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Sedative and motor incoordination effects of ethanol in mice lacking CD14, TLR2, TLR4, or MyD88

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Abstract

Background—In our companion article, we examined the role of MyD88-dependent signaling in ethanol consumption in mice lacking key components of this inflammatory pathway and observed differential effects on drinking. Here we studied the role of these same signaling components in the acute sedative, intoxicating, and physiological effects of ethanol. TLR4 has been reported to strongly reduce the duration of ethanol-induced sedation, though most studies do not support its direct involvement in ethanol consumption. We examined TLR4 and other MyD88 pathway molecules to determine signaling specificity in acute ethanol-related behaviors. We also studied other GABAergic sedatives to gauge the ethanol specificity and potential role for GABA in ethanol's sedative and intoxicating effects in the mutant mice.

Methods—Loss of righting reflex (LORR) and recovery from motor incoordination were studied following acute injection of ethanol or other sedative drugs in male and female control C57BL/6J mice vs. mice lacking CD14, TLR2, TLR4 (C57BL/10ScN), or MyD88. We also examined ethanol-induced hypothermia and blood ethanol clearance in these mice.

Results—Male and female mice lacking TLR4 or MyD88 showed reduced duration of ethanol-induced LORR and faster recovery from ethanol-induced motor incoordination. MyD88 KO mice had slightly faster recovery from ethanol-induced hypothermia compared to control mice. None of the mutants differed from control mice in the rate of blood ethanol clearance. There were no genotype differences in the duration of gaboxadol-induced LORR, and only mice lacking TLR4 were less sensitive to the sedative effects of pentobarbital. Faster recovery from diazepam-induced motor incoordination was observed in CD14, TLR4, and MyD88 null mice of both sexes.

Conclusions—TLR4 and MyD88 were key mediators of the sedative and intoxicating effects of ethanol and GABAergic sedatives, indicating a strong influence of TLR4-MyD88 signaling on GABAergic function. Despite the involvement of TLR4 in ethanol's acute behaviors, it did not regulate ethanol consumption in any drinking model as shown in our companion article.

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Collectively, our studies demonstrate differential effects of TLR-MyD88 components in the acute vs. chronic actions of ethanol.

Keywords

loss of righting reflex and motor incoordination; TLR4 deficient (C57BL/10ScN) mice; TLR2 KO; CD14 KO; MyD88 KO

Introduction

Alcohol consumption in animal models of excessive drinking and human alcoholics has been linked with increased innate immune signaling (Blednov et al., 2011; Crews and Vetreno, 2016). Although deletion of certain immune-related genes reduces ethanol consumption (Mayfield et al., 2013, 2016; Robinson et al., 2014), the specificity of ethanol's interaction with individual inflammatory mediators is not known. Most studies have focused on either the toxic, neurodegenerative effects of persistent activation of immune pathways by chronic ethanol or the effects of inhibiting these pathways on ethanol consumption. Few studies have compared the effects of acute alcohol on other behaviors or physiological actions following manipulation of different immune signaling components.

Our companion paper (Blednov et al., submitted) provides novel evidence that deletion of specific components of the MyD88-dependent pathway can produce opposite actions on ethanol intake by decreasing (e.g., TLR2 or CD14 KO mice) or increasing (MyD88 KO mice) drinking. Deletion of TLR4 had no effect on drinking as previously reported in knockout mice (Alfonso-Loeches et al., 2010; Pascual et al., 2011). Based on these differential actions, we further examined the effects of these same signaling components on acute ethanol-related behaviors.

Previous studies showed that the duration of ethanol-induced loss of righting reflex (LORR) was decreased in mice lacking TLR2, TLR4, or MyD88 (Corrigan et al., 2015; Harris et al., in press; Wu et al., 2012). We hypothesized that MyD88 pathway components are also involved in other behaviors and compared the effects of ethanol and other sedatives (gabaxoxol, pentobarbital, and diazepam) on the duration of LORR and recovery from acute motor incoordination in mice lacking CD14, TLR2, TLR4, or MyD88. Considering the well known actions of ethanol on GABAergic function and the evidence that acute ethanol potentiates GABAergic synaptic transmission via CD14/TLR4 signaling in mouse neurons (Bajo et al., 2014), other GABAergic sedatives were tested to determine the ethanol-specificity and the role for GABA-mediated signaling in the sedative and intoxicating effects. In addition, we examined potential genotype differences in ethanol-induced hypothermia and blood ethanol clearance because these physiological factors can affect the behavioral phenotypes.

