} = 143.6$, $p < 0.0001$ respectively). Tukey post hoc analysis calculated cells treated with LPS exhibited significantly greater cytotoxicity (lower absorption) compared to vehicle-treated cells across all concentrations of (+)-Naloxone in the neutral red assay. LPS treatment resulted in greater cellular injury (higher absorbance) between 0 – 200uM of (+)-Naloxone compared to vehicle treated cells in the lactate dehydrogenase assay (post hoc analysis).

There was an additional main effect of (+)-Naloxone's concentration on absorption in the neutral red and lactate dehydrogenase assays (effect of concentration, $F_{(5, 55)}$ = 11.33, $p < 0.0001$ and $F_{(5, 55)}$ = 60.82, $p < 0.0001$ respectively). 200 – 600uM of (+)-Naloxone significantly reduced LPS-induced cytotoxicity and injury as inferred by increased and decreased absorbance in the neutral red and lactate dehydrogenase assays respectively (post hoc analysis). However, higher concentrations of (+)-Naloxone (600 – 800uM) increased cytotoxicity (decreased absorbance) in vehicle-treated cells in the neutral red assay. This effect was not observed in the lactate dehydrogenase assay. This indicates the highest concentrations of (+)-Naloxone induce a small but statistically significant amount of cytotoxicity. Overall, the results suggest (+)-Naloxone attenuates LPS-induced cell death and injury. However, higher concentrations of (+)-Naloxone additionally cause cell death.

Given that (+)-Naloxone reduced markers of LPS-induced cytotoxicity and injury, the next series of experiments were designed to reinforce the observations by Wang et al., (2016) who demonstrated the biased antagonistic nature of (+)-Naloxone at TLR4. To infer biased antagonism cell supernatant and lysate were collected and the expression of IL-1β, TNFα, IFNβ and CCL5 were assessed (figure 12). Again, IFNβ could not be detected in the cell supernatant nor could CCL5 in the supernatant or lysate using western blots.

A two-way ANOVA determined a significant effect of treatment on the supernatant and lysate expression of IL-1β and TNFα and the lysate expression of IFNβ ($p < 0.05$, see supplementary material for precise statistical information). Cells treated with LPS exhibited significantly greater expression of IL-1β and TNFα in the cell supernatant and lysate across all concentrations of (+)-Naloxone compared to vehicle (post hoc analysis). Furthermore, cells treated with LPS exhibited significantly greater IFNβ

expression compared to vehicle treated cells. However, this effect was only observed in the absence of (+)-Naloxone (x = 0) (Tukey post hoc analysis).

The concentration of (+)-Naloxone additionally influenced the expression of supernatant and lysate IL-1β and TNFα ($p < 0.05$ for all variables, see supplementary materials for precise statistical information). Post hoc analysis determined (+)-Naloxone decreased the LPS-induced expression of IL-1β and TNFα in the supernatant and lysate between the 200 and 800uM. By contrast, the concentration of (+)-Naloxone did not significantly modify the expression of IFNβ (effect of concentration, $F_{(5, 35)} = 1.19$, $p = 0.36$). However, post hoc analysis determined a significant reduction in LPS-induced IFNβ expression at 200 and 800uM of (+)-Naloxone compared to vehicle* (absence of (+)-Naloxone (x = 0). Collectively, the results suggest (+)-Naloxone reduces the expression of both MyD88 and TRIF cytokines.

To explore whether (+)-Naloxone exhibits biased antagonism at the transcription factor level, the expression NFκB p65 and IRF3 was assessed (figure 13). IRF3 but not NFκB exhibited a significant effect of treatment (effect of treatment, $F_{(1, 7)} = 11.62$ $p = 0.042$ and $F_{(1, 7)} = 8.23$, $p = 0.064$ respectively) as inferred by a two-way ANOVA. Post hoc analysis determined however, LPS significantly increased the expression of NFκB p65 and IRF3 in the absence of (+)-Naloxone (x = 0) compared to vehicle treated cells. Furthermore, the expression of NFκB p65 and IRF3 were not significantly modified by the concentration of (+)-Naloxone (effect of concentration, $F_{(5, 35)} = 3.12$, $p = 0.05$ and $F_{(5, 35)} = 1.73$, $p = 0.19$ respectively). There was no post hoc differences for NFκB p65 at any concentration of (+)-Naloxone. However, 100 – 200uM of (+)-Naloxone significantly attenuated LPS-induced IRF3 expression compared to a vehicle* pretreatment (absence of (+)-Naloxone, x = 0).

9.5.2 Pharmacological comparison of LPS:RS and (+)-Naloxone

The preceding results implied (+)-Naloxone attenuated aspects of both MyD88 and TRIF pathways. Consequently, we next sought to determine whether (+)-Naloxone exhibits similar antagonistic properties at TLR4 compared to LPS:RS. Both LPS:RS and (+)-Naloxone are hypothesised to bind to the same pocket in MD2 which prevents the activation and recruitment of TLR4 and its downstream signalling pathways (Hutchinson et al., 2010). Therefore, these drugs are hypothesised to produce similar antagonistic outcomes. The dose of LPS:RS used was based upon previous studies performed in this laboratory demonstrating attenuation of TLR4 signalling at 200ng/mL (Hutchinson et al., 2010). The dose of (+)-Naloxone used (200uM) represents the lowest concentration at which we observed attenuation of LPS-induced cell death, injury and the expression of MyD88 and TRIF-dependent cytokines.

The effects of LPS:RS and (+)-Naloxone on cellular cytotoxicity and injury were assessed using neutral red and the lactate dehydrogenase assay (figure 14). A two-way ANOVA determined the absorption values in the neutral red and lactate dehydrogenase assays were significantly modified by treatment (LPS vs vehicle (herein referred to as media) (effect of treatment, $F_{(1, 30)} = 653.9$, $p < 0.0001$ and $F_{(1, 5)} = 2782$, $p < 0.0001$ respectively). Again, cells treated with LPS exhibited significantly greater amounts of cytotoxicity and injury as inferred by higher and lower absorption values in the lactate dehydrogenase and neutral red assays respectively (Tukey post hoc). Furthermore, the absorption values in the neutral red and lactate dehydrogenase assays were significantly affected by pretreatment (LPS:RS vs (+)-Naloxone vs vehicle) (effect of pretreatment, $F_{(2, 30)} = 12.13$, $p = 0.0001$ and $F_{(2, 10)} = 103.7$, $p < 0.0001$ respectively). Compared to vehicle, both LPS:RS and (+)-Naloxone significantly increased absorption in the neutral red (reduced cytotoxicity) with no difference observed between the two TLR4 antagonists (post hoc analysis). Similarly,

both antagonists reduced absorbance in the lactate dehydrogenase assays compared to vehicle (reduced injury). However, there was a significant difference between the two antagonists, with (+)-Naloxone reducing absorption to a greater extent than LPS:RS. Collectively, both TLR4 antagonists block LPS-induced increases in cell death and injury.

The expression of IL-1β, TNFα and IFNβ was again assessed to determine whether LPS:RS and (+)-Naloxone attenuate similar aspects of both the MyD88 and TRIF pathways (figure 15). A two-way ANOVA determined the supernatant and lysate expression of IL-1β and TNFα and the lysate expression of IFNβ was significantly influenced by treatment (effect of treatment, $p < 0.05$, see supplementary material for precise statistical information). Cells treated with LPS exhibited significantly greater expression of these cytokines compared to media-treated cells (Tukey post hoc analysis). In addition, pretreatment significantly modified the expression of supernatant IL-1β and TNFα (effect of pretreatment, $F_{(2, 14)} = 11.54$, $p = 0.0011$ and $F_{(2, 14)} = 16.59$, $p = 0.0002$ respectively) and the lysate expression of IL-1β, TNFα and IFNβ (effect of pretreatment, $F_{(2, 14)} = 4.84$, $p = 0.033$; $F_{(2, 14)} = 8.87$, $p = 0.0061$; and $F_{(2, 14)} = 19.71$, $p = 0.0023$ respectively). Post-hoc analysis determined (+)-Naloxone significantly reduced LPS-induced increases in supernatant and lysate IL-1β, TNFα and IFNβ compared to vehicle treated cells. By contrast, LPS:RS only reduced the LPS increases in supernatant IL-1β and TNFα lysate expression compared to vehicle treated cells. LPS:RS did not attenuate IL-1β and IFNβ lysate and TNFα supernatant expression compared to vehicle treated cells. At these doses, both LPS:RS and (+)-Naloxone block proteins pertaining to MyD88 pathway. However, only (+)-Naloxone inhibits TRIF-related cytokine expression.

To infer whether (+)-Naloxone and LPS:RS attenuate the transcription of cytokines pertaining to the MyD88 and TRIF pathways, the expression of *Il1b*, *Tnfa*, *Ifnb* and *Ccl5* was assessed (figure 16). A significant effect of treatment was observed for *Il1b* (effect of treatment, $F_{(1, 3)} = 13.1$, $p = 0.036$) but not *Tnfa*, *Ifnb* and *Ccl5* expression (effect of treatment, $F_{(1, 3)} = 8.96$, $p = 0.058$; $F_{(1, 3)} = 5.40$, $p = 0.10$; and $F_{(1, 3)} = 2.09$, $p = 0.24$ respectively) as determined by a two-way ANOVA. Post hoc analysis determined LPS potentiated the expression of all cytokine genes relative to media in vehicle pretreated cells. Furthermore, only *Il1b* mRNA exhibited a significant effect of pretreatment ($F_{(2, 6)} = 6.33$, $p = 0.033$). *Tnfa*, *Ifnb* and *Ccl5* were unaltered by the pretreatment effect ($F_{(2, 6)} = 3.49$, $p = 0.099$; $F_{(2, 6)} = 1.06$, $p = 0.40$; and $F_{(1, 3)} = 2.09$, $p = 0.24$ respectively). Post hoc analysis determined LPS:RS significantly reduced the LPS-induced increases in *Il1b* and *Ccl5* mRNA compared to vehicle. (+)-Naloxone did not modify LPS-induced increases in any MyD88 or TRIF-related gene.

Given that the expression of cytokine protein and mRNA was attenuated by (+)-Naloxone and LPS:RS we next sought to determine whether these drugs block the expression of NFκB p65 and IRF3 (figure 17). A two-way ANOVA determined IRF3 but not NFκB p65 expression was significantly influenced by treatment (effect of treatment, $F_{(1, 7)} = 10.76$, $p = 0.046$ and $F_{(1, 7)} = 5.46$, $p = 0.10$ respectively). Post hoc analysis determined LPS significantly increased the expression of both transcription factors in vehicle pretreated cells. No other treatment related differences were observed. Further, NFκB p65 and IRF3 expression was not influenced by pretreatment (effect of pretreatment, $F_{(2, 14)} = 2.15$, $p = 0.20$ and $F_{(2, 14)} = 1.68$, $p = 0.26$ respectively). However, post hoc analysis determined both LPS:RS and (+)-Naloxone significantly reduced the LPS-induced increases of NFκB p65 and IRF3 compared to vehicle treated cells.

To determine whether the reductions in mRNA were attributable to a reduction in NFκB p65 and IRF3 activity, the degree of nuclear translocation was assessed (figure 18). The translocation of NFκB p65 but not IRF3 was significantly influenced by treatment (effect of treatment, $F_{(1, 3)} = 23.54$, $p = 0.016$ and $F_{(1, 3)} = 4.38$, $p = 0.13$ respectively) (two-way ANOVA). Post hoc analysis determined LPS significantly increased translocation of NFκB p65 and IRF3 into the nucleus in vehicle pretreated cells. Similarly, NFκB p65 but not IRF3 was significantly influenced by pretreatment (effect of pretreatment, $F_{(2, 6)} = 7.72$, $p = 0.021$ and $F_{(2, 6)} = 3.69$, $p = 0.090$ respectively). Post-hoc analysis determined both LPS:RS and (+)-Naloxone reduced the LPS-induced translocation of NFκB p65 and IRF3 into the nucleus compared to vehicle pretreatment. There was no post hoc difference between (+)-Naloxone and LPS:RS-treated cells suggesting both antagonists similarly prevent the actions of transcription factors pertaining to MyD88 and TRIF-dependent pathways.

9.5.3 Figures

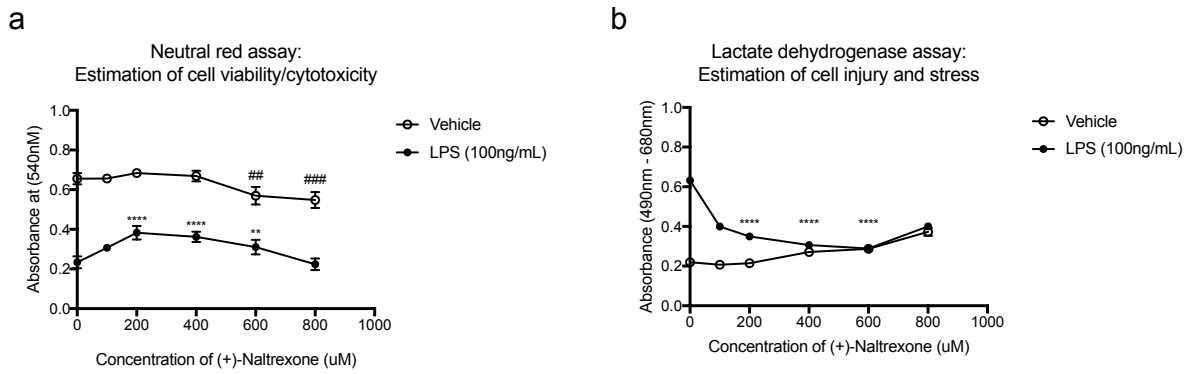


Figure 11 (+)-Naloxone reduced LPS-induced BV2 cells cytotoxicity and injury as inferred by neutral red (a) or lactate dehydrogenase assays (b). Treating cells with (+)-Naloxone increases neutral red absorbance following stimulation with LPS (decreased cytotoxicity) (a) and reduces lactate dehydrogenases expression in the supernatant (decreased injury) (b). However, high doses of (+)-Naloxone additionally cause cytotoxicity in vehicle treated cells (a). All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6-12; ***p* <0.01, ****p* < 0.001, *****p* < 0.0001 (LPS), ## *p* <0.01, ###*p* < 0.001 (vehicle) from vehicle* (absence of (+)-Naloxone, x = 0).

