Alcohol-induced sedation and synergistic interactions between alcohol and morphine: A key mechanistic role for Toll-Like Receptors and MyD88-dependent signalling

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Abstract

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Conflict of Interest
None

Increasing evidence demonstrates induction of proinflammatory Toll-like receptor (TLR) 2 and TLR4 signaling by morphine and, TLR4 signaling by alcohol; thus indicating a common site of drug action and a potential novel innate immune-dependent hypothesis for opioid and alcohol drug interactions. Hence, the current study aimed to assess the role of TLR2, TLR4, MyD88 (as a critical TLR-signalling participant), NF-κB, Interleukin-1β (IL-1β; as a downstream proinflammatory effector molecule) and the μ opioid receptor (MOR; as a classical site for morphine action) in acute alcohol-induced sedation (4.5 g/kg) and alcohol (2.5 g/kg) interaction with morphine (5 mg/kg) by assessing the loss of righting reflex (LORR) as a measure of sedation. Wild-type male Balb/c mice and matched genetically-deficient TLR2, TLR4, and MyD88 strains were utilized, together with pharmacological manipulation of MOR, NF-κB, TLR4 and Interleukin-1β. Alcohol induced significant LORR in wild-type mice; this was halved by MyD88 and TLR4 deficiency, and surprisingly nearly completely eliminated by TLR2 deficiency. In contrast, the interaction between morphine and alcohol was found to be MOR-, NF-κB-, TLR2- and MyD88-dependent, but did not involve TLR4 or Interleukin-1β. Morphine-alcohol interactions caused acute elevations in microglial cell counts and NF-κB-p65 positive cells in the motor cortex in concordance with wild-type and TLR2 deficient mouse behavioral data, implicating neuroimmunopharmacological signaling as a pivotal mechanism in this clinically problematic drug-drug interaction.

Keywords

Introduction

Alcohol increases the risk of heroin-related deaths (Hickman et al., 2008; Levine et al., 1995), not due to any pharmacokinetic interaction (Darke et al., 1997; Fugelstad et al., 2003), but due to a pharmacodynamic interaction. Knowledge of the mechanism of such a synergistic interaction between alcohol and opioids in the central nervous system (CNS) is surprisingly limited (White and Irvine, 1999), but is generally considered to be alcohol-induced potentiation of sedation by morphine. The molecular mechanisms implicated in the sedative effects of alcohol include neuronal gamma-aminobutyric acid (GABA) receptors, the N-methyl-D-aspartate (NMDA) receptor, and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signalling (Boyce-Rustay and Holmes, 2005; Sharko and Hodge, 2008; Wand et al., 2001). In contrast, morphine can rapidly and differentially modulate the binding affinity of GABA for its receptors in various brain regions (Sivam et al., 1982; Ticku and Huffman, 1980), or alternatively directly cause sedation (Craft and Leitl, 2006; Kissin et al., 1991) due to activation of the mu (μ) opioid receptor (MOR) (Craft and Leitl, 2006; Kissin et al., 1991). However, other non-neuronal mechanisms of alcohol- and opioid-induced sedation may be involved.

Recent appreciation of the non-neuronal CNS actions of opioids and alcohol has led to the discovery of xenobiotic-induced proinflammatory reactivity of glia, through Toll-like receptors (TLRs) and myeloid differentiation primary response gene 88 (MyD88)-dependent

pathways. Opioid-induced TLR4 activation was first identified as a modifier of opioid analgesia (Hutchinson et al., 2008a; Hutchinson et al., 2010b; Wang et al., 2012) and reward (Hutchinson et al., 2012), with TLR2 subsequently shown to play a similar role (Li et al., 2010; Li et al., 2009; Zhang et al., 2011). An intriguing parallel research track developed for alcohol where alcohol-induced sedation and motor dysfunction occurred in a TLR4-, MyD88-, and IL-1β-dependent fashion (Wu et al., 2012; Wu et al., 2011). Therefore, this common TLR-dependent neuroimmune convergence by alcohol and opioids may shed light on a new mechanism that contributes to the pharmacodynamic interaction between alcohol and opioids.

Hence, we hypothesized that: a) TLR2 is involved in the sedative effects of alcohol; and b) that TLR2, TLR4, MyD88, NF-κB, Interleukin-1 receptor, and MOR signaling are collectively involved in the synergistic sedative effects induced by alcohol plus morphine.

Thus, the aim of this study was, firstly, to examine if mice genetically deficient in TLR2 had decreased sedation to alcohol alone, since such an effect was found in all the other TLR-related genetic deficient mouse strains in our previous experiments (Wu et al., 2012). Secondly, we aimed to characterize the effect of genetic deficiency of TLR2, TLR4 or MyD88 on the interaction between alcohol and morphine. Thirdly, we aimed to determine the effect of (+)-naloxone (the MOR-inactive isomer but TLR4 signaling inhibitor (Hutchinson et al., 2010a; Hutchinson et al., 2008b)), (−)-naloxone (the MOR antagonist and TLR4 signaling inhibitor), IL-1ra (Interleukin-1 receptor antagonist) and IKK-NBD (a NF-κB inhibitor (Ghosh et al., 2007a)) on combined alcohol- and morphine-induced sedation. Examination of the impact of these genetic and pharmacological manipulations on acute microglial response within the motor cortex was also examined to provide a mechanistic cellular link to the altered behavioral responses.