Materials and Methods

Mice

Generation of *Cd14* (B6.129S4-*Cd14^{tm1Frm}*/J, stock #003726), *Tlr2* (B6.129-*Tlr2^{tm1Kir}*/J, stock #004650), and *Myd88* (B6.129P2(SJL)-*Myd88^{tm1.1Defr}*/J, stock #009088) knockout

(KO) mice were described previously (Moore et al., 2000; Wooten et al., 2002; Hou et al., 2008). The C57BL/10ScN mouse was used as a TLR4 deficient model (B6.B10ScN-*Tlr4^{lps-del}*/JthJ, stock #007227). This strain is homozygous for a deletion allele *Tlr4^{lps-del}* that causes spontaneous deletion of TLR4, resulting in absence of TLR4 mRNA and protein. All mutant strains were purchased from Jackson Laboratories and were backcrossed on a C57BL/6J genetic background more than 6 generations, and thus the C57BL/6J inbred strain is an appropriate control for these studies (<https://www.jax.org/jax-mice-and-services/customer-support/technical-support/breeding-and-husbandry-support/considerations-for-choosing-controls>). C57BL/6J mice were taken from a colony maintained at The University of Texas at Austin (original breeders were purchased from Jackson Laboratories, Bar Harbor, ME). Mice were group-housed 4 to 5 to a cage based on genotype and sex. The humidity and temperature of the rooms were kept constant and they were maintained a 12/12 hour light/dark cycle with lights on at 7 AM. Food and water were available *ad libitum*. Behavioral testing began when the mice were at least 2 months old, and mice were weighed every 4–6 days. All experiments were conducted in isolated behavioral testing rooms in the Animal Resource Center at UT Austin with a reversed light/dark cycle to avoid external distractions. Before beginning experiments, mice were moved to their experimental room and remained there for 1–2 weeks to adapt to the new light/dark cycle. All experiments were approved by the university's Institutional Animal Care and Use Committee.

Drugs

Injectable ethanol (Aaper Alcohol and Chemical, Shelbyville, KY) solutions were prepared in 0.9% saline (20%, v/v). Gaboxadol (Sigma-Aldrich, St. Louis, MO), diazepam (Sigma-Aldrich), and pentobarbital (Sigma-Aldrich RBI, Natick, MA) were dissolved in 0.9% saline. All drugs were injected intraperitoneally (i.p.) at 0.01 ml/g of body weight.

Loss of Righting Reflex (LORR)

Sensitivity to the depressant effects of ethanol (3.6 g/kg, i.p.), gaboxadol (55 mg/kg, i.p.), and pentobarbital (50 mg/kg, i.p.) were determined using the LORR (sleep time) assay in mice. These drug doses were based on previous studies (Blednov et al., 2013, 2014; Boehm et al., 2004). When mice became ataxic, they were placed in the supine position in V-shaped plastic troughs until they were able to right themselves three times within 30 s. Sleep time was defined as the time from being placed in the supine position until they regained their righting reflex.

Rotarod

Mice were trained on a fixed speed rotarod (Economex; Columbus Instruments, Columbus, OH) at 5 rpm, and training was considered complete when mice were able to remain on the rotarod for 60 s. Every 15 minutes after injection of ethanol (2 g/kg i.p.) or diazepam (5 mg/kg i.p.), mice were placed back on the rotarod and latency to fall was measured until mice were able to remain on the rotarod for 60 s.