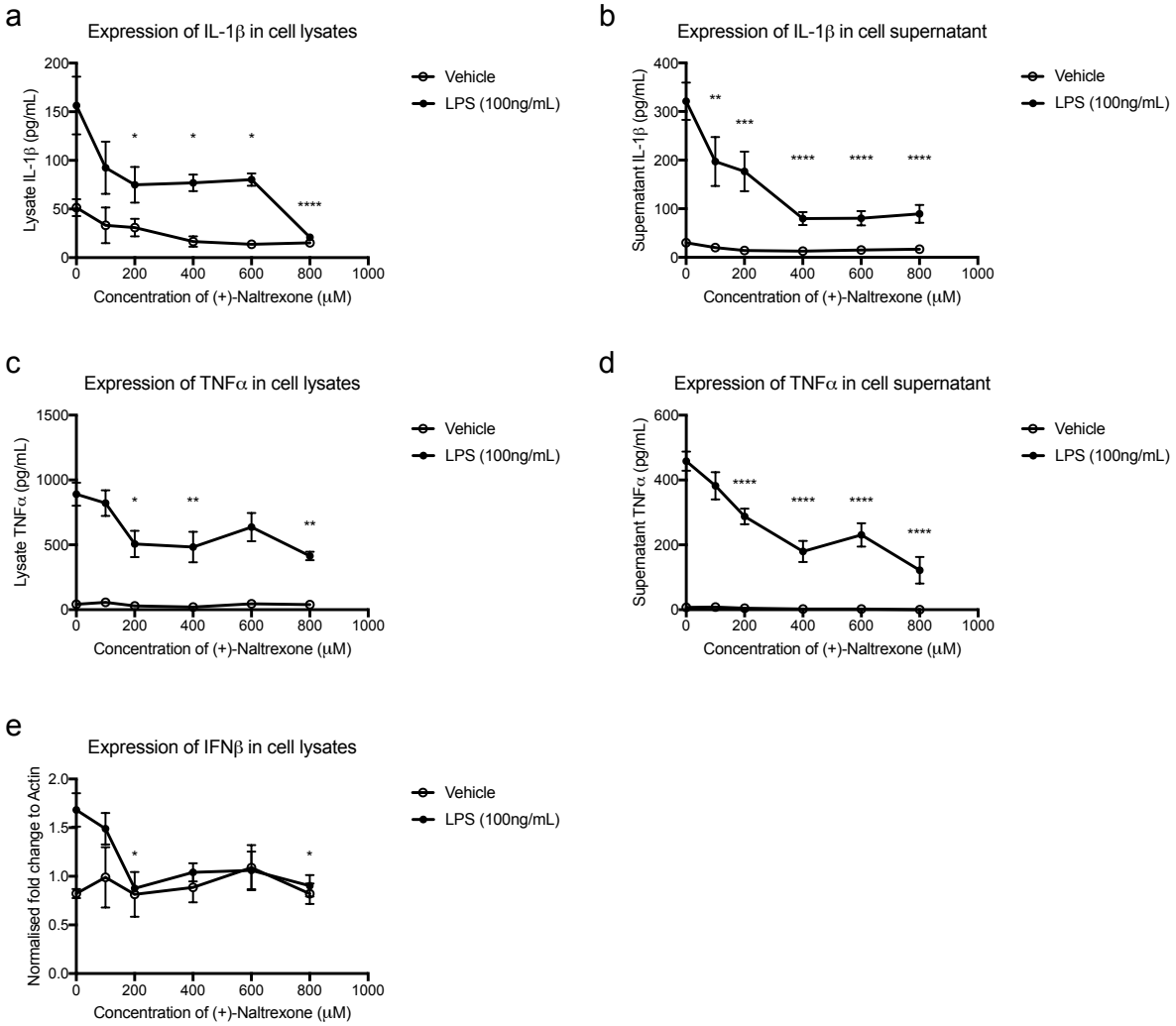


Figure 12 (+)-Naloxone reduced LPS-induced IL-1β (a, b), TNFα (c, d) and IFNβ (e) expression from cell supernatant and lysates. (+)-Naloxone dose-dependently reduced LPS-induced IL-1β and TNFα expression from cell lysates and supernatant. (+)-Naloxone reduced LPS-induced IFNβ expression at 200uM and 800uM, however, there was no main effect of concentration. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=8; ** p <0.01, *** p < 0.001, **** p < 0.0001 from vehicle* (absence of (+)-Naloxone, x = 0).

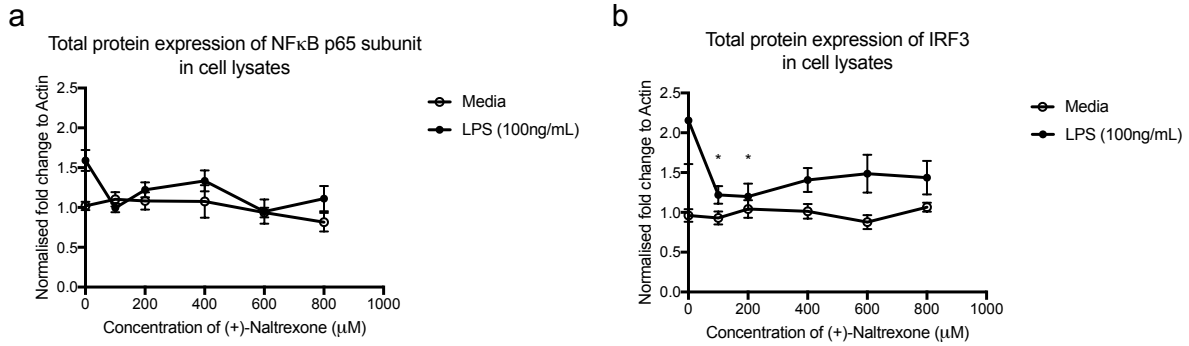


Figure 13 (+)-Naloxone did not alter NFκB p65 subunit expression (a) but reduced IRF3 expression (b) from cell lysates. (+)-Naloxone did not reduce LPS-induced increases in NFκB p65 subunit expression (a). However, 100 – 200uM of (+)-Naloxone reduced LPS-induced IRF3 expression (b). All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6-8; *p <0.05, from vehicle* (absence of (+)-Naloxone, x = 0).

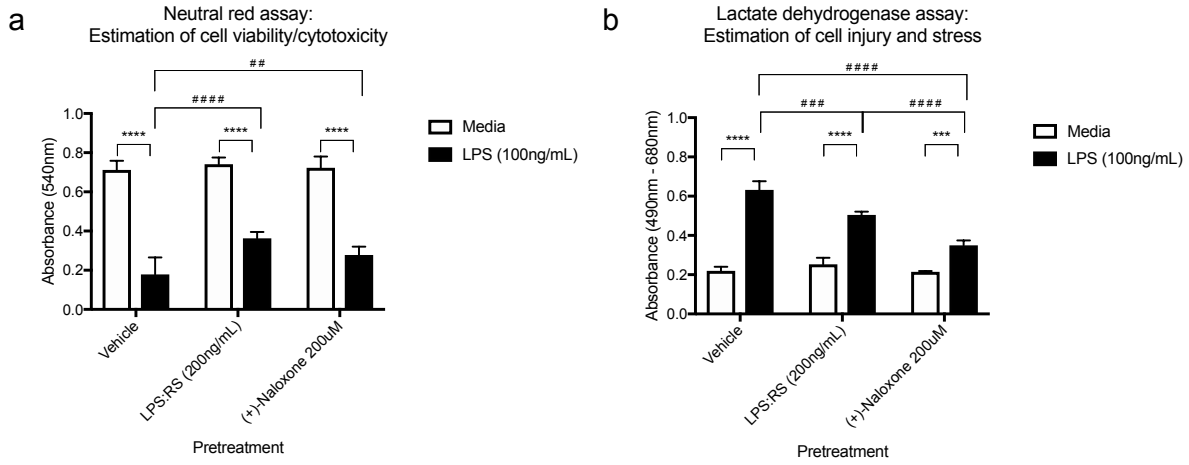


Figure 14 TLR4 antagonists LPS:RS and (+)-Naloxone reduced LPS-induced cytotoxicity (a) and injury (b). Both LPS:RS and (+)-Naloxone increased absorbance in the neutral red assay (a) and reduced absorbance in the lactate dehydrogenase assay (b) following LPS treatment (indicating a reduction in cytotoxicity and injury respectively). However, (+)-Naloxone further reduced lactate dehydrogenase expression compared to LPS:RS. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6-8; ****$p < 0.0001$ (between treatments); ##$p < 0.01$, ###$p < 0.001$, #### $p < 0.0001$ (between pretreatments).

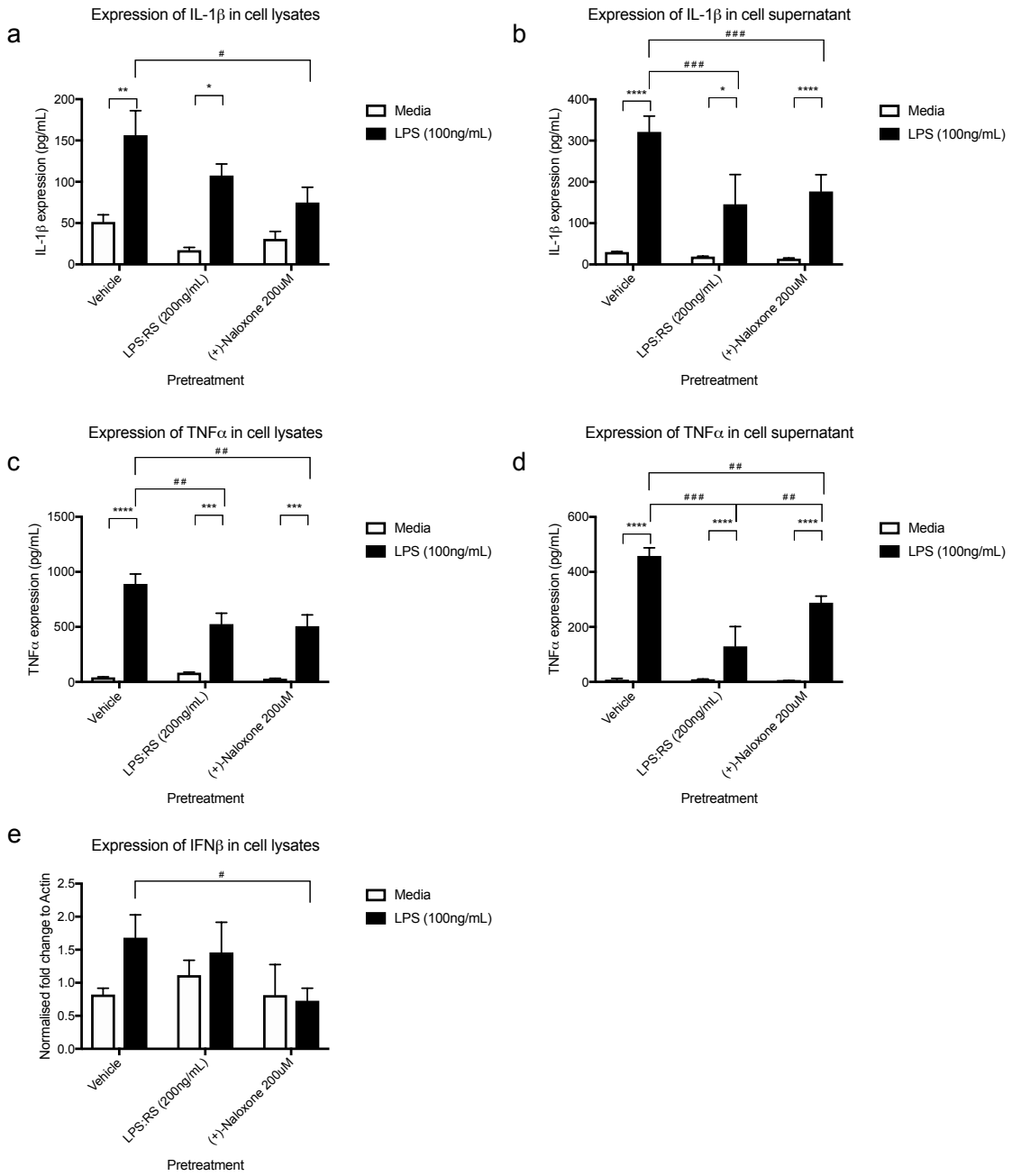


Figure 15 TLR4 antagonists LPS:RS and (+)-Naloxone reduced LPS-induced IL-1β (a, b), TNFα (c, d) and IFNβ (e) expression from BV2 supernatant and lysates. LPS:RS and (+)-Naloxone reduced LPS-induced IL-1β expression in cell supernatant and TNFα expression from both cell lysate and supernatant. (+)-Naloxone additionally reduced LPS-induced IL-1β and IFNβ expression from cell lysates relative to vehicle control. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6-8; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (between treatment); #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (between pretreatment).