Materials and Methods

Animals

Pathogen-free male Balb/c wild-type mice, and mice with null mutations in *Tlr2* (*Tlr2−/−*), *Tlr4* (*Tlr4−/−*), and *Myd88* (*Myd88−/−*) (all 8–14 weeks old; weighing 18–26 g; n = 4–8) were used in the experiments. These genetically-deficient mice, back crossed onto Balb/c for more than ten generations, were sourced from Prof. Akira (Osaka University, Osaka, Japan), and the *Tlr4*, and *Myd88* null mutant mice were kindly supplied by Dr. Simon Phipps (University of Queensland, QLD, Australia) and Prof. Paul Foster (University of Newcastle, NSW, Australia). Mice were housed under SPF conditions 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. Every effort was taken to Replace, Refine and Reduce animal usage in the design and conduct of these experiments.

Drugs

Human IL-1ra (Kineret®, Amgen Inc, Thousand Oaks, CA, USA) was purchased from the Queen Elizabeth Hospital Pharmacy (Woodville, SA, Australia). (+)-Naloxone was supplied

by Dr. Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD, USA). (−)-Naloxone was purchased from Sigma (St. Louis, MO, USA). Morphine was obtained from Fauldings Australia (Adelaide, SA, Australia). Alcohol (ethanol) was obtained from Chem-Supply (99.5%; Gillman, SA, Australia). Sodium chloride was obtained from Merck (99.5%, Darmstadt, Germany). The IKK-NBD peptide was obtained from Enzo LifeSciences (Exeter, United Kingdom).

Morphine (5 mg/kg), (+)-naloxone (60 mg/kg) (Wu et al., 2012), (−)-naloxone (60 mg/kg), IL-1ra (100 mg/kg) (Wu et al., 2012) and IKK-NBD (0.75 mg/kg) (Ghosh et al., 2007a) were injected intraperitoneally (*i.p.*) at 0.1 mL/kg. Alcohol was injected at a dose of 4.5 g/kg and 2.5 g/kg to assess the sedative effect induced by alcohol alone and the co-administration of alcohol and morphine, respectively. The volume for injection of alcohol (20%, v/v, *i.p.*) varied as it was based on animal weights and was 25 g on average, (range: 22–30 g); thus the volume for injection of alcohol was 0.40 mL (range: 0.35–0.48 mL) at 2.5 g/kg and 0.71 mL (range: 0.63–0.86 mL) at 4.5 g/kg of alcohol. Saline (0.9% sodium chloride) was used as the vehicle control.

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

After drug treatment, mice were placed into separate cages with bedding. The duration of LORR was measured as the time from LORR to the time of righting themselves 3 times in 30 s (waking up) (Wu et al., 2012; Wu et al., 2011). Animals were continually monitored for LORR. This behavioral response is considered a measure of the sedation induced by the treatments.

Experimental procedures

A. Acute Dose of Alcohol Alone on LORR—To determine LORR induced by alcohol alone in wild-type, *Tlr2*−/−, *Tlr4*−/− and *MyD88*−/− mice, saline was administered at −30 min followed by alcohol (4.5 g/kg) at 0 min. Assessment of the impact of pharmacological TLR4 blockade on alcohol-induced LORR in these four mice strains was conducted by administering (+)-naloxone (60 mg/kg i.p.) at −30 min followed by the alcohol at 0 min with LORR monitored until righting reflex was recovered.

B. Alcohol and morphine-induced LORR: Pharmacological antagonist assessment—To examine in wild-type mice the effects of acute morphine on alcohol-induced LORR, mice were injected with morphine (5 mg/kg, −35 min) then saline at −30 min then alcohol (2.5 g/kg) or saline at 0 min. The reduction in alcohol dosage from the acute alcohol alone study was to accommodate for the synergistic effects of alcohol-morphine administration and thus avoid a ceiling pharmacological effect and prevent overdose deaths in these mice.

To assess the influence of IL-1ra, (+)-naloxone, (−)-naloxone and IKK-NBD on alcohol-induced LORR following morphine co-administration, wild-type mice were injected with morphine (−35 min), followed by IL-1ra, (+)-naloxone, (−)-naloxone or IKK-NBD administration (−30 min). Alcohol (2.5 g/kg) being administered at 0 min.

C. Alcohol and morphine-induced LORR: Genetic knockout assessment—To examine the effects of morphine on alcohol-induced sedation across different genetically deficient mouse strains, *Tlr2–/–*, *Tlr4–/–* and *Myd88–/–* mice were dosed with morphine (5 mg/kg, –35 min) then saline (–30 min) and alcohol (2.5 g/kg) at 0 min. These data were compared to their wild-type counterpart data from study B.

D. Immunohistochemical assessment of the molecular alterations induced by alcohol and morphine—To further elucidate the cellular mechanisms underlying the behavioral observations presented here, immunohistochemistry of Iba1 and NF-κB expression in the motor cortex 30 min after alcohol exposure was examined in wild-type, *Tlr2–/–* and *Tlr4–/–* mice. Motor cortex was chosen as a region of interest owing to these and other studies examining alcohol-induced impairment of gross motor function (see Figure 1). The same morphine and alcohol dosing protocol that induced LORR in studies B and C was employed. Thirty minutes after alcohol administration, animals (n = 3 group, [morphine vs saline] by [alcohol vs saline]) were humanely sacrificed and transcardially perfused with 10% formalin. Brains were sectioned coronally (2 mm) using a mouse brain blocker (Kopf, Tujunga, CA, USA) and embedded in paraffin wax. Using a microtome (Microm, Walldorf, Germany), 5 μm sections were cut from each brain at Bregma –1.5 to allow examination of the motor cortex. Three sections per animal were labelled with either rabbit anti-NF-κB-p65 (1:1000, Santa Cruz, Santa Cruz, CA, USA) or goat anti-ionised calcium-binding adaptor molecule-1 (Iba1) (1:1000, Sigma, St Louis, Missouri, USA). Following overnight incubation, slides were then incubated with the appropriate secondary antibody (Dako 1:250) for 30 min followed by incubation with SPC (1:1000) for 1 h. For negative controls primary antibodies were omitted. Bound antibody was then detected with 3,3-diaminobenzidine tetrahydrochloride (DAB) in the presence of hydrogen peroxide and sections counterstained with haematoxylin. Slides were digitally scanned (Nanozoomer, Hanamatsu) and viewed with the associated software (NDP view). Four serial 40x images were taken from each section and digitally reconstructed into a montage to allow manual counting of Iba1 and NFκB-p65 positive cells using the cell count software associated with ImageJ. Morphology of microglial cells was also evaluated qualitatively, and representative images of typical appearance collated.