Hypothermia

Mice were separated into individually ventilated hypothermia chambers to acclimate for 60 min. Mice were then removed from the chambers to record baseline temperatures with a glycerol-lubricated probe (1.2 mm ball $\times$ 2cm length; Senterok Thermalert TH-8) that was inserted into the rectum for 5 s. Immediately following baseline recording, each mouse was injected with ethanol (3.6 g/kg, i.p.) and placed back in its chamber. Rectal temperatures were monitored over 5 h with measurements taken every 30 min following ethanol injection.

Ethanol clearance

Animals were injected with a single dose of ethanol (4 g/kg, i.p.), and blood samples were taken from the retro-orbital sinus 30, 60, 120, 180, and 240 min after injection. Samples (~20 μ l) were collected in capillary tubes and centrifuged for 6 min at 3100g using a Haematospin 1400 centrifuge (Analox Instruments, London, UK). The plasma samples were stored at -20°C until BECs were determined in 5- μ l aliquots using an AM1 Alcohol Analyzer (Analox Instruments). The machine was calibrated every 15 samples using an industry standard, and BECs were determined using commercially available reagents according to the manufacturer's instructions. Samples were averaged from duplicate runs and expressed as mg/dl.

Statistical Analysis

Data are reported as the mean $\pm$ S.E.M. The statistics software program GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used to perform one or two-way ANOVAs, Student's t-tests, and Bonferroni or Dunnett's post hoc analyses. Data from male and female mice were analyzed separately.

Results

Loss of righting reflex

The duration of LORR was measured following the injection of ethanol, gaboxadol, or pentobarbital in mice lacking CD14, TLR2, TLR4, or MyD88 vs. control mice. For ethanol (3.6 g/kg), there was a shorter duration of LORR in TLR4 and MyD88 deficient mice of both sexes (Fig. 1A,D). All male and female mutant mice had shorter duration of LORR after injection of gaboxadol (55 mg/kg) (Fig. 1B,E). Male and female mice lacking TLR4 had reduced duration of pentobarbital (50 mg/kg)-induced LORR, but no other genotype differences were found (Fig. 1C,F).

Motor incoordination

Acute administration of ethanol (2 g/kg) produced motor incoordination in all genotypes. No differences in the recovery from ethanol-induced motor incoordination were found in CD14 KO and TLR2 KO male mice vs. control mice (Fig. 2A,B), while TLR4 and MyD88 mutant males recovered faster than controls (Fig. 2C,D). Similar to male mice, no differences in the recovery from ethanol-induced motor incoordination were found in CD14 KO and TLR2 KO female mice vs. controls (Fig. 3A,B), while TLR4 and MyD88 mutant females recovered from ethanol-induced motor incoordination faster than control mice (Fig. 3C,D).

Acute administration of diazepam (5 mg/kg) produced motor incoordination in all genotypes. CD14, TLR4, and MyD88 mutant male mice recovered faster than control mice (Fig. 4). No differences in the recovery from diazepam-induced motor incoordination were found between male TLR2 KO and control mice (Fig. 4B). Although male MyD88 KO mice recovered faster than controls (Fig. 4D), this may be due to the impaired ability of diazepam to initially induce motor incoordination in the KO mice (see 15 min time point). Female mice of all genotypes showed greater impairment in motor incoordination after acute administration of diazepam. Similar to males, CD14 and MyD88 KO female mice recovered from diazepam-induced motor incoordination faster than control mice (Fig. 5A,D). Although the recovery in female mice lacking TLR4 was not significantly different from control mice, there was a significant genotype $\times$ time interaction effect (Fig. 5C). As observed in male mice, no differences in the recovery from diazepam-induced motor incoordination were found between female TLR2 KO and control mice (Fig. 5B).

