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

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Running title: TLR4 and MyD88 contribute to alcohol actions

Summary

BACKGROUND AND PURPOSE

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

EXPERIMENTAL APPROACH

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

KEY RESULTS

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

CONCLUSIONS AND IMPLICATIONS

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

Keywords

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

Abbreviations

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

Introduction

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

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

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

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

Methods

Animals

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

Drugs, Doses and Solutions

Endotoxin-free (+)-naloxone was kindly provided by Dr. Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland, USA). Alcohol was obtained from Chem-Supply (99.5%, Gillman, South Australia, Australia). All other reagents and chemicals were of analytical grade quality. The receptor and channel nomenclature used in the paper follows Alexander *et al.* (2009).

For animal behavioural studies, (+)-naloxone was injected intraperitoneally (*i.p.*) to the mice at 0.01 $mL \cdot g^{-1}$. The volume for injection of alcohol (20%, v/v, *i.p.*) varied and was based on animal weight and dose of alcohol. The weight of mice was 25 g on average, and ranged from 22 to 30 g.

Thus, the volume for injection of alcohol was 0.32 mL (range: 0.28-0.38 mL) at 2.0 $g{\cdot}kg^{-1}$, 0.40 mL (range: 0.35-0.48 mL) at 2.5 $g{\cdot}kg^{-1}$, 0.55 mL (range: 0.49-0.67 mL) at 3.5 $g{\cdot}kg^{-1}$, and 0.71 mL (range: 0.63-0.86 mL) at 4.5 $g{\cdot}kg^{-1}$ of alcohol. Endotoxin-free saline (0.9% sodium chloride) was used as the vehicle control.

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

Alcohol-induced sedation and motor impairment

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

Following a dose of saline (-30 min), single alcohol doses of 2.5, 3.5 or 4.5 $g{\cdot}kg^{-1}$ were administered (0 min) once only to groups of WT, *Tlr4-/-*, and *Myd88-/-* mice and alcohol-induced sedation, as measured by the duration of LORR, was recorded from 0 min. Subsequently, an ED_{50} (median effective dose) value of alcohol was estimated (see Statistical analysis).

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

Effect of (+)-naloxone treatment

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

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

In vitro and ex vivo molecular studies

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

Mixed hippocampal cells were isolated as previously described (Wu *et al.*, 2011) from naïve drug-free WT, *Tlr4-/-*, and *Myd88-/-* mice (n = 3 each). To analyze the effect of alcohol on IκBα levels, and the influence of (+)-naloxone, cells were treated with (+)-naloxone (153 µM, 50 µg·mL^{-1}), or RPMI 1640 at 37°C, 5% CO_2 for 30 min, prior to stimulation with alcohol (50 mM) or RPMI 1640 for a further 30 min and relative IκBα protein levels were investigated by western blotting.

Regulation of brain p38, JNK, and ERK phosphorylation by alcohol

To examine the effects of alcohol *in vitro*, hippocampal cells from each naïve mouse (WT or *Tlr4-/-*) were prepared as previously described (Wu *et al.*, 2011). Cells were stimulated with 50 mM alcohol [based on previous studies (Alfonso-Loeches *et al.*, 2010)] or vehicle at 37°C, 5% CO_2 for 10 min.

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

Alcohol pharmacokinetics

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

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

Behavioural testing

LORR (sedation)

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

Rotarod (motor co-ordination)

The rotarod apparatus (Orchid Scientifics, Nashik, India) with a 3-cm-diameter dowel was set at a fixed speed of $4.3x10^{-3}$ x g (16 rpm). The latency to fall was recorded as the duration that the mice remained on the rod, with a maximum cutoff latency of 180 s.