To determine differences in the intensity of NF-κB-p65 staining, images were processed using Photoshop, converted to grayscale and the intensity of staining determined. All NF-κB-p65 positive cells within the motor cortex of the section were analysed for integrated density with background staining then subtracted and an average of each of these cells then taken for each animal, to negate the effects of variability of number of NF-κB positive cells between groups. Backgrounds were defined as the intensity of grey in areas without cells. Investigators who were blinded to treatment assignments conducted all quantifications.

Statistical analysis

Analysis of the alcohol-induced LORR, morphine-alcohol-induced LORR, Iba1 positive cells, NF-κB-p65 and the average integrated density of NF-κB-p65 per positive cell data were conducted in RStudio (Version 0.97.551; R version 3.0.1 (Team, 2013)). Linear models of the multi-way relationships were created in RStudio. Post hoc contrasts were

conducted using the multcomp R package and the general linear hypothesis (glht) analysis which allows for simultaneous tests for general linear hypotheses, and multiple comparisons of means with Tukey contrasts and single-step adjusted p-values (Hothorn et al., 2008). GraphPad Prism 6.0.1 (GraphPad Software Inc., San Diego, CA, USA) was used for graphing. P-values (adjusted for repeated sampling where appropriate) of 0.05 or less were considered statistically significant. Graphically, all data are presented as mean ± SEM.

Results

A. High dose alcohol LORR: TLR2 and TLR4 signaling contribute to high dose alcohol-induced LORR

Alcohol (4.5 g/kg) produced profound loss of righting reflex in wild-type animals with statistically significant main effects of strain ($F_{3,46} = 17.0$; $P < 0.0001$), and a strain:(+)-naloxone interaction ($F_{3,47} = 10.7$; $P < 0.0001$) on alcohol-induced LORR time (Figure 2A). As expected mice given saline at −30 min and saline at 0 min did not lose righting reflex (data not shown). Post hoc analysis revealed significant reductions in alcohol-induced LORR in saline+alcohol treated *Tlr2*−/− (mean = 88%), *Tlr4*−/− (55%) and *Myd88*−/− (46%) animals when compared with saline+alcohol treated wild-type mice ($P < 0.001$; Figure 2A). Alcohol-induced LORR in *Tlr2*−/− mice was reduced significantly greater compared to *Tlr4*−/− and *Myd88*−/− mice ($P < 0.02$, Figure 2A). Pharmacological TLR4 blockade with (+)-naloxone significantly reduced alcohol-induced LORR (45%) in wild-type animals ($P = 0.002$), but failed to alter the alcohol-induced LORR in *Tlr4*−/− and *Myd88*−/− animals ($P > 0.74$). However, (+)-naloxone treatment in *Tlr2*−/− mice caused a significant increase ($P = 0.036$) in alcohol-induced LORR to times not statistically significantly different to saline treated *Tlr4*−/− or *Myd88*−/− animals ($P > 0.97$; Figure 2A).

B. Morphine:Alcohol synergistic interactions: Potentiation of the duration of alcohol-induced LORR by morphine is inhibited by (−)-naloxone and IKK-NBD in wild-type mice

A low dose of morphine (5 mg/kg) in wild-type mice significantly increased the duration of alcohol-induced LORR when compared to alcohol alone by 313% ($t = 3.7$; $P = 0.01$) or morphine alone ($t = 4.9$; $P < 0.001$) (Figure 2B) (multi-way ANOVA with post hoc analysis). This synergistic effect of morphine on alcohol-induced LORR was blocked by MOR antagonism using (−)-naloxone ($t = 4.9$; $P < 0.001$) and by NF-κB inhibition (IKK-NBD; $t = 3.4$; $P = 0.02$) but not by pharmacological TLR4 blockade with (+)-naloxone ($t = 0.38$; $P = 0.99$) or with the recombinant human IL-1ra ($t = 0.61$; $P = 0.99$) (Figure 2B).

C. Morphine:Alcohol synergistic interactions: Key roles of TLR2 and MyD88 signaling in synergistic Morphine+Alcohol induced LORR

To further examine the influence of innate immune receptor signaling on morphine+alcohol interactions, strains of mice deficient in TLR2, TLR4 and MyD88 were administered morphine or saline followed by alcohol. Multi-way ANOVA with post hoc analysis of alcohol- and morphine-alcohol-induced LORR revealed that morphine significantly potentiated alcohol-induced LORR in wild-type mice ($t = 5.4$, $P < 0.001$) and *Tlr4*−/− mice ($P < 0.019$) but not in *Tlr2*−/− or *Myd88*−/− mice ($P < 0.001$; Figure 2C).