Hypothermia

The acute hypothermic responses to ethanol (3.6 g/kg, injected at time 0) in male and female control and mutant mice are shown in Table 1. Maximum reduction in body temperature occurred 60–120 min after injection of ethanol followed by a gradual recovery period over the next 5–6 h. No genotype differences in ethanol-induced hypothermic responses were found in male and female mice lacking CD14, TLR2, or TLR4 compared to control mice. Male and female mice lacking MyD88 recovered from ethanol-induced hypothermia slightly faster than control mice [effect of genotype in males and females, respectively: $F_{(1,10)} = 6.8$ and $F_{(1,13)} = 4.9$; $P < 0.05$; two-way ANOVA]. This would not account for the reduced ethanol-induced sedation given that these mice regain righting reflex within 60 min, and no differences in hypothermia are observed during this time period. Also, the genotype effect in MyD88 KO female mice is likely due to initial differences in body temperature. For example, female mice lacking MyD88 or TLR2 had significantly increased core body temperature measured at time 0 compared to control mice (Table 1). A similar trend was observed in female mice lacking TLR4 but the genotype effect was not significant. Comparison of body temperature (time 0) using Student's t-tests showed that all three mutant strains were different from female control mice ($P < 0.05$ for TLR4 mutant; $P < 0.01$ for TLR2 and MyD88 mutants). The last column of Table 1 depicts the slopes of the curves during recovery from ethanol-induced hypothermia (measured from 60 to 360 min) in control and mutant mice; however, one-way ANOVA revealed no significant genotype effects on slope in male or female mice.

Ethanol clearance

BECs were measured 30, 60, 120, 180, and 240 min after injection of ethanol (4 g/kg). There were no differences in the rates of clearance of blood ethanol in male and female control and mutant mice and no differences in slopes (Fig. 6).

Discussion

Our companion paper provides novel evidence that deletion of individual signaling components of the MyD88 pathway has selective effects on ethanol consumption (Blednov

et al., submitted). MyD88 signaling is also implicated in the acute sedative and intoxicating effects of ethanol as shown in the current study. However, different pathway mediators were involved in the different behaviors. Our previous study of the interleukin-1 receptor type 1 (IL-1R1) pathway provides additional support that another inflammatory pathway using the MyD88 adapter protein can regulate distinct ethanol-mediated behaviors (Blednov et al., 2015). For example, ethanol consumption was not altered in IL-1R1 KO mice, but IL-1R1 signaling was involved in ethanol-induced sedation and motor incoordination as well as withdrawal severity (Blednov et al., 2015). The ethanol- and benzodiazepine-induced LORR phenotypes in IL-1R1 KO mice were similar to those observed in the TLR4 and MyD88 mutant mice shown here, and both studies suggest that a GABAergic mechanism may contribute to the altered sedative effects in these mutant strains. Although different proinflammatory pathways culminate in a common endpoint of elevated levels of cytokines, we have shown that simply inhibiting various pathway components does not always yield the same phenotype. Some, but not all, components are important in ethanol behaviors, and deletion of individual mediators can produce distinct behavioral responses to ethanol. Notably, some pathway components were linked with ethanol drinking while others were involved in ethanol's acute sedative and intoxicating effects. The behavioral tests that we used would produce either acutely elevated blood alcohol levels following i.p. injection or lower levels of chronically elevated blood alcohol following voluntary drinking. The short-term vs. long-term ethanol exposure times could determine the impact (if any) of neuroimmune/inflammatory responses or the particular pathways involved. These pathway components may also regulate normal brain function independent of inflammatory signaling.

In the present study, we consistently found that male and female mice lacking TLR4 or MyD88 were less sensitive to ethanol's acute sedative and intoxicating effects, whereas CD14 KO and TLR2 KO male and female mice did not differ from control mice in these tests (Table 2). All mutants studied were less sensitive to the sedative effects of gaboxadol, while only TLR4 mutant mice were less sensitive to pentobarbital-induced sedation. Inhibition of TLR4-MyD88 signaling also increased recovery from the motor incoordination induced by diazepam (Table 2). Our companion study showed that TLR4 did not regulate ethanol consumption in male or female mice and MyD88 had a complex sex-dependent role (Blednov et al., submitted), but the current study supports a strong involvement of these components in the acute depressant actions of ethanol and other GABAergic sedative drugs (e.g., gaboxadol, diazepam).