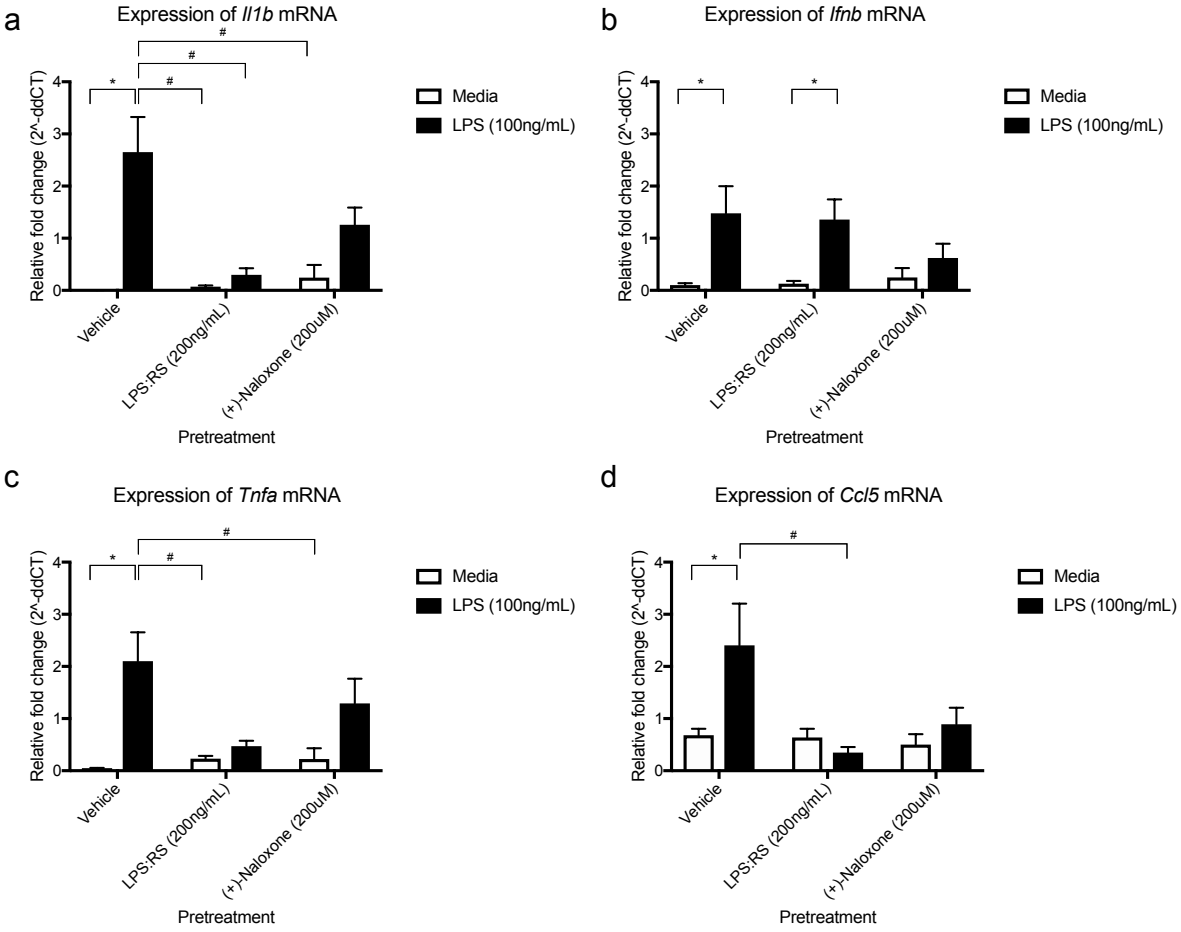


Figure 16 TLR4 antagonists, LPS:RS and (+)-Naloxone reduced LPS-induced *Il1b* (a) mRNA but only LPS:RS reduced *Ifnb* (b), *Tnfa* (c) and *Ccl5* (d) mRNA expression in BV2 cells. Pretreating cells with LPS:RS prevented the LPS-induced increases among cytokine mRNA compared to vehicle pretreated cells. (+)-Naloxone did not reduce LPS-induced cytokine mRNA compared to vehicle pretreated cells. However, the drug prevented LPS-induced increases as inferred by a lack of difference between media and LPS in (+)-Naloxone pretreated cells. All data was analysed using a two-way ANOVA with Bonferroni post-hoc. Summary values represented as mean±SEM; n=4; *$p < 0.05$, within group differences; #$p < 0.05$ between group differences.

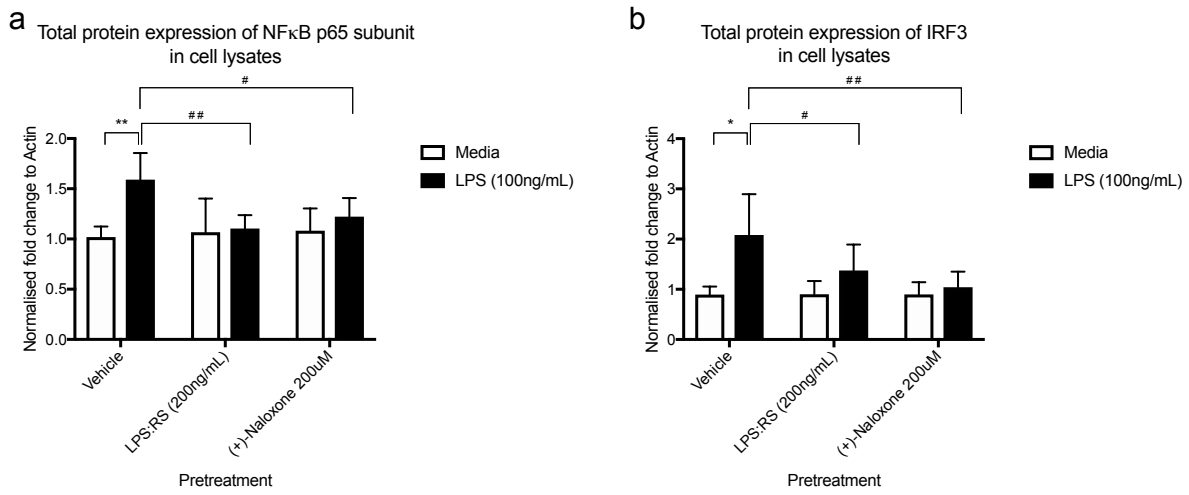


Figure 17 TLR4 antagonists, LPS:RS and (+)-Naloxone reduced LPS-induced NFκB p65 subunit and IRF3 expression in BV2 cells. LPS:RS and (+)-Naloxone reduced the LPS-induced increase in NFκB p65 and IRF3 expression compared to vehicle pretreated cells. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=6-8; *$p < 0.05$, **$p < 0.01$ (between treatments); #$p < 0.05$, ##$p < 0.01$ (between pretreatments).

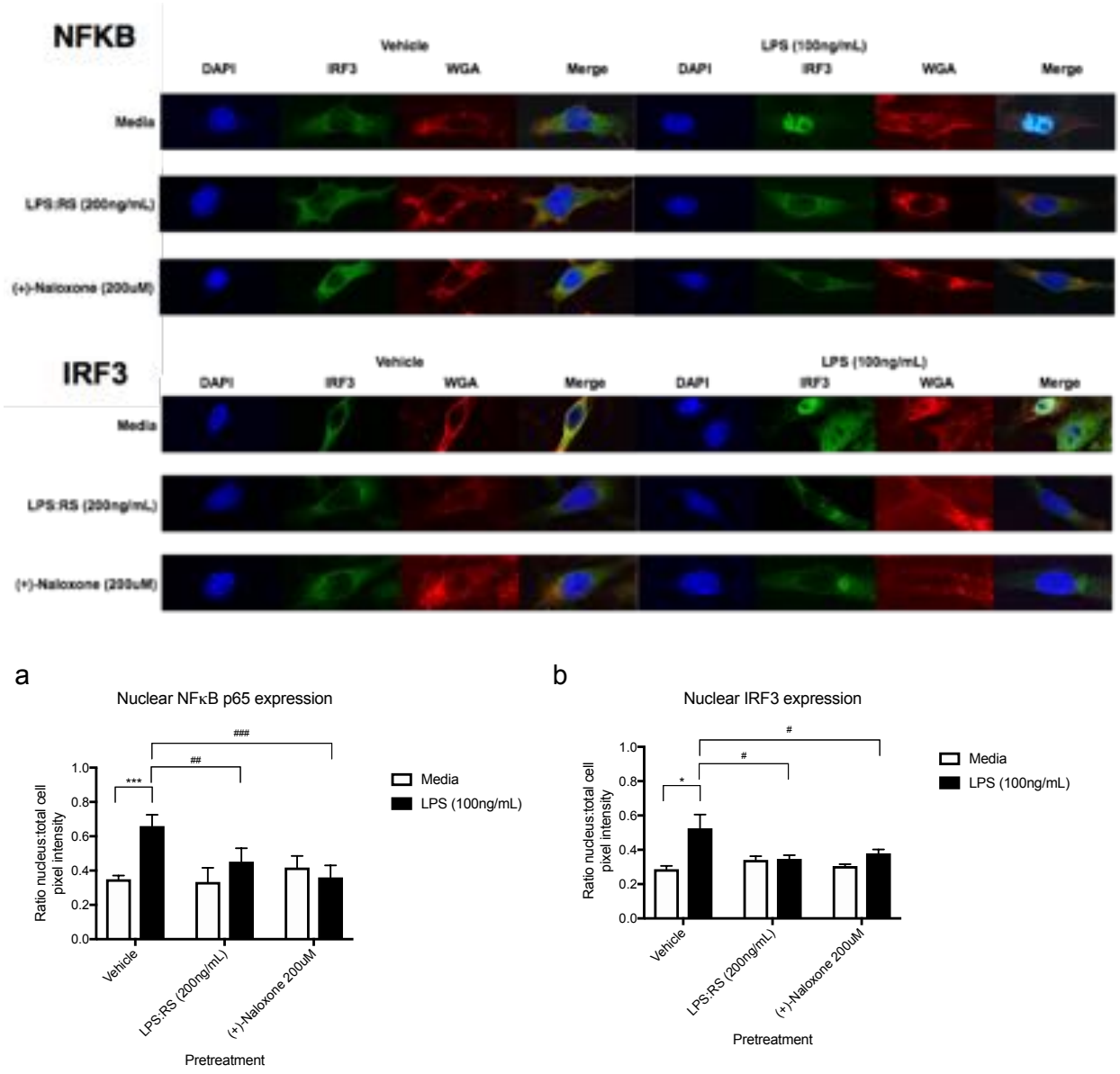


Figure 18 TLR4 antagonists, LPS:RS and (+)-Naloxone reduced LPS-induced NFκB p65 subunit (a) and IRF3 (b) translocation to the nucleus in BV2 cells. LPS:RS and (+)-Naloxone reduced the LPS-induced increase in NFκB p65 and IRF3 translocation as inferred by post hoc analysis. However, there was no main effect of pretreatment on translocation. The representative images of a and b and the corresponding RGB profile plot and colocalisation output from Fiji are shown above. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=4 *p < 0.05, ***p < 0.001 (between treatments); #p < 0.05, ##p < 0.01, ###p < 0.001 (between pretreatments).

9.6 Discussion

The mechanism and outcomes pertaining to (+)-Naloxone antagonism of TLR4 is complex with discrepancies between *in vitro* and *in vivo* findings. For example, *in vitro* experiments suggest (+)-Naloxone is a biased TLR4 antagonist exclusively attenuating the TRIF pathway (Wang et al., 2016). By contrast, *in vivo* experiments demonstrate (+)-Naloxone attenuates IL-1β expression and increases in reactive gliosis (Northcutt et al., 2015; Hutchinson et al., 2010). Consequently, an aim of this study was to further characterise (+)-Naloxone's ability to antagonise a TLR4-induced immune response and to compare it to a well-established TLR4 antagonist LPS:RS. The results presented in this study demonstrate (+)-Naloxone reduced LPS-induced IL-1β, TNFα, IFNβ and IRF3 expression across a range of concentrations with attenuation typically occurring between 200 – 800uM. However, there was no main effect of (+)-Naloxone concentration on the expression of NFκB p65 subunit. Furthermore, when compared to LPS:RS, (+)-Naloxone exhibited a similar antagonistic profile. Both compounds reduced cytokines pertaining to the MyD88 and TRIF dependent pathways primarily at a protein and not at the mRNA level. Furthermore, both compounds prevented NFκB p65 and IRF3 translocation into the nucleus suggesting they are pan rather than biased TLR4 antagonists.

The results presented in this study confirm and contrast the existing *in vitro* and *in vivo* studies assessing the actions of (+)-Naloxone (Wang et al., 2016; Northcutt et al., 2015). This study showed (+)-Naloxone successfully attenuated LPS-induced increases in MyD88 and TRIF pathway products as inferred by a reduction in IL-1β, TNFα, IFNβ and IRF3 expression in BV2 cells. These findings contrast those presented by Wang et al., (2016). In their study, (+)-Naloxone attenuated TNFα and IFNβ but did not attenuate LPS-induced IL-1β expression. They further demonstrated

(+)-Naloxone decreased IRF3 but not NFκB p65 expression. This led the authors to conclude that (+)-Naloxone exhibited biased antagonism.

The differential results may be due to the concentration of LPS administered to BV2 cells. Wang et al., (2016) administered 200ng/mL compared to 100ng/mL of LPS used in this study. This caveat is important when considering how low affinity antagonists such as (+)-Naloxone work. Given that LPS induces a greater MyD88 response compared to the TRIF response, it is hypothesised that the large MyD88 mediated by 200ng/mL of LPS is sufficient to overcome the weak antagonistic abilities of (+)-Naloxone. Consequently, only the TRIF pathway appears to be attenuated by (+)-Naloxone. However, at lower doses of LPS, a situation in which the MyD88 pathway may not be as engaged, (+)-Naloxone may be able to antagonise both the MyD88 and TRIF pathways. This would potentially explain the differential results obtained in this study and those by Wang et al., (2016) and why this drug attenuates an *in vivo* IL-1β expression (Northcutt et al., 2015).

Given that (+)-Naloxone attenuated aspects of both the MyD88 and TRIF pathways, it was compared to LPS:RS, a well characterised TLR4 antagonist. These two drugs are hypothesised to bind to the same pocket in MD2 thereby preventing activation of TLR4 and its signalling pathways (Hutchinson et al., 2010). While these two drugs exhibit different potencies (LPS:RS and (+)-Naloxone require nanomolar and micromolar concentrations to antagonise TLR4 respectively) they still bind to the same area in MD2. It was therefore thought, that they may function in a similar manner and cause comparable outcomes in regard to modifying a TLR4-based immune response. The results in this study suggest that (+)-Naloxone and LPS:RS attenuate MyD88-dependent cytokines IL-1β and TNFα and the upregulation and translocation of NFκB

p65 in response to LPS. Similarly, both compounds reduced markers of TRIF-activation as inferred by a reduction in IRF3 translocation and expression.