D. Morphine:Alcohol synergistic interactions: Morphine+Alcohol acutely increase Iba1 microglial counts in the motor cortex

Morphine and alcohol treatments caused significant alterations in Iba1 microglia counts in the motor cortex, that were dependent on strain ($F_{2,24} = 4.0$; P = 0.03) and morphine-alcohol interactions ($F_{1,24} = 5.3$; P = 0.03; multi-way ANOVA [alcohol by morphine by strain]) (Figure 3A). Morphine+alcohol caused significant increases in wild-type Iba1 cell counts compared to saline+alcohol (t = 3.7, P = 0.04) and saline+saline (t = 3.9, P = 0.03) treated wild-type; and compared to morphine+alcohol treated *Tlr2−/−* animals (t = 3.7, P = 0.04) (Figure 3C). In addition to microglial number, microglial morphology was evaluated, with the typical appearance of microglia within layer 5 of the motor cortex (Figure 4). Morphine +alcohol treatment in WT and *Tlr4−/−* mice led to a typical `bushy' appearance of the microglia suggestive of heightened reactivity, which was not seen in the *Tlr2−/−* mice, or in mice treated with morphine or alcohol alone (Figure 4).

E. Morphine:Alcohol synergistic interactions: Alcohol +Morphine acutely increases NF-κB-p65 in Iba1 microglia in the motor cortex

Activation of NF-κB-p65 signaling in the motor cortex in wild-type, *Tlr2−/−* and *Tlr4−/−* mice is shown in Figure 3B. Once again, morphine and alcohol treatments caused significant changes in NF-κB-p65 cell counts that were dependent on drug (morphine [$F_{1,24} = 6.6$, P = 0.02], alcohol [$F_{1,24} = 4.8$, P = 0.04]) and drug strain interactions (morphine:strain [$F_{2,24} = 6.6$, P = 0.005] and alcohol:strain [$F_{2,24} = 4.0$, P = 0.03]). Morphine+alcohol caused significant increases in wild-type in NF-κB-p65 positive cell counts compared to wild-type saline+alcohol (t = 4.0, P = 0.02) and saline+saline treated wild-type mice (t = 5.6, P < 0.001) (Figure 3D). Moreover, this occurred in a TLR2- and TLR4-dependent fashion as no significant changes in NF-κB-p65 positive cells were observed between saline+saline and morphine+alcohol treated *Tlr2−/−* (t = 0.5, P = 1.0) or *Tlr4−/−* mice (t = 0.8, P = 1.0; Figure 3D). Interestingly, significantly lower cell counts were seen when comparing morphine +alcohol between wild-type and *Tlr2−/−* (t = 3.7, P = 0.04) and *Tlr4−/−* (t = 3.8, P = 0.03) animals.

Further examination of the integrated density of the NF-κB-p65 positive cells demonstrated that morphine ($F_{1,24} = 5.0$, P = 0.04), strain ($F_{2,24} = 36.6$, P < 0.0001), and an alcohol:strain ($F_{2,24} = 8.2$, P = 0.002) interactions significantly influenced the straining (Figure 3E). There was a significant increase in the integrated density of staining induced by morphine+alcohol in wild-type animals compared to saline+saline treatments (t = 5.2, P < 0.01), a response not observed in *Tlr2−/−* (t = 0.5, P = 1.0) or *Tlr4−/−* (t = 0.7, P = 1.0) mice. *Tlr2−/−* mice displayed significantly reduced NF-κB-p65 staining density under all four treatments compared with wild-type (morphine+saline (t = 6.2, P = 0.01); saline+alcohol (t = 5.2, P = 0.01) and *Tlr4−/−* mice (morphine+alcohol [t = 3.9, P = 0.02]; saline+saline [t = 4.6, P < 0.01]; Figure 3E).

Discussion

Here we show that TLRs and their associated signaling pathways are novel contributors to acute alcohol- and morphine+alcohol-induced cellular and behavioral responses. Firstly, a

novel role for TLR2 was established in the acute sedative effects of alcohol, with near complete protection from alcohol-induced LORR in *Tlr2*−/− mice. However, an intriguing and complex TLR2-TLR4 interaction was uncovered as the *Tlr2*−/− mice LORR protection was halved by combining pharmacological TLR4 blockade in *Tlr2*−/− mice. Secondly, a key role for innate immune TLR signaling in the synergistic drug-drug interaction elicited by combining morphine and alcohol was also established. Specifically, TLR2-MyD88-dependent NF-κB signaling was found to contribute to the synergistic drug-drug interaction of alcohol- and morphine-induced LORR, as demonstrated pharmacologically and through immunohistochemical staining. Finally the role of microglia were assessed in wild-type and TLR deficient strains, with an increase in microglial number associated with a change in morphology suggestive of activation in morphine+alcohol treated wild-type mice, that was not seen in the *Tlr2*−/− mice. However, discordant results were observed in *Tlr4*−/− mice, which did not have an increase in microglial number, although microglia appeared to adopt an activated phenotype, despite a behavioural response to morphine+alcohol administration (Table 2 results summary). Nonetheless, these results uncover a new potential non-neuronal cellular target of drug-drug interactions and reaffirm our previous hypothesis that xenobiotics such as morphine and alcohol (or their metabolites (Lewis et al., 2010; Lewis et al., 2013), act as xenobiotic-associated molecular patterns (XAMPs) to activate TLRs causing altered pharmacodynamic responses (Hutchinson et al., 2012).