The overall effects of ethanol and diazepam in the mutants were similar (Table 2), suggesting that altered GABAergic responses may be a common mechanism as discussed earlier for IL-1R1 KO mice (Blednov et al., 2015). Electrophysiological studies showed that acute ethanol potentiates GABAergic transmission in mouse central amygdala neurons via CD14/TLR4 signaling (Bajo et al., 2014), and also support a role for IL-1R in acute ethanol-induced facilitation of GABAergic synaptic currents (Bajo et al., 2015). The electrophysiological evidence corroborates our previous and current behavioral data showing that MyD88-related components potentially regulate the acute and chronic effects of ethanol via GABAergic signaling. Interestingly, electrophysiological evidence also supports distinct signaling roles for proinflammatory/neuroimmune molecules in basal GABAergic transmission (Bajo et al., 2014; Bajo et al., 2015). It is also possible that ethanol directly

stimulates TLR4-MyD88 signaling as has been proposed for opioid-induced activation of TLR4 (Hutchinson et al., 2010). An alternative possibility is that ethanol activates this pathway by releasing endogenous activators of TLR4 such as HMGB1 (Zou and Crews, 2014; Crews et al., 2013).

Our results in male and female mice are consistent with previous studies showing that ethanol-induced duration of LORR decreased in male TLR4 KO mice or mice treated with the selective TLR4 inhibitor, ($\pm$)-naloxone (Wu et al., 2011; Corrigan et al., 2015). Thus, two different genetic mouse models (TLR4 null mutants and B6.B10ScN mice), as well as pharmacological inhibition of TLR4 in mice, confirm that TLR4 is involved in the acute sedative effects of ethanol; and we provide further evidence that male and female mice do not differ in the acute sedative or intoxicating effects. Different genetic and pharmacological mouse models have also provided corroborating evidence that TLR4 is not a critical determinant of ethanol drinking (Alfonso-Loeches et al., 2010; Bajo et al., 2016; Pascual et al., 2011) (Blednov et al., submitted), and we confirmed these findings in female mice. These results are further supported by an extensive body of research in male and female rats and mice, showing that genetic or pharmacologic manipulation of TLR4 does not regulate ethanol drinking, but the acute sedative effect of ethanol is decreased in TLR4 KO rats (Harris et al., in press). Including our work in male and female B6.B10ScN mice, a role for TLR4 in ethanol's acute sedative effects (but not drinking) has now been validated in several different male and female rodent models. Although null mutations may induce compensatory molecular changes in the periphery and/or the CNS, our findings have been validated by studies using pharmacological inhibitors of TLR4 (described above). Previous TLR4 studies did not compare ethanol with other sedatives, but we now show that TLR4 is also involved in the sedative effects of GABAergic drugs such as gaboxadol and pentobarbital, and does not specifically mediate ethanol-induced sedation.

In addition to TLR4, we demonstrate a key role for MyD88 in the sedative and intoxicating effects of ethanol in male and female mice, which is consistent with the findings reported by Wu et al. (2012) in male MyD88 KO mice. Our work further implicates TLR4-MyD88 signaling in the motor incoordination effects of diazepam and suggests that this pathway mediates the acute depressant effects of ethanol and other GABAergic sedatives in mice of both sexes.

Our results do not support a role for TLR2 in ethanol-induced sedation in contrast to the study by Corrigan et al. (2015), which showed that LORR was almost completely eliminated in TLR2 KO mice. It should be noted that the TLR2 KO mice used in these studies were generated by different groups using different genetic backgrounds (Wooten et al., 2002; Corrigan et al., 2015) and may differ in developmental and compensatory changes associated with genetic deletion. Corrigan et al. (2015) used TLR2 KO mice on a Balb/c background, whereas the TLR2 KO mice used in our study were on a C57BL/6J background. However, the TLR4 KO and MyD88 KO mice studied in Wu et al. (2012) were on a Balb/c background and we observed similar LORR effects using mice on a C57BL/6J background. Although we found that TLR2 was not involved in the sedative response to ethanol, it was involved in gaboxadol-induced sedation (as were the other immune mediators that we studied). In contrast to the differential effects on voluntary ethanol consumption, the acute

sedative and intoxicating effects of ethanol (or other GABAergic drugs) were regulated in a similar manner by several MyD88 pathway components, suggesting a role for overall cytokine signaling in these behaviors.