However, (+)-Naloxone significantly reduced the TRIF pathway to a greater extent compared to LPS:RS, as inferred by post hoc analysis. For example, (+)-Naloxone reduced LPS-induced increases in IFNβ protein, *ifnb* mRNA and IRF3 expression. This effect was either absent or not as pronounced in LPS:RS treated cells. This conclusion partly supports the results obtained by neutral red and lactate dehydrogenase assays which measured cell cytotoxicity and injury respectively. (+)-Naloxone reduced LPS-induced cytotoxicity and injury more than LPS:RS. LPS-induced apoptosis occurs by multiple pathways involving MyD88 and TRIF pathways. However, recent research has placed more emphasis on the TRIF pathway as a key mediator underlying apoptosis (Jung et al., 2005; Ruckdeschel et al., 2004). This supports the hypothesis that (+)-Naloxone attenuates the TRIF pathway to a greater extent than LPS:RS as (+)-Naloxone reduced markers of cell injuy/death to a greater extent than LPS:RS at 200ng/mL. To confirm this hypothesis however, equal concentrations of LPS:RS and (+)-Naloxone should be compared. The concentration of LPS:RS used in this study is approximately 10 – 20nM and is therefore significantly less than (+)-Naloxone.

Collectively, these studies provide us with further insight into how (+)-Naloxone influences a neuroimmune response *in vitro* and can be used to potentially infer how it is acting in the context of an alcohol-induced neuroimmune response. Overall, the results suggest (+)-Naloxone attenuates aspects of both MyD88 and TRIF pathways with a potentially greater ability to attenuate the TRIF pathway compared to traditional TLR4 antagonists. However, whether this effect is due to the concentration of LPS:RS remains to be determined. Therefore, future studies should examine a range of LPS,

LPS:RS and (+)-Naloxone concentrations to better understand and compare how these agonists and antagonists function at TLR4.

9.7 Acknowledgments

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9.9 Supplementary material

Table 1. Primer sequence used in qPCR

Gene	**Forward Primer 5' – 3'**	**Reverse Primer 5' – 3'**
Ccl5 – Chemokine (C-C motif) ligand 5	CTTCCCTGTCATTGCTTGCTC	CCGAGTGGGAGTAGGGGATT
Ifnb – Interferon beta 1,	TGGGAGATGTCCTCAACTGC	CCAGGCGTAGCTGTTGTACT
Il1b – Interleukin 1 beta	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
Tnfa – Tumour necrosis factor alpha	GACCCTCACACTCAGATCATCTTC	CCTCCACTTGGTGGTTTGCT

9.9.1 Statistics for in-text figures

Figure 2 IL-1β (a – b), TNFα (c – d) and IFNβ (e) expression following 4 or 24 h of stimulation with vehicle, LPS (100ng/mL) or alcohol (100mM). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Supernatant IL-1β expression*

Time, $F_{(1, 5)} = 0.49$, $p = 0.51$

Treatment, $F_{(2, 10)} = 1137$, $p < 0.0001$

Interaction (time x treatment), $F_{(2, 10)} = 0.59$, $p = 0.57$

(b) *Lysate IL-1β expression*

Time, $F_{(1, 5)} = 96.15$, $p = 0.002$

Treatment, $F_{(2, 10)} = 352.6$, $p < 0.0001$

Interaction (time x treatment), $F_{(2, 10)} = 357.5$, $p < 0.0001$

(c) *Supernatant TNFα expression*

Time, $F_{(1, 5)} = 370$, $p < 0.0001$

Treatment, $F_{(2, 10)} = 352.6$, $p < 0.0001$

Interaction (time x treatment), $F_{(2, 10)} = 357.5$, $p < 0.0001$

(d) *Lysate TNFα expression*

Time, $F_{(1, 5)} = 6.36$, $p = 0.053$

Treatment, $F_{(2, 10)} = 6433$, $p < 0.0001$

Interaction (time x treatment), $F_{(2, 10)} = 8.78$, $p = 0.0065$

(e) *Lysate IFNβ expression*

Time, $F_{(1, 5)} = 17.05$, $p = 0.015$

Treatment, $F_{(2, 10)} = 1.17$, $p = 0.36$

Interaction (time x treatment), $F_{(2, 10)} = 0.82$, $p = 0.48$

Figure 3 *Il1b* (a), *Ifnb* (b), *Tnfa* (c) and *Ccl5* (d) mRNA expression following 4 or 24h of stimulation with vehicle, LPS (100ng/mL) or alcohol (100mM). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Il1b mRNA expression*

Time, $F_{(1, 2)} = 0.31$, $p = 0.64$

Treatment, $F_{(2, 6)} = 7.86$, $p = 0.041$

Interaction (time x treatment), $F_{(2, 6)} = 0.37$, $p = 0.71$

(b) *Ifnb mRNA expression*

Time, $F_{(1, 2)} = 0.011$, $p = 0.93$

Treatment, $F_{(2, 6)} = 29.94$, $p = 0.0039$

Interaction (time x treatment), $F_{(2, 6)} = 9.45$, $p = 0.031$

(c) *Tnfa mRNA expression*

Time, $F_{(1, 2)} = 3.65$, $p = 0.20$

Treatment, $F_{(2, 6)} = 10.48$, $p = 0.026$

Interaction (time x treatment), $F_{(2, 6)} = 0.061$, $p = 0.94$

(d) *Ccl5 mRNA expression*

Time, $F_{(1, 2)} = 10.97$, $p = 0.08$

Treatment, $F_{(2, 6)} = 0.19$, $p = 0.83$

Interaction (time x treatment), $F_{(2, 6)} = 2.05$, $p = 0.24$

Figure 4 The expression of NFκB p65 subunit (a) and IRF3 (b) following 4 or 24h of stimulation with vehicle, LPS (100ng/mL) or alcohol (100mM). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 expression*

Time, $F_{(1, 4)} = 5.04$, $p = 0.088$

Treatment, $F_{(2, 8)} = 12.78$, $p = 0.032$

Interaction (time x treatment), $F_{(2, 8)} = 8.88$, $p = 0.0093$

(b) *IRF3 expression*

Time, $F_{(1, 4)} = 17.05$, $p = 0.015$

Treatment, $F_{(2, 8)} = 1.17$, $p = 0.36$

Interaction (time x treatment), $F_{(2, 8)} = 0.822$, $p = 0.48$

Figure 5 Translocation of NFκB p65 subunit (a) and IRF3 (b) at 0.5 and 2 h respectively following stimulation with vehicle, LPS (100ng/mL) or alcohol (100mM). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 expression*

Cellular compartment, $F_{(1, 4)} = 6.38$, $p = 0.065$

Treatment, $F_{(2, 8)} = 4$, $p = 0.062$

Interaction (cellular compartment x treatment), $F_{(2, 8)} = 29.3$, $p = 0.0002$

(b) *IRF3 expression*

Cellular compartment, $F_{(1, 4)} = 54.67$, $p = 0.0018$

Treatment, $F_{(2, 8)} = 4.1$, $p = 0.063$

Interaction (cellular compartment x treatment), $F_{(2, 8)} = 172.4$, $p < 0.001$

Figure 6 Estimation of cell cytotoxicity and injury using following or 24h of stimulation with vehicle, LPS (100ng/mL) or alcohol (100mM). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Neutral red assay*

Time, $F_{(1, 8)} = 68.47$, $p < 0.0001$

Treatment, $F_{(2, 6)} = 68.26$, $p < 0.0001$

Interaction (time x treatment), $F_{(2, 6)} = 3.05$, $p = 0.075$

(b) *Lactate dehydrogenase assay*

Time, $F_{(1, 8)} = 46.59$, $p = 0.0064$

Treatment, $F_{(2, 6)} = 89.05$, $p < 0.0001$

Interaction (time x treatment), $F_{(2, 6)} = 292.4$, $p < 0.0001$

Figure 7 Alcohol reduces LPS-induced IL-1β (a, b), TNFα (c, d) but not IFNβ (e) expression from BV2 supernatant and lysates. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Supernatant IL-1β expression*

Pretreatment, $F_{(1, 5)} = 150.3$, $p < 0.0001$

Treatment, $F_{(2, 10)} = 112.3$, $p < 0.0001$

Interaction (Treatment x treatment), $F_{(2, 10)} = 115.3$, $p < 0.0001$

(b) *Lysate IL-1β expression*

Pretreatment, $F_{(1, 5)} = 16.84$, $p = 0.0093$

Treatment, $F_{(2, 10)} = 29.05$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 16.8$, $p = 0.0006$

(c) *Supernatant TNFα expression*

Pretreatment, $F_{(1, 5)} = 179$, $p < 0.0001$

Treatment, $F_{(2, 10)} = 1085$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 178.7$, $p < 0.0001$

(d) *Lysate TNFα expression*

Pretreatment, $F_{(1, 5)} = 352$, $p < 0.0001$

Treatment, $F_{(2, 10)} = 558$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 401.2$, $p < 0.0001$

(e) *Lysate IFNβ expression*

Pretreatment, $F_{(1, 5)} = 4.03$, $p = 0.12$

Treatment, $F_{(2, 10)} = 3.18$, $p = 0.15$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 0.052$, $p = 0.83$

Figure 8 Alcohol reduces LPS-induced mRNA expression of *Il1b* (a), *Ifnb* (b), and *Tnfa* (c) but not *Ccl5* (d). All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Il1b mRNA expression*

Pretreatment, $F_{(1, 3)} = 13.87$, $p = 0.034$

Treatment, $F_{(1, 3)} = 13.71$, $p = 0.034$

Interaction (time x treatment), $F_{(1, 3)} = 13.44$, $p = 0.035$

(b) *Ifnb mRNA expression*

Pretreatment, $F_{(1, 3)} = 73.93$, $p = 0.033$

Treatment, $F_{(1, 3)} = 8.56$, $p = 0.061$

Interaction (time x treatment), $F_{(1, 3)} = 21.72$, $p = 0.019$

(c) *Tnfa mRNA expression*

Pretreatment, $F_{(1, 3)} = 22.39$, $p = 0.018$

Treatment, $F_{(1, 3)} = 17.38$, $p = 0.025$

Interaction (time x treatment), $F_{(1, 3)} = 21.41$, $p = 0.019$

(d) *Ccl5 mRNA expression*

Pretreatment, $F_{(1, 3)} = 29.34$, $p = 0.012$

Treatment, $F_{(1, 3)} = 0.017$, $p = 0.90$

Interaction (time x treatment), $F_{(1, 3)} = 0.56$, $p = 0.50$

Figure 9 Alcohol nor LPS alters the expression NFκB p65 subunit (a) and IRF3 (b) from BV2 cell lysates. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 expression*

Pretreatment, $F_{(1, 5)} = 0.96$, $p = 0.38$

Treatment, $F_{(2, 10)} = 4.73$, $p = 0.095$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 0.26$, $p = 0.63$

(b) *IRF3 expression*

Pretreatment, $F_{(1, 5)} = 2.54$, $p = 0.21$

Treatment, $F_{(2, 10)} = 13.47$, $p = 0.035$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 1.77$, $p = 0.27$

Figure 10 Alcohol has no effect on LPS-induced cell cytotoxicity but reduces cellular injury as inferred by neutral red (a) Lactate dehydrogenase assays (b) respectively. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Neutral red assay*

Pretreatment, $F_{(1, 5)} = 0.44$, $p = 0.57$

Treatment, $F_{(2, 10)} = 16.14$, $p = 0.056$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 0.00054$, $p = 0.98$

(b) *Lactate dehydrogenase assay*

Pretreatment, $F_{(1, 5)} = 21.90$, $p = 0.0054$

Treatment, $F_{(2, 10)} = 415.9$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 97.65$, $p = 0.0002$

Figure 11 (+)-Naloxone modulates LPS-induced BV2 cells cytotoxicity and injury as inferred by neutral red (a) or Lactate dehydrogenase assays (b). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Neutral red assay*

Concentration, $F_{(5, 55)} = 11.33$, $p < 0.0001$

Treatment, $F_{(1, 11)} = 578.3$, $p < 0.0001$

Interaction (concentration x treatment), $F_{(5, 55)} = 6.45$, $p < 0.0001$

(b) *Lactate dehydrogenase assay*

Concentration, $F_{(5, 55)} = 60.82$, $p < 0.0001$

Treatment, $F_{(1, 11)} = 143.6$, $p < 0.0001$

Interaction (concentration x treatment), $F_{(5, 55)} = 59.86$, $p < 0.0001$

Figure 12 (+)-Naloxone reduced LPS-induced IL-1β (a, b), TNFα (c, d) and IFNβ (e) expression from cell supernatant and lysates. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Lysate IL-1β expression*

Concentration, $F_{(5, 35)} = 10.69$, $p < 0.0001$

Treatment, $F_{(1, 7)} = 70.89$, $p < 0.0001$

Interaction (concentration x treatment), $F_{(5, 35)} = 18.92$, $p < 0.0001$

(b) *Supernatant IL-1β expression*

Concentration, $F_{(5, 35)} = 7.73$, $p = 0.002$

Treatment, $F_{(1, 7)} = 51.79$ $p < 0.0001$

Interaction (concentration x treatment), $F_{(5, 35)} = 2.24$, $p = 0.0822$

(c) *Lysate TNFα expression*

Concentration, $F_{(5, 35)} = 20.04$, $p < 0.0001$

Treatment, $F_{(1, 7)} = 141.6$, $p < 0.0001$

Interaction (concentration x treatment), $F_{(5, 35)} = 18.92$, $p < 0.0001$

(d) *Supernatant TNFα expression*

Concentration, $F_{(5, 35)} = 3.87$, $p = 0.0098$

Treatment, $F_{(1, 7)} = 2066$, $p < 0.0001$

Interaction (concentration x treatment), $F_{(5, 35)} = 3.12$, $p = 0.0253$

(e) *Lysate IFNβ expression*

Concentration, $F_{(5, 35)} = 1.19$, $p = 0.36$

Treatment, $F_{(1, 7)} = 11.25$, $p = 0.044$

Interaction (concentration x treatment), $F_{(5, 35)} = 2.29$, $p = 0.098$

Figure 13 (+)-Naloxone had no effect and reduced the expression NFκB p65 subunit (a) and IRF3 (b) expression from cell lysates respectively. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 subunit expression*