Firstly, this study has demonstrated that the acute neurological effects of alcohol are dependent on both TLR4 and TLR2 signaling. This builds on our previous work indicating that treatment with IL-1ra and minocycline reduced alcohol-induced sedation in mice, as measured by the LORR (Wu et al., 2012; Wu et al., 2011). As both the TLR2 and TLR4 signalling pathways leads to the production of IL-1β (Wu et al., 2012; Wu et al., 2011), it can be hypothesized that activation of TLR2 and TLR4 signalling pathways, promoting IL-1β production by microglia, are key participants in the pharmacodynamic actions of alcohol. Alcohol has been shown to recruit TLR2 and TLR4 to lipid rafts in microglial cells, with an associated release of pro-inflammatory cytokines (Fernandez-Lizarbe et al., 2013) TLR4 signalling has previously been linked to other behavioural changes following alcohol exposure including acute changes in motor function (Wu et al., 2012) and development of conditional learning and memory deficits in the object recognition task following chronic alcohol consumption (Pascual et al., 2011).

It is intriguing that *Tlr2*−/− mice display significantly less acute alcohol-induced LORR than the other strains tested, indicating TLR2 is a vital participant in this acute alcohol pharmacodynamic response. However, complex TLR2/4 and MyD88 interactions are also evident in this acute alcohol response, as pharmacological TLR4 blockade with (+)-naloxone in *Tlr2*−/− mice increases their LORR, although this still remains less than that in untreated wild-type mice. This complexity is further emphasized by the increase in LORR in response to alcohol in *Myd88*−/− mice compared to *Tlr2*−/− mice, despite TLR2 signalling being MyD88 dependent.

This highlights the underlying complexity of the relationship between alcohol and the innate immune system. Complex interactions in innate immune signaling have been hypothesized recently (Nish and Medzhitov, 2011), with opportunities for cooperation, complementation

and compensation in the signaling cascade. Indeed, it has been demonstrated that ethanol promotes interaction between TLR4 and TLR2 following their recruitment into lipid rafts which act to stabilise TLRs and form signalling platforms (Fernandez-Lizarbe et al., 2013). Our results suggest that in the genetic absence of TLR2, TLR4 signaling by alcohol is ineffective, whilst in the absence of TLR4 (genetic or pharmacological), TLR2 signaling can continue to produce some, but reduced acute alcohol effects. However, in the case of the *Myd88−/−* or *Tlr2−/−* mice given (+)-naloxone, we are presented with a unique situation where the action of a third unknown signaling factor, biased signaling or an acutely activated compensatory mechanism appears to be contributing to the alcohol response. Further investigations of these acute proinflammatory TLR-dependent contributions to the acute actions of alcohol are warranted, as how these signaling interactions occur and the mechanism(s) remains to be determined. Moreover, the developmental consequences of the genetic absence of innate immune genes needs to be considered as contributing to the different responses observed here.

Nonetheless, having demonstrated that the behavioural response to alcohol is immune dependent, we sought to determine whether innate immune signalling was promoting the interaction between alcohol and morphine. We were able to demonstrate that this interaction appears to be dependent on MOR, TLR2 and MyD88-NFκB-signaling, as *Tlr2−/−* and *Myd88−/−* mice and MOR or IKK-NBD blockade protects against morphine+alcohol LORR. In contrast targeting IL-1β and TLR4 are without effect (see Table 2 for a summary).

To address the possible underlying cellular mechanisms at play in morphine+alcohol LORR, alterations in microglial cell number (Iba1 positive cells) and NF-κB-p65 expression were examined in the motor cortex 30 min after drug administration. In wild-type mice significant morphine+alcohol-induced increases in Iba1 positive microglia were observed in the motor cortex. This rapid and substantial elevation in Iba1 positive cells may reflect both recruitment of Iba1 positive cells as well as possible elevations in Iba1 expression by resident populations. However, this is a very immediate effect and will require further research to elucidate the precise mechanism.

The importance of NF-κB signalling in this interaction was also investigated, both pharmacologically through the administration of the IKK-NBD peptide, and immunohistochemically through evaluation of NF-κB-p65 staining. Treatment with the IKK-NBD peptide significantly reduced LORR. It should be noted that previous studies utilizing IP injection of the NDD-IKK peptide demonstrate that it is able to enter into the brain where it inhibits activation of NF-κB signaling (Ghosh et al., 2007b). The importance of this component of the signaling cascade was highlighted by the NF-κB-p65 changes seen in wild-type mice, which corresponded to a synergistic increase in LORR in wild-type animals. Moreover, in *Tlr2−/−* mice, where there was complete blockade of LORR, no such elevations in microglial Iba1 cell number, NF-κB-p65 positive cells or staining intensity occurred, thus agreeing with the behavioral responses, compared to wild-type mice. However, in *Tlr4−/−* mice that displayed a significant morphine+alcohol response there was no change in the number of NF-κB-p65 positive cells nor staining intensity, although the latter may have been masked by an elevation in basal NF-κB-p65 staining intensity.

Thus, whilst a simple NF-κB-p65 activation can potentially explain the synergistic morphine +alcohol interaction in wild-type versus *Tlr2−/−* mice, the same cannot be said for the *Tlr4−/−* strain. This suggests that non-immune signaling may play a greater role in *Tlr4−/−* mice than in wild-type mice. This may relate to activation of MOR, as (−)-naloxone was found to be effective at reducing LORR in WT mice or it is possible that the genetic deletion of TLR4 has allowed other novel pathways to become involved in the promotion of LORR in response to morphine+alcohol, which would require further investigation. Nevertheless, in contrast to the effects of morphine or alcohol alone involving both TLR2 and TLR4, the current results suggest that the co-administration of morphine and alcohol in wild-type mice elicits the sedative effect via the TLR2-MyD88-NF-κB signaling cascade, highlighting a new XAMP-induced innate immune signal that alters drug responses.