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

Molecular and chemical analyses

Western blotting

The preparation of cellular lysates was performed as described previously (Lousberg *et al.*, 2010). Briefly, mixed hippocampal cells were incubated on ice for 10 min, collected by centrifugation (2264 x g, 4 °C, 5 min), and washed with ice-cold Dulbeccos Phosphate Buffered Saline (DPBS, Invitrogen, Carlsbad, CA, USA). Cell pellets were resuspended in modified radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM sodium orthovanadate, 20 mM $Na_4P_2O_7$, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, and complete EDTA-free protease inhibitor cocktail] for 15 min on ice. Following cell lysis, lysates were clarified via centrifugation (18894 x g, 4°C, 5 min) and stored until analysis at -80°C. Protein concentration was determined by BCA assay prior to western blot.

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

Cytometric Bead Array

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

NAD-ADH assay (alcohol quantification)

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

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

Statistical analysis

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

ED_{50} was calculated from the dose-response curves, which were developed from the data of duration of alcohol-induced LORR. To fit these data, nonlinear regression (Graphpad Prism 5.02) was used, where Minimum was set as 0, and Maximum was the maximum duration of LORR. This resulted in estimate of ED_{50}, Slope, and Maximum.

$$Y=\frac{\text{Minimum}+(\text{Maximum}-\text{Minimum})}{1+\text{Slope}\times 10^{(\log ED_{50}-X)}}$$

The areas under the alcohol concentration-time curves (AUCs) from 0.25 to 3 h post-alcohol administration were calculated using the linear trapezoidal rule. Slopes of the alcohol concentration-time curves were calculated by linear regression, which followed zero-order kinetics.

Results

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

Tlr4-/- and *Myd88-/-* mice exhibit a shorter duration of alcohol-induced LORR

After a single dose of alcohol, the sedative effect of alcohol was dose-dependent as an increase in dose led to an increase in the duration of LORR in WT, *Tlr4-/-*, and *Myd88-/-* mice (p < 0.0001). Both *Tlr4-/-* and *Myd88-/-* mice displayed a shorter duration of alcohol-induced LORR than WT mice (p < 0.0001) at both 3.5 $g{\cdot}kg^{-1}$ (*Tlr4-/-*, 16 ± 4 min, p < 0.001, n = 9; *Myd88-/-*, 31 ± 8 min, p < 0.001, n = 10; WT, 73 ± 3 min, n = 13) and 4.5 $g{\cdot}kg^{-1}$ (*Tlr4-/-*, 56 ± 6 min, p < 0.001, n = 8; *Myd88-/-*, 78 ± 19 min, p < 0.01, n = 8; WT, 123 ± 3 min, n = 6) of alcohol (Fig. 1a). The ED_{50} of alcohol was 3.4 ± 0.1 [95% confidence interval (95% CI), 3.2-3.6] and 4.2 ± 0.2 (95% CI, 3.8-4.6) $g{\cdot}kg^{-1}$ for WT and *Myd88-/-* mice, respectively, and more than 4.5 $g{\cdot}kg^{-1}$ (the highest dose used) for *Tlr4-/-* mice.

Mice deficient in TLR4 or MyD88 recover more quickly from alcohol-induced motor impairment

As shown in Fig. 1b, the latency to fall-off decreased from 180 s prior to treatment, to less than 3 s in all treatment groups after alcohol administration (2.0 $g{\cdot}kg^{-1}$), with a gradual improvement over the monitoring time.

Both *Tlr4-/-* and *Myd88-/-* mice displayed a shorter recovery time from alcohol-induced decreases in rotarod performance compared to WT mice (*Tlr4-/-*, p = 0.002, n = 9, two-way ANOVA; p < 0.001 at 20 min, and p < 0.05 at 30, 40 and 50 min with Bonferroni post hoc test;

Myd88-/-, $p = 0.030$, n = 6, two-way ANOVA; $p < 0.001$ at 20 min, and $p < 0.05$ at 30, 40 and 50 min with Bonferroni post hoc test; WT, n = 9, Fig. 1b).