Concentration, $F_{(5, 35)} = 3.12$, $p = 0.05$

Treatment, $F_{(1, 7)} = 8.23$, $p = 0.064$

Interaction (concentration x treatment), $F_{(5, 35)} = 1.55$, $p = 0.23$

(b) *IRF3 expression*

Concentration, $F_{(5, 35)} = 1.73$, $p = 0.19$

Treatment, $F_{(1, 7)} = 11.62$ $p = 0.042$

Interaction (concentration x treatment), $F_{(5, 35)} = 2.05$, $p = 0.13$

Figure 14 TLR4 antagonists LPS:RS and (+)-Naloxone reduced LPS-induced cytotoxicity (a) and injury (b). All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Neutral red assay*

Pretreatment, $F_{(2, 30)} = 12.13$, $p = 0.0001$

Treatment, $F_{(1, 30)} = 653.9$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 30)} = 6.41$, $p = 0.0048$

(b) *Lactate dehydrogenase assay*

Pretreatment, $F_{(2, 10)} = 103.7$, $p < 0.0001$

Treatment, $F_{(1, 5)} = 2782$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 10)} = 49.35$, $p < 0.0001$

Figure 15 TLR4 antagonists LPS:RS and (+)-Naloxone reduced LPS-induced IL-1β (a, b), TNFα (c, d) and IFNβ (e) expression from BV2 supernatant and lysates. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Lysate IL-1β expression*

Pretreatment, $F_{(2, 14)} = 4.84$, $p = 0.033$

Treatment, $F_{(1, 7)} = 82.16$, $p = 0.003$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 1.42$, $p = 0.29$

(b) *Supernatant IL-1β expression*

Pretreatment, $F_{(2, 14)} = 11.54$, $p = 0.0011$

Treatment, $F_{(1, 7)} = 17.17$, $p = 0.0043$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 8.23$, $p = 0.0043$

(c) *Lysate TNFα expression*

Pretreatment, $F_{(2, 14)} = 8.87$, $p = 0.0061$

Treatment, $F_{(1, 7)} = 59.13$, $p = 0.0006$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 9.47$, $p = 0.0049$

(d) *Supernatant TNFα expression*

Pretreatment, $F_{(2, 14)} = 16.59$, $p = 0.0002$

Treatment, $F_{(1, 7)} = 69.58$, $p < 0.0001$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 17.78$, $p < 0.0001$

(e) *Lysate IFNβ expression*

Pretreatment, $F_{(2, 14)} = 19.71$, $p = 0.0023$

Treatment, $F_{(1, 7)} = 12.35$, $p = 0.039$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 2.82$, $p = 0.14$

Figure 16 TLR4 antagonists, LPS:RS and (+)-Naloxone reduce LPS-induced *Il1b* (a) mRNA but only LPS:RS reduces *Ifnb* (b), *Tnfa* (c) and *Ccl5* (d) mRNA expression in BV2 cells All data was analysed using a two-way ANOVA with Bonferonni post hoc.

(a) *Il1b mRNA expression*

Pretreatment, $F_{(2, 6)} = 6.33$, $p = 0.033$

Treatment, $F_{(1, 3)} = 13.1$, $p = 0.036$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 8.81$, $p = 0.016$

(b) *Ifnb mRNA expression*

Pretreatment, $F_{(2, 6)} = 1.06$, $p = 0.40$

Treatment, $F_{(1, 3)} = 5.40$, $p = 0.10$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 3.60$, $p = 0.094$

(c) *Tnfa mRNA expression*

Pretreatment, $F_{(2, 6)} = 3.49$, $p = 0.099$

Treatment, $F_{(1, 3)} = 8.96$, $p = 0.058$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 5.84$, $p = 0.039$

(d) *Ccl5 mRNA expression*

Pretreatment, $F_{(2, 6)} = 4.84$, $p = 0.05$

Treatment, $F_{(1, 3)} = 2.09$, $p = 0.24$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 4.34$, $p = 0.068$

Figure 17 TLR4 antagonists, LPS:RS and (+)-Naloxone reduced LPS-induced NFκB p65 subunit expression but had no effect on IRF3 expression in BV2 cells. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 subunit expression*

Pretreatment, $F_{(2, 14)} = 2.15$, $p = 0.20$

Treatment, $F_{(1, 7)} = 5.46$, $p = 0.10$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 8.11$, $p = 0.02$

(b) *IRF3 expression*

Pretreatment, $F_{(2, 14)} = 1.68$, $p = 0.26$

Treatment, $F_{(1, 7)} = 10.76$, $p = 0.046$

Interaction (pretreatment x treatment), $F_{(2, 14)} = 2.96$, $p = 0.13$

Figure 18 TLR4 antagonists, LPS:RS and (+)-Naloxone reduced LPS-induced NFκB p65 subunit (a) and IRF3 (b) translocation to the nucleus in BV2 cells. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 subunit expression*

Pretreatment, $F_{(2, 6)} = 7.72$, $p = 0.021$

Treatment, $F_{(1, 3)} = 23.54$, $p = 0.016$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 24.92$, $p = 0.0012$

(b) *IRF3 expression*

Pretreatment, $F_{(2, 6)} = 3.69$, p = 0.090

Treatment, $F_{(1, 3)} = 4.38$, p = 0.13

Interaction (pretreatment x treatment), $F_{(2, 6)} = 8.74$, p = 0.016

9.9.2 Supplementary figures

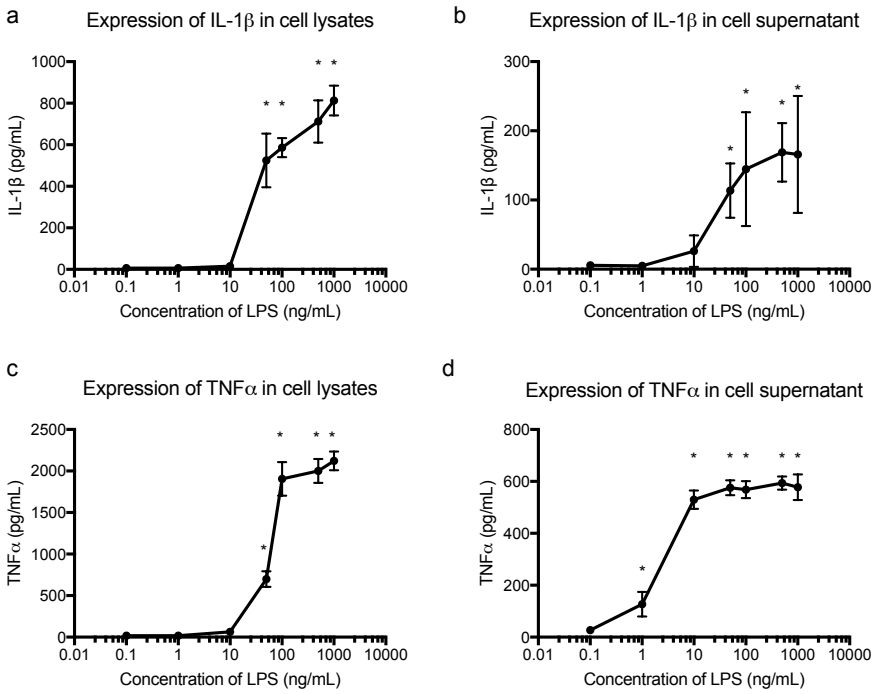

Figure s1 LPS dose-dependently increases the lysate and supernatant of IL-1β (a, b) and TNFα (c, d) from BV2 cells. All data was analysed using a one-way ANOVA. Summary values represented as mean±SEM; n=12

*$p < 0.0001$ compared to LPS concentration = 0ng/mL

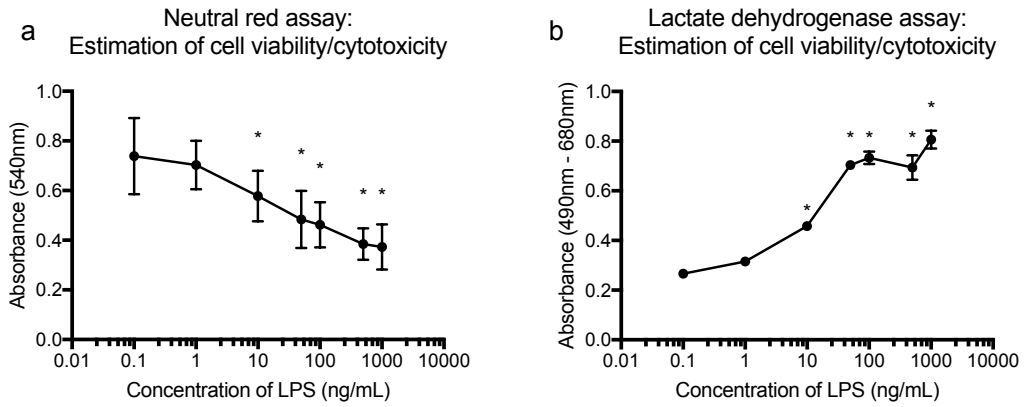

Figure s2 LPS dose-dependently reduces markers of cell viability (a) and increases markers of cell injury (b). Increasing the concentration of LPS significantly reduced absorbance in the neutral red assay (indicative of increased cytotoxicity). By contrast, increasing the concentration of LPS increased absorbance in the lactate dehydrogenase assays indicating increased cellular release of lactate dehydrogenase. All data was analysed using a one-way ANOVA. Summary values represented as mean±SEM; n=4-12; *$p < 0.0001$ compared to LPS concentration = 0ng/mL

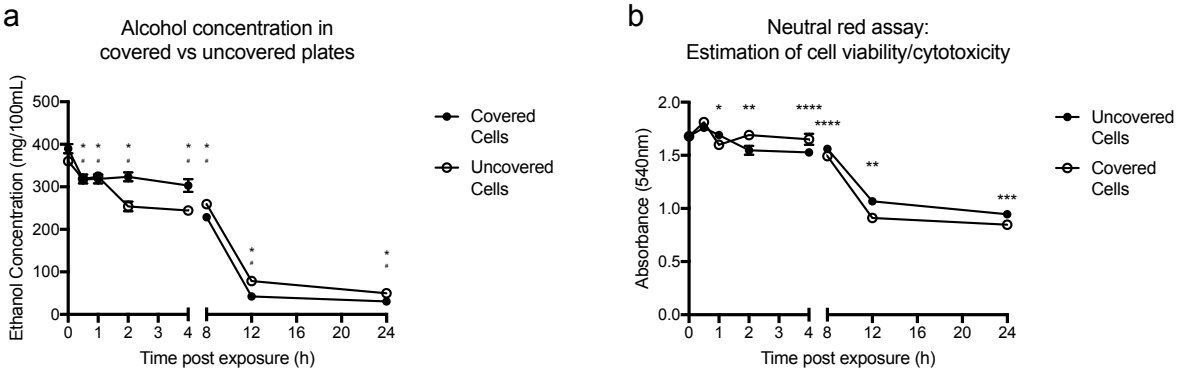

Figure s3 The concentration of alcohol (a) and cell viability/cytotoxicity (b) in plates that are covered with a plate seal compared to unsealed plates. Alcohol evaporates or is metabolised by approximately 12hs following its administration to BV2 cells. Applying a plate seal to reduce ethanol evaporation does not alter the rate at which this occurs (a) however, it does increase cytotoxicity (decrease the absorbance) in the neutral red assay. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=4-8

a *$p < 0.001$ from T_0 from covered cells; # $p < 0.001$ from T_0 uncovered cells

b *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$ uncovered compared to covered

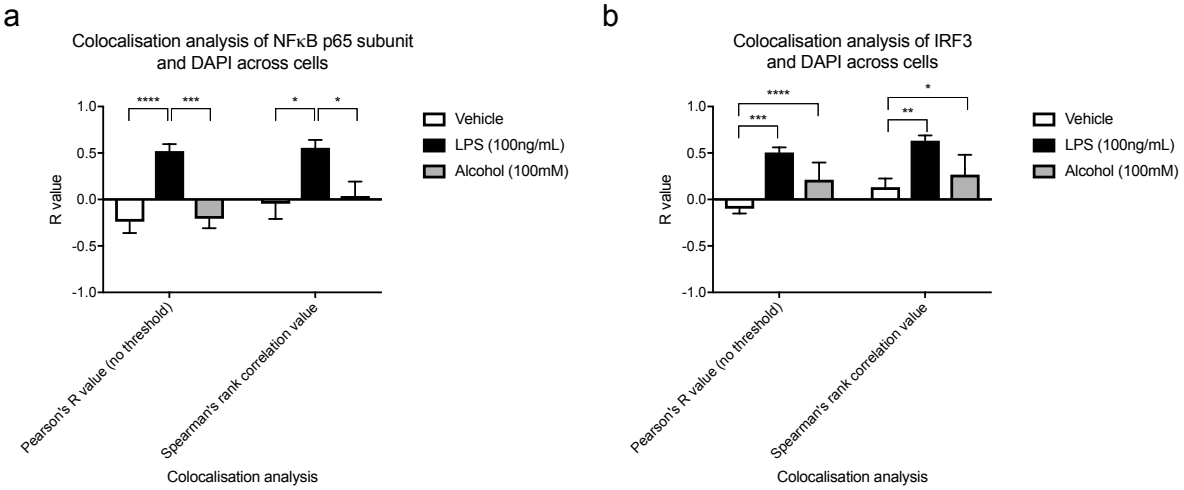

Figure s4 Validation of nuclear translocation using Pearson's coefficient and Spearman's Rank Value colocalisation analysis for NFκB p65 subunit (a) and IRF3 (b) translocation to the nucleus in BV2 cells. LPS resulted in significant colocalisation of the DAPI and NFκB p65 or IRF3 (405nm and 488nm) channels compared to alcohol and vehicle treated cells. Alcohol induced significantly greater colocalisation between IRF3 and DAPI compared to vehicle. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=4; * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$