The exact mechanisms behind how morphine+alcohol act synergistically via activation of TLR2 to promote sedation is yet to be fully elucidated. In glial cells the net result of the TLR2 signaling cascades is Myd88 dependent NF-κB activation leading to the robust and transient transcription of a myriad of pro-inflammatory factors including cytokines (TNF-α, IL-6), chemokines and immune receptors, promoting glial reactivity (Kumar et al., 2009). Apart from cytokines, reactive glial cells also release a variety of other substances such as cytokines, prostaglandins, brain-derived neurotrophic factor, adenosine triphosphate (ATP) and glutamate (Kettenmann et al., 2013), which can all modulate neuronal activity, although the mechanisms remain poorly understood. Of note inhibitory GABA neurotransmission is enhanced by the chemokine (C-X-C motif) ligand 12 (CXCL12) (Heinisch and Kirby, 2010) and IL-6 (De Laurentiis et al., 2000), providing a potential mechanism for enhanced neuronal inhibition. The prior exposure to morphine may prime microglia to have an exaggerated proinflammatory cytokine response to the subsequent effects of alcohol, as seen by the appearance of activated microglia and upregulation of NF-κB. This leads to an enhanced release of cytokines and chemokines from microglia, potentially exaggerating the behavioral response. Several mechanisms have been proposed as the basis for this exaggerated response by primed microglia including enhanced NF-κB activation and chromatin remodelling, where histone acetylation and phosphorylation make cytokine gene promoters more readily accessible (Pestka and Zhou, 2006).

It should also be noted that apart from modulating neuronal signaling via microglial cross-talk, stimulation of TLRs on neurons themselves may also directly affect neuronal function. An increase in TLR4 expression has been noted within hippocampal neurons in a model of kainate induced seizures, with TLR4 blockade reducing seizure intensity, suggesting a role in modulating excitatory neurotransmission (Maroso et al., 2010). Activation of MOR also plays an important role in the synergistic effects of morphine+alcohol, given the reversal of LORR following treatment with (−)-naloxone. Both alcohol and morphine interact with MOR, with the acute anxiolytic and locomotor effects of alcohol prevented in μ-opioid receptor knockout mice (Ghozland et al., 2005), whilst morphine induced sedation has been linked to activation of the MOR (Craft et al, 2006; Kissen et al, 1991). This suggests that there is complex interplay of microglia-neuronal crosstalk that can be triggered by activation of both TLR2 and MOR signaling pathways promoted by release of a host of factors

including cytokines, chemokines and neurotransmitters that ultimately leads to the change of behavior noted in these mice.

In conclusion, it is apparent that TLR2-MyD88-NF-κB signaling is important in the acute sedative effects of alcohol and the interaction between alcohol and morphine in mice. Given the novel XAMP sensitivity of, at minimum the TLR2 pathway, this may prove crucial in explaining novel xenobiotic-alcohol synergistic interactions. As such, a unique opportunity has presented itself whereby novel pharmacological targeting of the TLR2-MyD88-NF-κB signaling pathway may beneficially avoid these predominantly unwanted synergistic drug-drug interactions.

Acknowledgements

Abbreviations

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

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Figure 1.
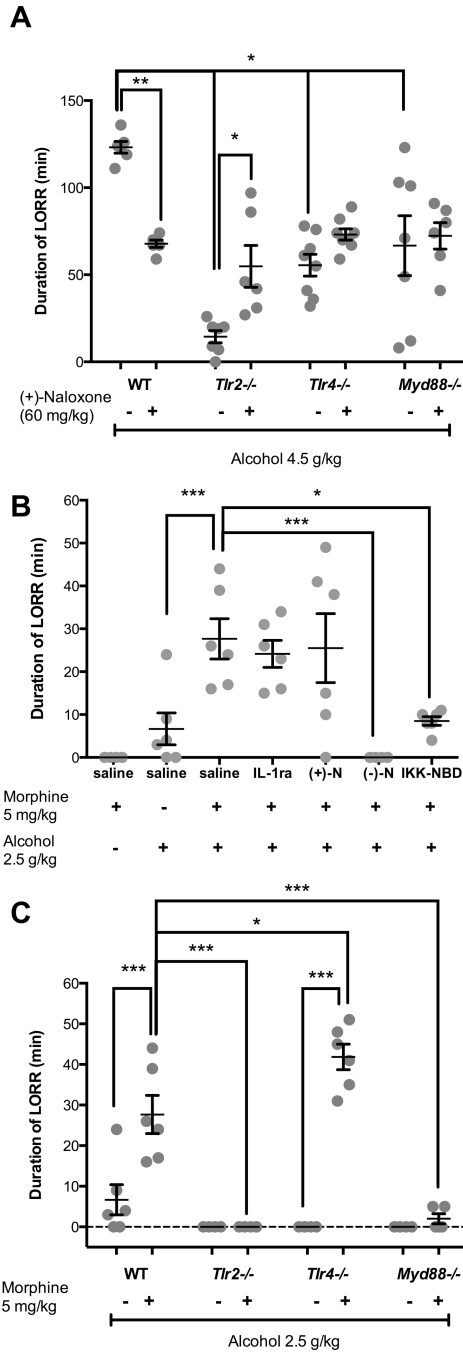Representative image of the region of interest for immunohistochemical cell counts. Scale bar = 1mm