Alcohol-induced behavioural changes are reduced by (+)-naloxone treatment

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

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

Shorter recovery time from alcohol-induced motor impairment in (+)-naloxone-treated WT mice

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

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

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

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

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

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

In the *ex vivo* brain samples, lower phosphorylated JNK was observed in hippocampus of *Tlr4-/-* mice when compared to WT (genotype, $p = 0.023$; alcohol treatment, $p = 0.62$). In contrast, no significant difference was found in phosphorylated p38, phosphorylated ERK, or total p38 in hippocampal samples (genotype, $p = 0.23$, 0.061, and 0.057, respectively; alcohol treatment, $p =$

0.60, 0.31, and 0.42, respectively). No significant effect of alcohol or of genotype was observed in phosphorylated p38, phosphorylated JNK, phosphorylated ERK, or total p38 in cerebellum samples (alcohol treatment, p = 0.17, 0.78, 0.64, and 0.98, respectively; genotype, p = 0.86, 0.34, 0.45, and 0.88, respectively) (data not shown).

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

No significant differences in serum or brain alcohol concentrations (n = 4-5) were observed between (+)-naloxone-treated [(+)-naloxone/WT] vs. saline-treated WT mice (saline/WT) (serum, $p = 0.35$; brain, $p = 0.24$), or between WT mice and either *Tlr4-/-* (saline/*Tlr4-/-*) (serum, $p = 0.053$; brain, $p = 0.099$) or *Myd88-/-* mice (saline/*Myd88-/-*) (serum, $p = 0.075$; brain, $p = 0.15$) (Fig. 4a and c).

The serum alcohol AUC values of saline/WT, (+)-naloxone/WT, saline/*Tlr4-/-*, and saline/*Myd88-/-* groups were 9.8, 10.1, 9.2, and 9.3 $mg \cdot mL^{-1} \cdot h$, respectively, and the slopes of the concentration-time curves were -0.47 ± 0.06, -0.46 ± 0.08, -0.45 ± 0.10, and -0.67 ± 0.06 h^{-1}, respectively. The brain alcohol AUCs of saline/WT, (+)-naloxone/WT, saline/*Tlr4-/-*, and saline/*Myd88-/-* groups were 345, 398, 327, and 387 $mg \cdot 100\ mg^{-1} \cdot h$, respectively, and the slopes of the concentration-time curve were -0.18 ± 0.05, -0.25 ± 0.10, -0.14 ± 0.07, and -0.24 ± 0.08 h^{-1}, respectively.

Serum and brain samples (n = 4-5) were also collected when the mice awoke after each treatment. Significant differences in the alcohol concentrations were found in serum samples ($p = 0.019$; 95% CI, 392-469, 430-490, 368-434, and 399-574 $mg \cdot 100\ mL^{-1}$ in saline/WT, (+)-naloxone/WT, saline/*Tlr4-/-*, and saline/*Myd88-/-* groups, respectively; Fig. 4b), but not in brain samples (p =

0.88; 95% CI, 106-206, 80-187, 62-234, and 59-257 mg·100 mg^{-1} in saline/WT, (+)-naloxone/WT, saline/*Tlr4-/-*, and saline/*Myd88-/-* groups, respectively; Fig. 4d) tested with one-way ANOVA. However, no significant difference was observed between the null mutant groups or (+)-naloxone-treated WT group and saline-treated WT controls with Bonferroni's multiple comparison test.

Discussion and conclusions

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

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

Although the activation of brain TLR4 signalling, including MAPK and NFκB pathways, by alcohol exposure has been demonstrated *in vitro* after acute alcohol exposure (Blanco *et al.*, 2005;

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

To further explore the link between our behavioural findings and TLR4-MyD88 signalling, we analyzed a number of cell signalling proteins that could be up-regulated by TLR4 signalling in the cerebellum and hippocampus. The cerebellum was chosen as it is generally considered to control motor activity (Valenzuela *et al.*, 2010) in the brain regions influenced by alcohol (Vilpoux *et al.*, 2009), and we assessed the modification of motor function by alcohol in this study. The hippocampus was investigated since hippocampal microglial activation was induced by adolescent binge alcohol exposure in rats (McClain *et al.*, 2011). As attenuation of microglia, the prime component of the brain's immune system (Streit *et al.*, 2004), inhibited acute alcohol dose-induced sedation in mice (Wu *et al.*, 2011), the activation of TLR4-MyD88-NFκB signalling may occur in microglia.