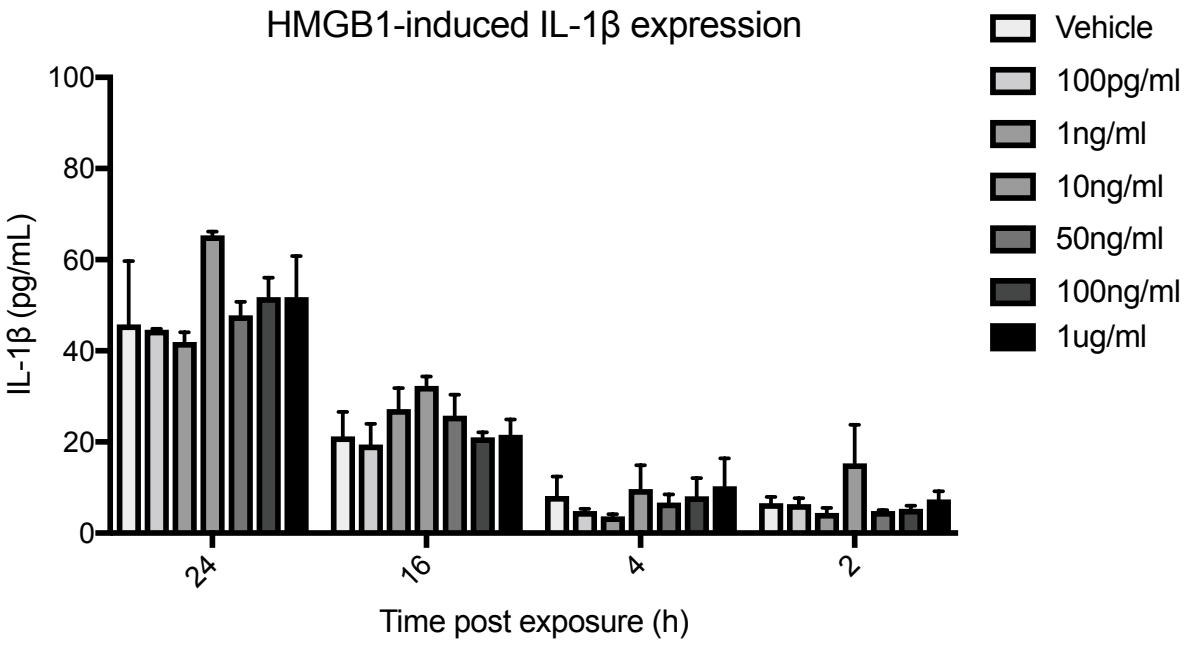

Figure s5 HMGB1(ab18650) failed to induce an IL-1β response from BV2 supernatant compared to media across multiple time points and concentrations. No post hoc differences were identified. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=3

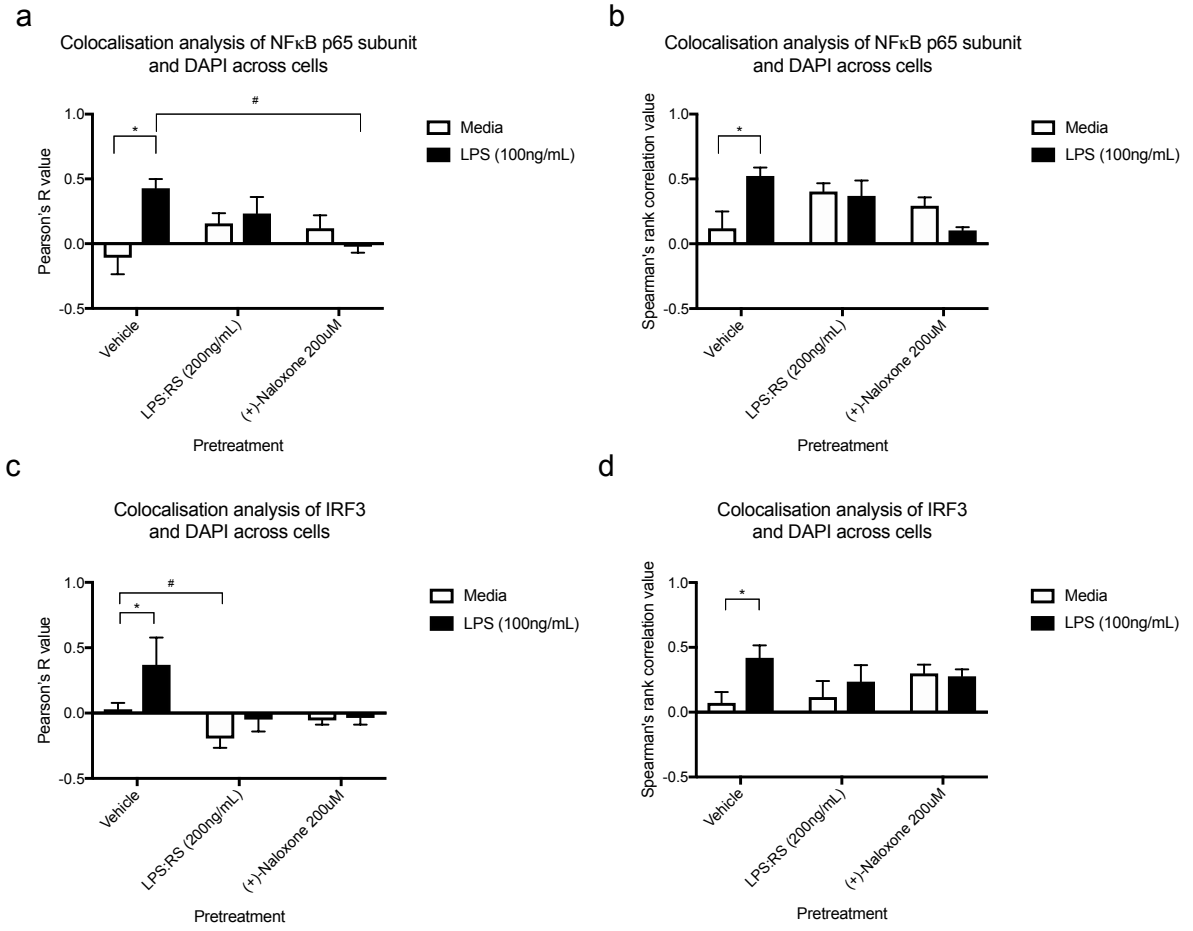

Figure s6 Validation of nuclear translocation using Pearson's coefficient and Spearman's Rank Value colocalisation analysis for NFκB p65 subunit (a, b) and IRF3 (c, d) translocation to the nucleus in BV2 cells. LPS treatment resulted in significant colocalisation between DAPI and NFκB p65 or IRF3 compared to vehicle treated cells. (+)-Naloxone significantly reduced LPS-induced colocalisation between NFκB p65 and DAPI compared to vehicle treated cells using the Pearson's colocalisation analysis. Cells pretreated with LPS:RS did not significantly modify colocalisation compared to vehicle. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=4; *$p < 0.05$ (within treatments); #$p < 0.05$ (within pretreatments).

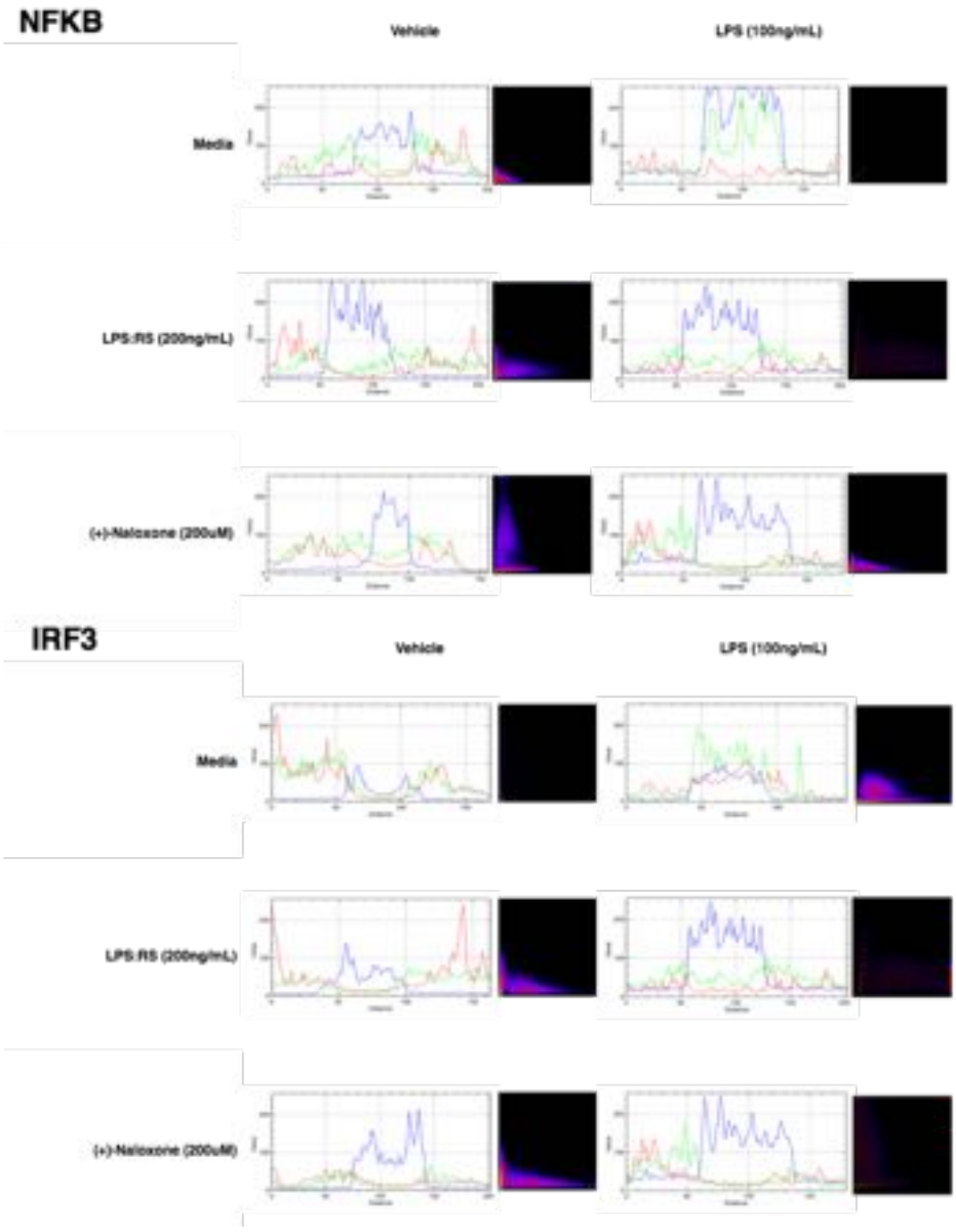

Figure s7 Representative RGB profile plots and colocalisation analysis outputs from Fiji of cells pretreated with vehicle, (+)-Naloxone or LPS:RS and treated with LPS or media.