Figure 2.
(A) Decreased duration of loss of righting reflex (LORR) induced by alcohol (4.5 g/kg) in *Tlr2−/− Tlr4−/−* and *Myd88−/−* mice compared to wild-type mice and the protective effect of (+)-naloxone (60 mg/kg) (n = 6 − 8). The effects of treatments with IL-1ra, (+)-naloxone, or (−)-naloxone and the genetic deficiency of innate immune genes in TLR signaling cascades on the increased duration of LORR interaction between morphine and alcohol. (B) In wild-type mice, (−)-naloxone and NK-κB inhibitor IKK-NBD blocked the potentiated duration of LORR effect of alcohol by morphine. (C) In *Tlr2−/−* and *Myd88−/−* mice, the

enhancement of alcohol-induced sedation by morphine was significantly decreased, but in *Tlr4−/−* mice the LORR response was enhanced (n = 4 – 6). Data are presented as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.
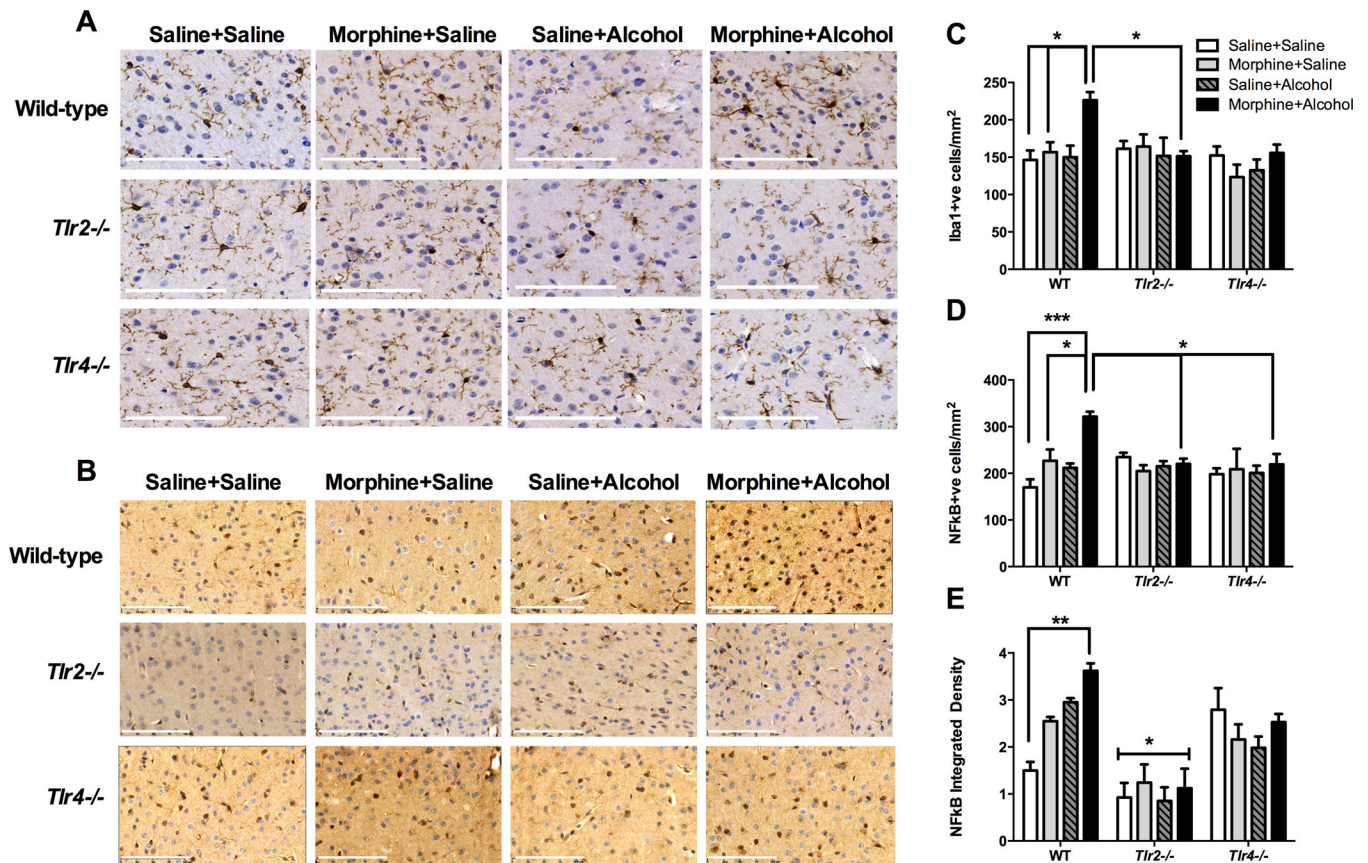
Figure 3.
Representative images of Iba1 (A) and NF-κB-p65 (B) staining in layer 5 of the motor cortex taken at 30 min following pharmacological treatments in each of the wild-type and innate immune genetic deficient mouse strains. (Images are representative of n = 3 – 4 per group) (Scale bar = 500 μm (A) or 100μm (B)). Immunohistochemical analysis of sections of the motor cortex and: (C) quantitative counts of the number of Iba1 positive cells expressed as cells/mm^2; (D) quantitative counts of the number of NF-κB-p65 positive cells expressed as cells/mm^2; and (E) calculation of the average integrated density of NF-κB-p65 positive cells. (n = 3 per group). * P < 0.05; ** P < 0.01; *** P < 0.001.
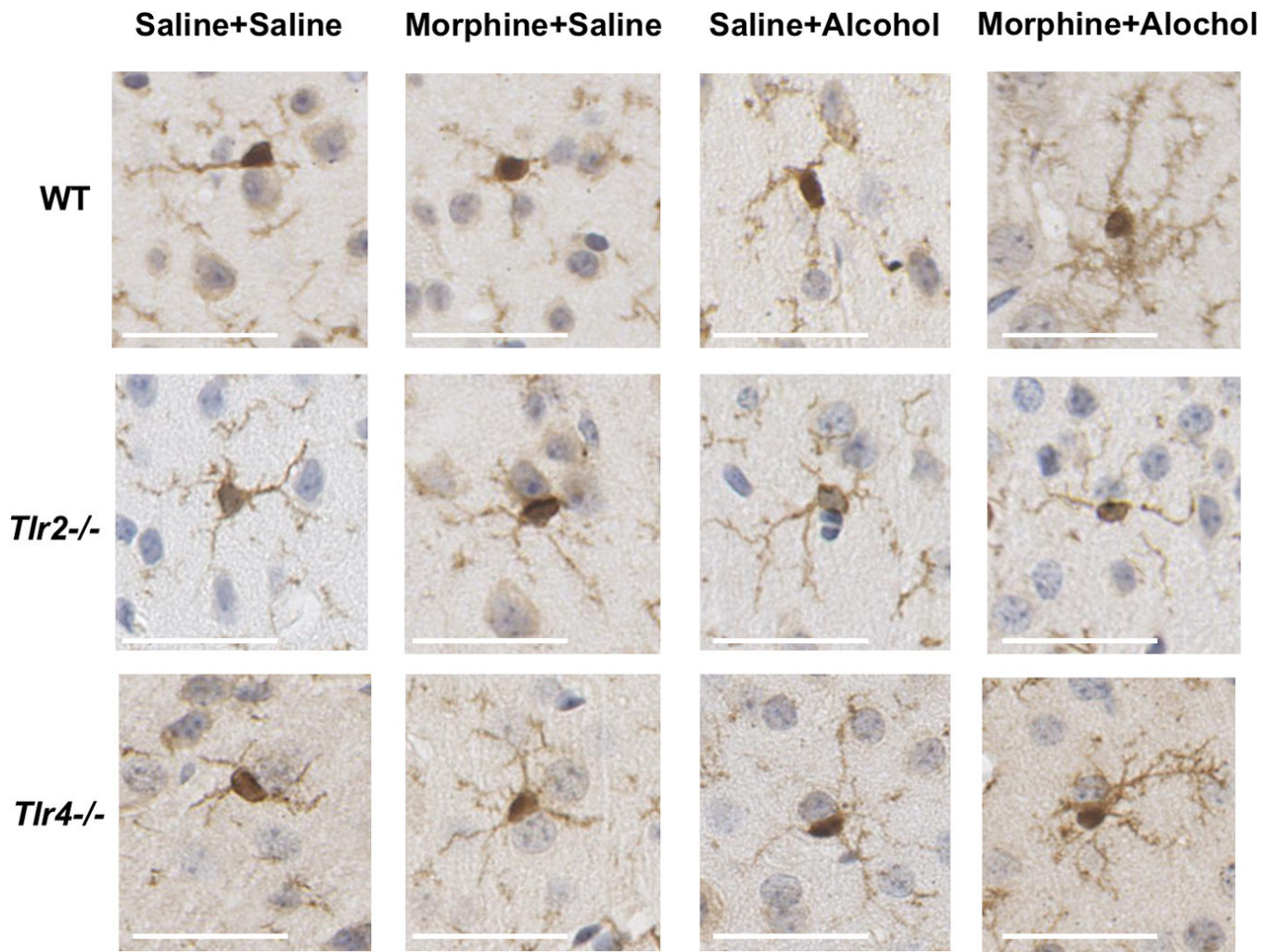
Figure 4.
Phenotypic changes of Iba1 positive microglia in layer 5 of the motor cortex taken at 30 min following pharmacological treatments in each of the wild-type and innate immune genetic deficient mouse strains (scale bar = 10μm).