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

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

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

Alcohol-induced behavioural changes are protected by (+)-naloxone treatment

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

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

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

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

TLR4-MyD88 signalling plays pivotal roles in acute behavioural actions of alcohol

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

In conclusion, the current study provides new evidence linking the contribution of TLR4-MyD88-dependent signalling to the behavioural response induced by acute alcohol administration. The consequences of blocking TLR4 signalling that support this theory include the inhibition of the influence of alcohol on IκBα protein levels, and the reduction in the sedative and motor effects of alcohol. Therefore, novel pharmacological strategies targeting TLR4 signalling, such as (+)-naloxone, may have important and highly relevant clinical application. Use of TLR4 antagonists would potentially also reduce alcohol-induced peripheral TLR4 signalling in the liver and gut (Szabo *et al.*, 2010).

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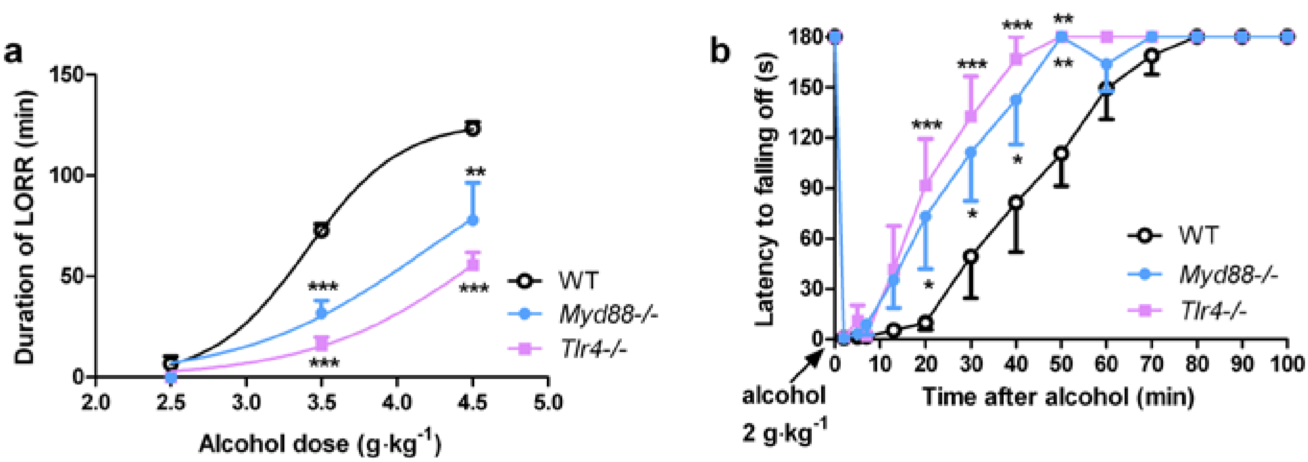
Conflicts of interest

None


a
Duration of LORR (min)
150
100
50
0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Alcohol dose (g·kg-1)
***
***
**
***
WT
Myd88-/-
Tlr4-/-
b
Latency to falling off (s)
180
150
120
90
60
30
0
0
10
20
30
40
50
60
70
80
90
100
Time after alcohol (min)
alcohol
2 g·kg-1
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*
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WT
Myd88-/-
Tlr4-/-