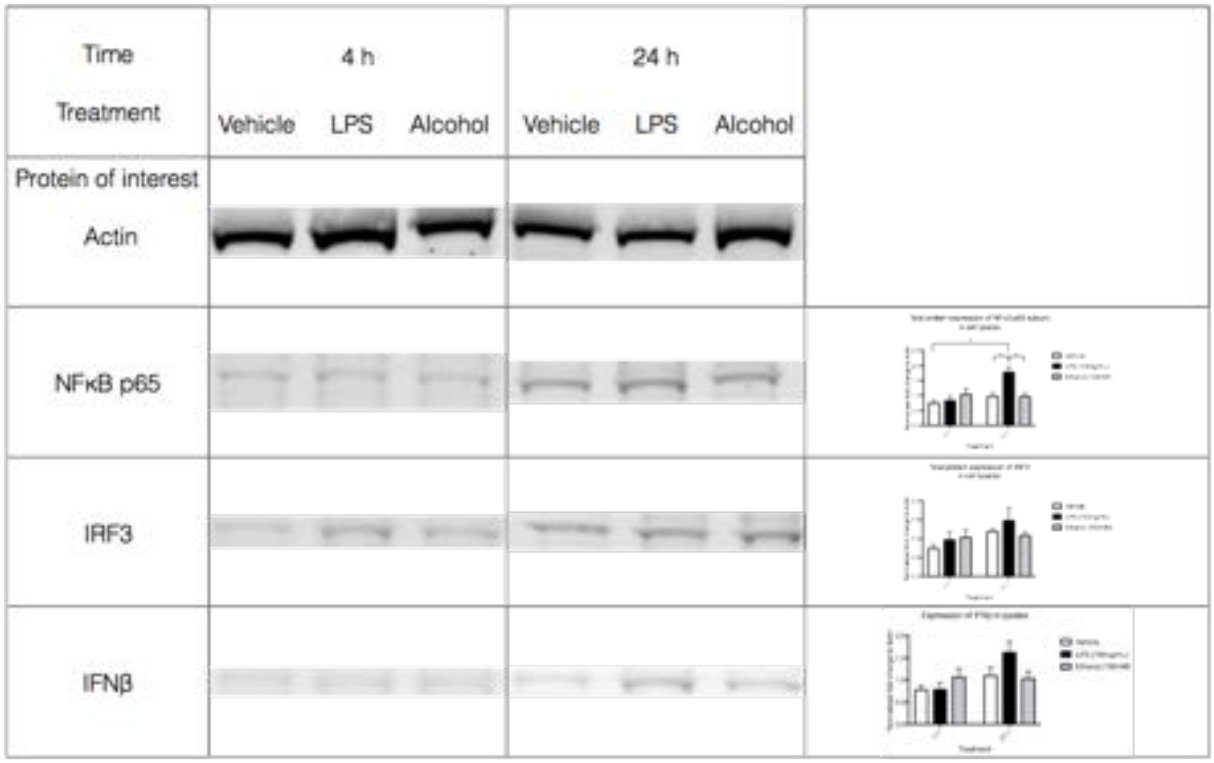

Figure s8 Representative western blots for Figures 2 and 4

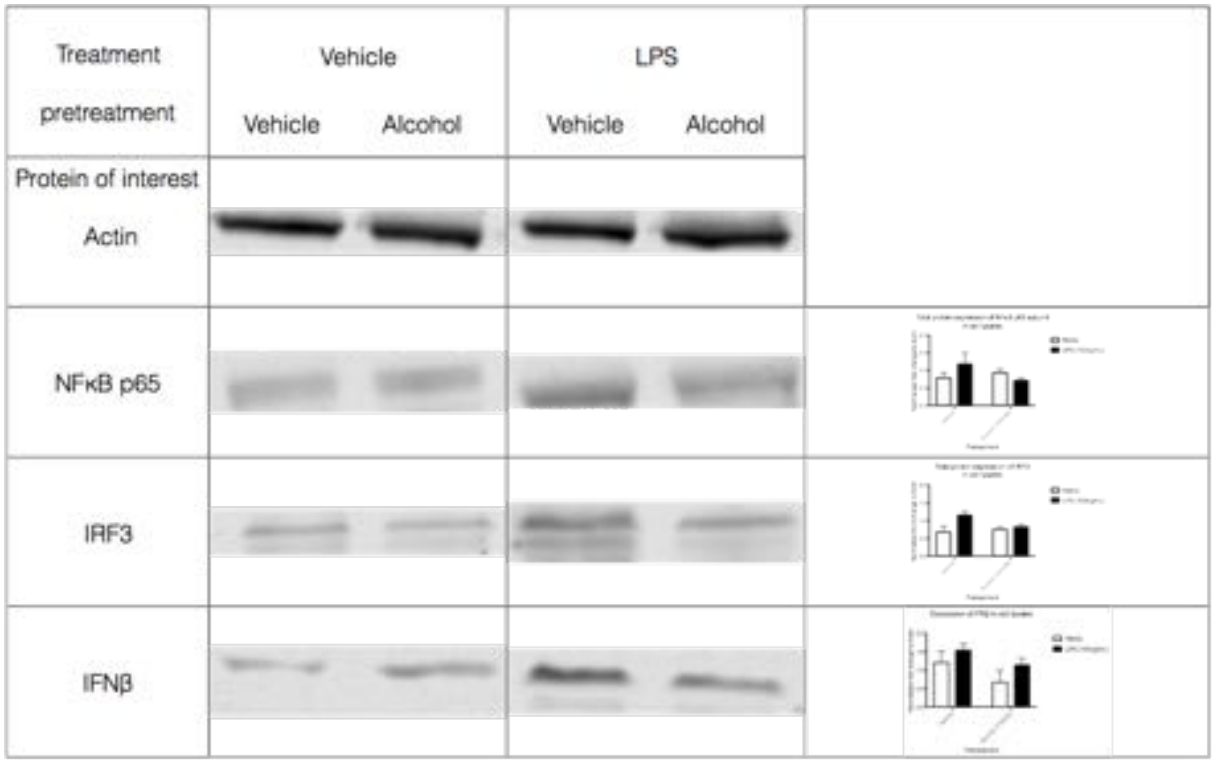

Figure s9 Representative western blots for Figures 7 and 9

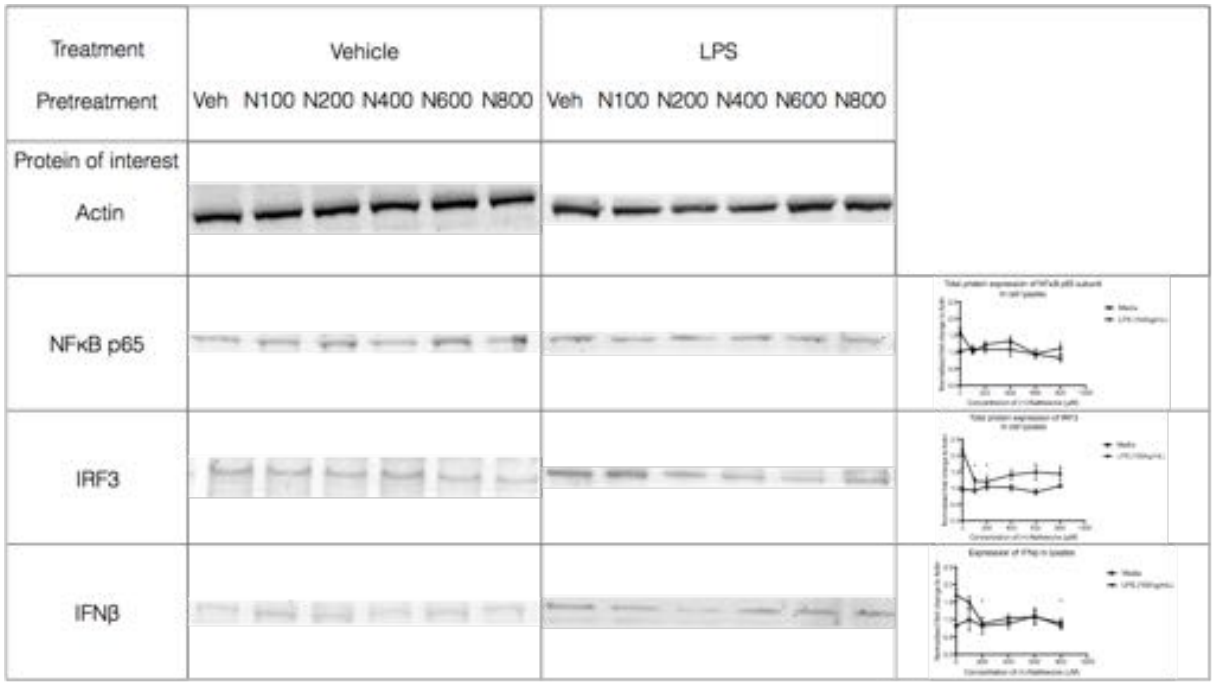

Figure s10 Representative western blots for Figures 12 and 13

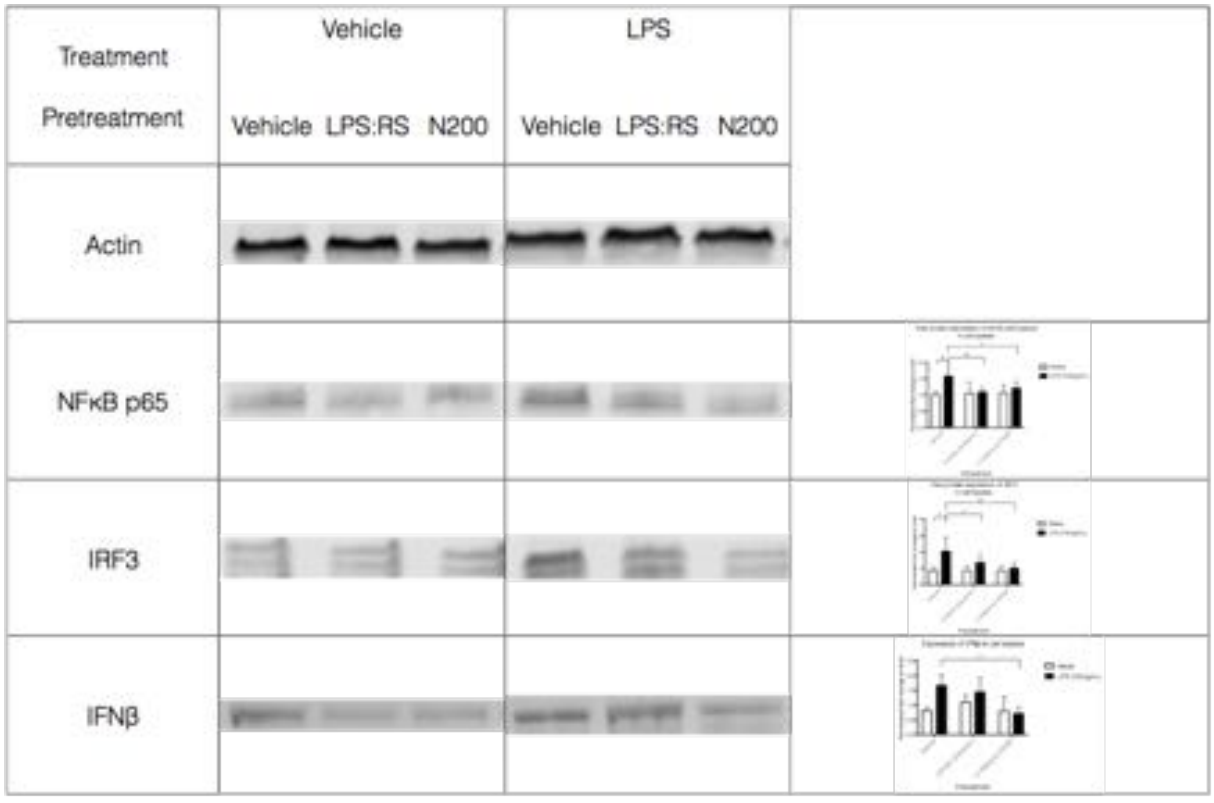

Figure s11 Representative western blots for Figures 15 and 17

9.9.3 Statistics for supplementary figures

Figure s1 LPS dose-dependently increases the lysate and supernatant of IL-1β (a, b) and TNFα (c, d) from BV2 cells. All data was analysed using a one-way ANOVA.

(a) IL-1β lysate

Effect of concentration, $F_{(7, 112)} = 238.2$, $p < 0.0001$

(b) IL-1β supernatant

Effect of concentration, $F_{(7, 112)} = 36.35$, $p < 0.0001$

(c) TNFα lysate

Effect of concentration, $F_{(7, 112)} = 645.2$, $p < 0.0001$

(d) TNFα supernatant

Effect of concentration, $F_{(7, 112)} = 1001$, $p < 0.0001$

Figure s2 LPS dose-dependently reduces markers of cell viability (a) and increases markers of cell injury (b). All data was analysed using a one-way ANOVA.

(a) *Neutral red assay*

Effect of concentration, $F_{(7, 120)} = 34.23$, $p < 0.0001$

(b) *Lactate dehydrogenase assay*

Effect of concentration, $F_{(7, 24)} = 78.96$, $p < 0.0001$

Figure s3 The concentration of alcohol (a) and cell viability/cytotoxicity (b) in plates that are covered with a plate seal compared to unsealed plates. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *Alcohol concentration assay*

Effect of condition, $F_{(1, 3)} = 10.03$, $p = 0.051$

Effect of time, $F_{(7, 21)} = 1108$, $p < 0.0001$

Interaction (condition x time), $F_{(7, 21)} = 46.54$, $p < 0.001$

(b) *Neutral red assay*

Effect of condition, $F_{(1, 3)} = 1.52$, $p = 0.34$

Effect of time, $F_{(7, 21)} = 1188$, $p < 0.0001$

Interaction (time x treatment), $F_{(7, 21)} = 45.59$, $p < 0.001$

Figure s4 Validation of nuclear translocation using Pearson's coefficient and Spearman's Rank Value colocalisation analysis for NFκB p65 subunit (a) and IRF3 (b) translocation to the nucleus in BV2 cells. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 subunit*

Pearson's correlation coefficient, $F_{(2, 24)} = 18.42$, $p < 0.0001$

Spearman's rank value, $F_{(2, 26)} = 5.72$, $p = 0.0087$

(b) *IRF3*

Pearson's correlation coefficient, $F_{(2, 24)} = 19.63$, $p < 0.0001$

Spearman's rank value, $F_{(2, 24)} = 8.00$, $p = 0.0022$

Figure s5 HMGB1(ab18650) failed to induce an IL-1β response from BV2 supernatant compared to media across multiple time points and concentrations. N All data was analysed using a two-way ANOVA with Tukey post hoc.

IL-1β supernatant

Effect of concentration, $F_{(6, 12)} = 7.32$, $p = 0.0018$

Effect of time, $F_{(3, 6)} = 52.28$, $p = 0.0001$

Interaction (concentration x time), $F_{(18, 36)} = 1.58$, $p = 0.11$

Figure s6 Validation of nuclear translocation using Pearson's coefficient and Spearman's Rank Value colocalisation analysis for NFκB p65 subunit (a, b) and IRF3 (c, d) translocation to the nucleus in BV2 cells. All data was analysed using a two-way ANOVA with Tukey post hoc.

(a) *NFκB p65 subunit Pearson's coefficient*

Effect of pretreatment, $F_{(2, 6)} = 1.46$, $p = 0.30$

Effect of treatment, $F_{(1, 3)} = 5.03$, $p = 0.11$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 7.82$, $p = 0.021$

(b) *NFκB p65 subunit Spearman's Rank Value*

Effect of pretreatment, $F_{(2, 6)} = 7.37$, $p = 0.024$

Effect of treatment, $F_{(1, 3)} = 1.04$, $p = 0.38$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 3.93$, $p = 0.081$

(c) *IRF3 Pearson's coefficient*

Effect of pretreatment, $F_{(2, 6)} = 1.04$, $p = 0.41$

Effect of treatment, $F_{(1, 3)} = 0.15$, $p = 0.73$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 9.34$, $p = 0.0144$

(d) *IRF3 Spearman's Rank Value*

Effect of pretreatment, $F_{(2, 6)} = 0.70$, $p = 0.53$

Effect of treatment, $F_{(1, 3)} = 8.77$, $p = 0.059$

Interaction (pretreatment x treatment), $F_{(2, 6)} = 3.35$, $p = 0.11$

Statement of authorships for Appendix

Title: TLR4$^{-/-}$ mice exhibit reduced alcohol preference compared to wildtype mice

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Principle author

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Dr Kenner Rice provided (+)-Naltrexone and assisted in the preparation of the manuscript.

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By signing the Statement of Authorship, each author certifies that the candidate's stated contribution to the publication is accurate; permission is granted for the candidate to include the publication in the thesis; and the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Appendix: TLR4$^{-/-}$ mice exhibit reduced alcohol preference compared to wildtype mice

10.1 Introduction

The aim of this experiment was to determine whether TLR4$^{-/-}$ mice (Balb/c background) and wildtype Balb/c mice exhibit differences in the "liking" and "wanting" components of alcohol reward.