Table 1

Summary of the main findings from past research (as indicated by #) (Wu et al., 2012; Wu et al., 2011) and the current study investigating the acute neurological effects of alcohol alone.

	Alcohol LORR
Wild-type	++++
Tlr2–/–	–––
Tlr4–/–	++
Myd88–/–	++
IL-1ra#	++
(+)-Naloxone	++
Minocycline#	++

LORR = Loss of righting reflex. M = morphine, A= Alcohol. Number of plus signs signifies approximate increase in the magnitude of response, whilst the minus signs signify blockade of the response.

Table 2

Summary of the main findings from this study investigating the effects of alcohol and morphine administration and the current study.

	Morphine+Alcohol LORR	Iba1 count	Iba1 Morphology	NF-κB count	NF-κb Intensity
Wild-type	++++	M+A ++++	M+A Activated	M+A ++++	M+A ++
Tlr2−/−	— —	— —	M+A Resting	— —	— —
Tlr4−/−	++++++	— —	M+A Activated	— —	— —*
Myd88−/−	— —				
IL-1ra	++++				
(+)-Naloxone	++++				
(−)-Naloxone	— —				
NF-κB-NBD	++				

LORR = Loss of righting reflex. M = morphine, A= Alcohol. Number of plus signs signifies approximate increase in the magnitude of response, whilst the minus signs signify blockade of the response.

*
Elevated levels of staining intensity basally compared to other groups, but no effect of drug treatment.