fig 1.jpg

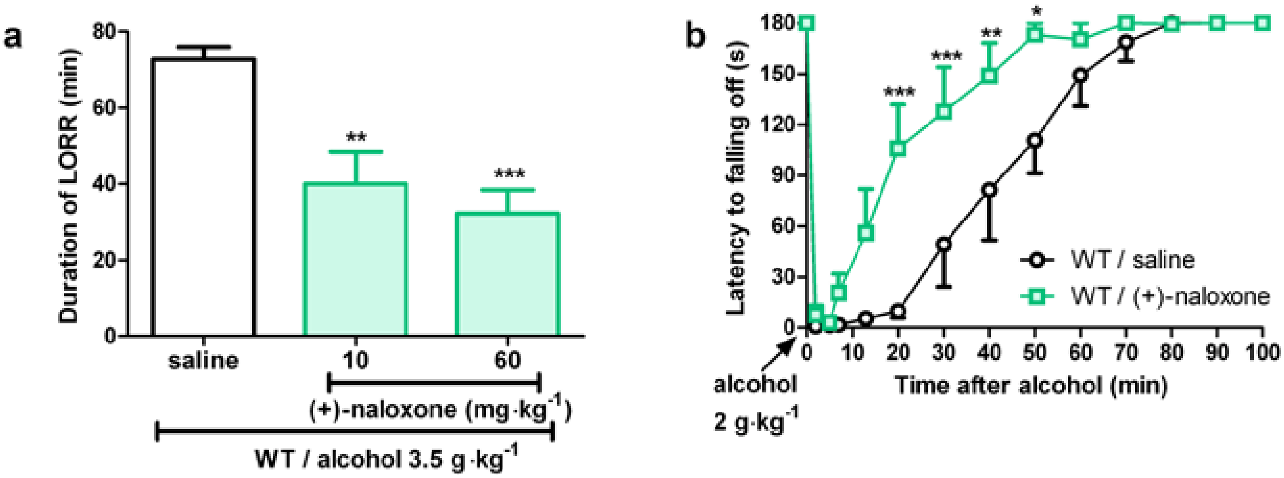
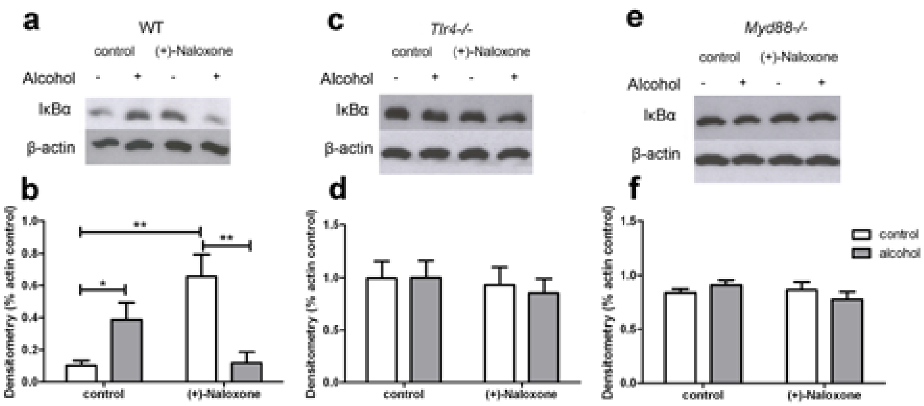

a
Duration of LORR (min)
80
60
40
20
0
**
***
saline
10
60
(+)-naloxone (mg·kg-1)
WT / alcohol 3.5 g·kg-1
b
Latency to falling off (s)
180
150
120
90
60
30
0
***
***
**
*
WT / saline
WT / (+)-naloxone
0
10
20
30
40
50
60
70
80
90
100
Time after alcohol (min)
alcohol
2 g·kg-1

fig 2.jpg


a
WT
control (+)-Naloxone
Alcohol - + - +
IκBα
β-actin
b
Densitometry (% actin control)
control
(+)-Naloxone
c
Tlr4-/-
control (+)-Naloxone
Alcohol - + - +
IκBα
β-actin
d
Densitometry (% actin control)
control
(+)-Naloxone
e
Myd88-/-
control (+)-Naloxone
Alcohol - + - +
IκBα
β-actin
f
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control
alcohol
control
(+)-Naloxone