10.2 Methods

10.2.1 Animals

Male (8 – 10-week-old) wildtype mice (Balb/c) from the University of Adelaide Laboratory Animal Services (Adelaide, SA, Australia) and male Balb/c mice with genetic knockout of TLR4 (*TLR4*$^{-/-}$) (originally sourced from Professor Akira (Osaka University, Osaka Japan) via Dr. Paul Foster from the University of Newcastle (Newcastle, NSW, Australia)) were used for the following experiments. Mice were housed in light/dark (12:12 hours) and temperature controlled rooms (23±3°C) with food and water available ad libitum. The light cycle began at 7am (ZT0) and concluded at 7pm (ZT12). Following seven days of acclimatisation, mice were handled by the experimenter for five days prior to experimentation. Mice were weighed daily throughout the handling and experimental periods. All animal care and experiments complied with the principles of the Australian Code of Practice for the care and use of animals for scientific purposes and were approved by the University of Adelaide's Animal Ethics Committee.

10.2.2 Drugs

Saccharin and quinine were purchased from Sigma Aldrich (St Louis, MO, USA). Ethanol (99.5%) (herein referred to as alcohol) was purchased from Chemsupply

(Gliman, SA, Australia). Oral gavages of alcohol were dosed at 1.5g/kg (25 per cent v/v) for conditioned place preference studies. Saline oral gavages were volume-matched. The dose of alcohol used in conditioned place preference was based upon the effective dose 50 from an unpublished conditioned place preference dose response curve.

(+)-Naltrexone, a TLR4-TRIF antagonist was synthesised and kindly supplied by Dr Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute of Alcohol Abuse and Alcoholism, Bethesda, MD, USA). (+)-Naltrexone was administered via intraperitoneal injections with doses ranging from 1 to 75 mg/kg (dose volume 10 ml/kg). Saline intraperitoneal injections were volume-matched.

10.2.3 Alcohol two-bottle choice

Alcohol drinking and preference was assessed using an 8 h two-bottle choice paradigm. Following 14 days of acclimatisation and handling, mice were placed into individual cages. After a further week of acclimatisation, mice were presented with two bottles containing water 2 h after the beginning of the dark cycle (ZT14) for 8 h. Two bottles of water were initially presented to mice in order to control for novelty-induced drinking. For the 8 h test, the bottles were removed ZT22, weighed and replaced with a single bottle of water randomised to either the left or right side of the cage for the remaining 16 h.

Following five days of drinking water from two bottles, mice were offered one bottle containing water and the other 3, 6, 9, 12, 15, 18, 21 or 42 per cent alcohol (v/v). The concentration and bottle position was randomised daily to prevent the acquisition of alcohol drinking and side preferences respectively. After 8 h bottles were removed

weighed and replaced by a water bottle randomly allocated to either side of the cage lid.

The amount of alcohol consumed was determined by the difference in bottle weights before and after drinking sessions. This enabled the calculation of the amount of alcohol consumed per kilogram bodyweight (grams/kilogram) and the preference ratio (alcohol intake ÷ (total water + alcohol intake)) for each mouse and averaged for each group per concentration of alcohol. An empty cage with two bottles was used to determine the rate of evaporation. The rate of evaporation was subtracted from the final weight of test bottles.

10.2.4 Saccharin and quinine two-bottle choice

Mice were also tested using the same 8 h paradigm above for saccharin (1, 15, 30, 45 and 60 mM) and quinine (0.001, 0.01, 0.1, 1 and 5 mM) preference. However, instead of the bottle of alcohol, mice received a bottle of saccharin or quinine and a bottle of water.

10.2.5 Conditioned place preference

Conditioned place preference was used to infer alcohol-seeking behaviour (Bardo & Bevins, 2000).

10.2.5.1 Apparatus

The conditioning apparatus consisted of two conditioning chambers (10.9 (length) x 9.3 (width) x 35 (height) cm) separated by a neutral chamber (16.6 x 4.8 x 35 cm). The neutral chamber contained black walls with grey flooring. The conditioning chambers differed in tactile and visual cues. The flooring of the conditioning chambers were either black plexiglass perforated holes (5 mm apart) or black plexiglass grids (5 mm apart).

The walls of each chamber were white or black. The combination of wall colour to floor texture was randomised for each cohort to prevent any inherent biases mice have for a specific texture x colour combination.

During conditioning, a sliding partition restricted access to only one chamber. Movement and time spent in each chamber was recorded using Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA).

10.2.5.2 Procedure

Day 1: Pretest. Mice were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

Day 2 to 9: Conditioning. Mice received an oral gavage of alcohol (1.5 g/kg) and placed within their conditioning chamber for 30 min on days 1, 3, 5 and 7. On days 2, 4, 6 and 8, mice received an oral gavage of saline and placed within the unconditioned chamber for 30 min. Mice received a total of four conditioning sessions with each drug (alcohol or saline).

Day 10: Test. Mice received an oral gavage of saline, were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

To infer whether the conditioning was successful, the time spent in the conditioned chamber during the post-test was subtracted from the time spent in the conditioned chamber during the pre-test.

10.2.6 Statistical analysis

Experiment 1: The effect of genotype on the intake and preference for alcohol, saccharin and quinine was analysed using a two-way ANOVA with Tukey post hoc (figure 1). The effect of genotype on alcohol induced conditioned place preference was analysed using a two-way ANOVA with Tukey post hoc (figure 2).

All summary values presented as mean ± standard error of mean (SEM). p-values ≤ 0.05 were considered statistically significant.

10.3 Results

TLR4$^{-/-}$ and wildtype mice underwent saccharin and quinine two-bottle choice tests to determine whether they exhibit basal differences in the "liking" component of reward and aversion behaviour (figure 1).

A two-way ANOVA determined a significant effect of concentration but not genotype on the intake of saccharin (effect of concentration, $F_{(4, 44)} = 22.48$, $p < 0.0001$; and effect of genotype, $F_{(1, 11)} = 0.38$, $p = 0.55$) (a). There was a significant interaction between genotype and concentration ($F_{(4, 44)} = 4.42$, $p = 0.043$). However, there were no post hoc differences. By contrast, concentration and genotype did not alter the preference for saccharin (effect of concentration, $F_{(4, 44)} = 1.73$, $p = 0.15$; and effect of genotype, $F_{(1, 11)} = 2.46$, $p = 0.14$) (b). There was no interactive effect ($F_{(4, 44)} = 0.17$, $p = 0.95$) or post hoc differences. Collectively, both TLR4$^{-/-}$ and wildtype mice exhibit similar preference for an innate inducer of the "liking" component of reward suggesting these mice do not differ in basal "liking" behaviour.

A two-way ANOVA determined the intake of quinine was significantly modified by concentration (effect of concentration, $F_{(4, 44)} = 149.8$, $p < 0.001$) but not genotype (effect of genotype, $F_{(1, 11)} = 0.19$, $p = 0.66$) nor was there an interactive effect between the two variables ($F_{(4, 44)} = 0.28$, $p = 0.90$) (c). Again, there were no post hoc

differences. Similarly, the preference for quinine was significantly influenced by concentration (effect of concentration, $F_{(4, 44)} = 3.57$, $p = 0.013$) but not genotype (effect of genotype, $F_{(1, 11)} = 0.58$, $p = 0.46$) (d). There were no interactive effects ($F_{(4, 44)} = 0.76$, $p = 0.55$) or post hoc differences. This suggests TLR4$^{-/-}$ and wildtype mice exhibit similarities in regards to taste and aversion.

To infer whether TLR4 alters the “liking” (and to some extent the “wanting”) component of reward TLR4$^{-/-}$ and wildtype mice underwent an alcohol two-bottle choice test. The intake of alcohol significantly influenced by concentration (effect of concentration, $F_{(7, 77)} = 106.9$, $p < 0.0001$) but not genotype (effect of genotype, $F_{(1, 11)} = 0.27$, $p = 0.61$) as inferred by a two-way ANOVA (e). There were no interactive effects ($F_{(7, 77)} = 1.03$, $p = 0.41$) or post hoc differences between the groups. By contrast, the preference for alcohol was modified by both concentration (effect of concentration, $F_{(7, 77)} = 10.25$, $p < 0.0001$) and genotype (effect of genotype, $F_{(1, 11)} = 37.71$, $p < 0.0001$) (f). However, no interaction ($F_{(7, 77)} = 0.72$, $p = 0.65$) was observed between the two variables. Tukey post hoc analysis determined TLR4$^{-/-}$ exhibited reduced alcohol preference at 3, 12 and 15 per cent alcohol compared to wildtype mice. The reduced preference but not intake of alcohol in TLR4$^{-/-}$ mice suggests they consume significantly more water during the 8 h test. However, whether this is due to altered taste or reward could not be determined.

To determine whether the reduced preference observed in the alcohol-two bottle choice test was likely due to alterations in thirst or reward, TLR4$^{-/-}$ and wildtype mice underwent conditioned place preference (figure 2). There was a significant effect of conditioning drug ($F_{(1, 10)} = 6.73$, $p = 0.027$) and genotype ($F_{(1, 10)} = 5.53$, $p = 0.040$) on the change in chamber time. Further there was a significant interaction between the two variables ($F_{(1, 10)} = 5.83$, $p = 0.036$). Tukey post hoc analysis determined wildtype

mice conditioned with alcohol spent significantly more time in the alcohol conditioned chamber compared to TLR4$^{-/-}$ mice.

Collectively, the results suggest TLR4$^{-/-}$ mice exhibit a reduction in the “wanting” of component of alcohol-reward compared to wildtype mice. However, it is unclear whether these mice exhibit alterations to the “liking” component of alcohol-induced reward.

10.3.1 Figures

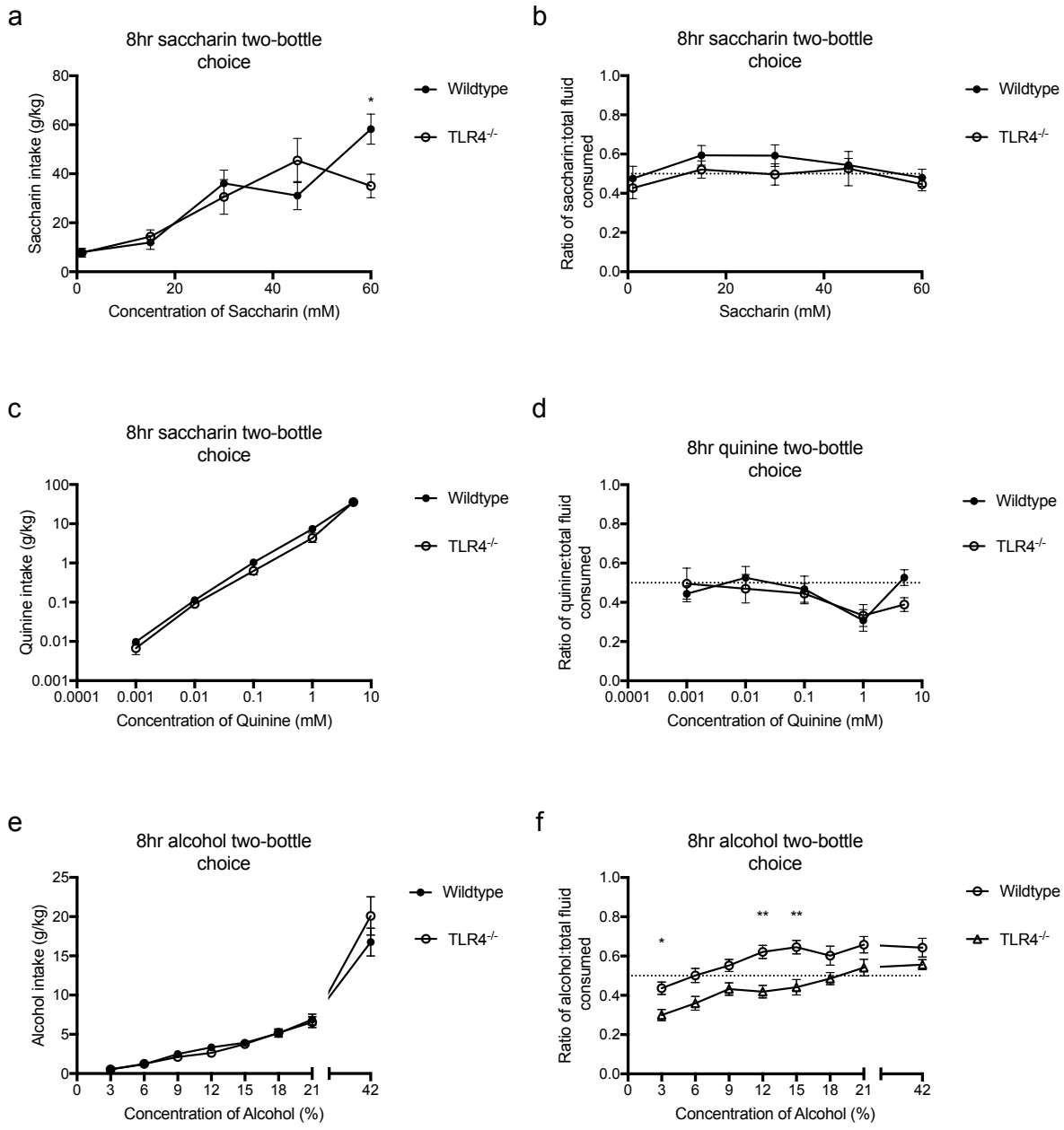

Figure 1 Genetic knockout of TLR4 reduces alcohol but not saccharin or quinine preference. TLR4$^{-/-}$ and wildtype (balb/c) mice exhibited similar levels of intake and preference for saccharin (a, b) and quinine (c, d). Interestingly, TLR4$^{-/-}$ mice exhibited similar intake of (e), but reduced preference (f) for alcohol compared to wildtype (Balb/c) mice. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=12, *$p < 0.05$; **$p < 0.01$.

Figure 2 Genetic knockout of TLR4 reduces alcohol-induced conditioned place preference. TLR4$^{-/-}$ mice exhibited significantly less change in conditioned chamber time post alcohol conditioning compared to wildtype (Balb/c) mice. All data was analysed using a two-way ANOVA with Tukey post hoc. Summary values represented as mean±SEM; n=10, *$p < 0.05$.

10.4 References

Bardo, M.T., Bevins, R.A., 2000. Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacology 153, 31–43. doi:10.1007/s002130000569

10.5 Acknowledgements

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