fig3.jpg

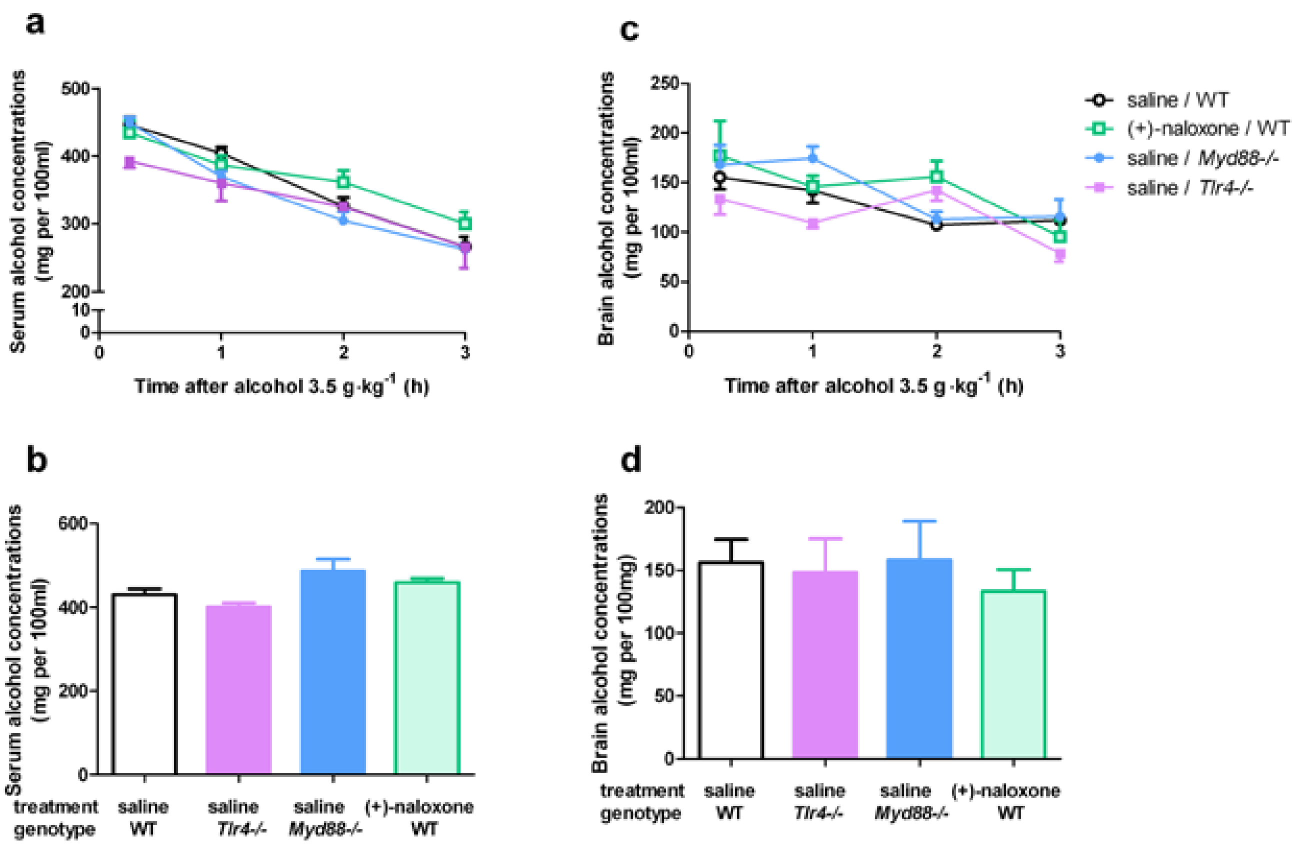
a
Serum alcohol concentrations (mg per 100ml)
Time after alcohol 3.5 g·kg-1 (h)
b
Serum alcohol concentrations (mg per 100ml)
treatment genotype
saline WT
saline Tlr4-/-
saline Myd88-/-
(+)-naloxone WT
c
Brain alcohol concentrations (mg per 100ml)
Time after alcohol 3.5 g·kg-1 (h)
saline / WT
(+)-naloxone / WT
saline / Myd88-/-
saline / Tlr4-/-
d
Brain alcohol concentrations (mg per 100mg)
treatment genotype
saline WT
saline Tlr4-/-
saline Myd88-/-
(+)-naloxone WT

fig4s.jpg