We studied ethanol-induced hypothermia and blood ethanol clearance because these physiological and pharmacokinetic effects can potentially impact the behaviors that we measured. Hypothermia can prolong ethanol's sedative effects, so we measured body temperature following the same dose of ethanol that was used in the LORR experiments. The rate of clearance of blood ethanol can also affect recovery from its sedative and intoxicating effects. However, there were no genotype differences in clearance or hypothermia that could account for the behavioral effects. Thus, the ethanol-induced behaviors measured in our two studies were not likely influenced by physiological or pharmacokinetic processes.

Collectively, our studies show that key mediators of the MyD88 inflammatory pathway are associated with distinct behavioral effects of ethanol. In general, TLR4 and MyD88 are implicated in the acute sedative and intoxicating effects of ethanol, while TLR2 and CD14 are implicated in ethanol drinking (although the effects of MyD88 effects are more complex as it may regulate drinking in male mice). Effects on ethanol consumption in some mutants depended on the sex of the mice (Blednov et al., submitted), but sex differences were not observed for the effects of mutations on the sedative and intoxicating effects of ethanol. Although the involvement of neuroimmune signaling in the different behaviors is unknown, it appears that GABAergic regulation is important in ethanol's acute actions. Our studies provide novel evidence that individual players in the MyD88 pathway are associated with distinct ethanol-related behaviors, suggesting that signaling components can be selectively targeted to mitigate the effects of ethanol or other sedative drugs.

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The authors declare no conflicts of interest.

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Figure 1. Duration of LORR induced by sedative drugs in mice lacking CD14, TLR2, TLR4, or MyD88

Effect of ethanol (3.6 g/kg, i.p.) injection in male mice (A) C57BL/6J, n= 34; CD14 and TLR2 KO, n= 16; B6.B10ScN, n= 11; MyD88 KO, n= 6 and female mice (D) C57BL/6J, n= 26; CD14 KO, n= 13; TLR2 KO, n= 15; B6.B10ScN, n= 14; MyD88 KO, n= 5. Effect of gaboxadol (55 mg/kg) injection in male mice (B) C57BL/6J, n= 11; CD14 KO, n= 10; TLR2 KO, n= 12; B6.B10ScN, n= 11; MyD88 KO, n= 17 and female mice (E) C57BL/6J, n= 11; CD14 KO, n= 10; TLR2 KO, n= 11; B6.B10ScN, n= 16; MyD88 KO, n= 13. Effect of pentobarbital (50 mg/kg) injection in male mice (C) C57BL/6J, n= 11; CD14 KO, n= 9, TLR2 KO, n= 12; B6.B10ScN, n= 11; MyD88 KO, n= 15 and female mice (F) C57BL/6J, n= 13; CD14 KO, n= 11; TLR2 KO, n= 14; B6.B10ScN, n= 18; MyD88 KO, n= 15. Values represent mean $\pm$ S.E.M. Data were analyzed by one-way ANOVA followed by Dunnett's

post hoc tests for multiple comparisons (***) $P < 0.001$ compared to control). EtOH = ethanol; LORR = loss of righting reflex.

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Figure 2. Recovery from ethanol-induced motor incoordination in male mice lacking CD14, TLR2, TLR4, or MyD88

Data represent time (s) on the rotarod after injection of ethanol (2 g/kg, i.p.). The same group of control mice is represented in A–D (C57BL/6J mice, $n = 14$). (A) CD14 KO mice, $n = 6$ [$F_{(1,18)} = 0.04$, $P > 0.05$, effect of genotype; $F_{(9,162)} = 184$, $P < 0.001$, effect of time; $F_{(9,162)} = 0.3$, $P > 0.05$, genotype $\times$ time interaction]. (B) TLR2 KO mice, $n = 6$ [$F_{(1,18)} = 0.22$, $P > 0.05$, effect of genotype; $F_{(9,162)} = 168$, $P < 0.001$, effect of time; $F_{(9,162)} = 0.4$, $P > 0.05$, genotype $\times$ time interaction]. (C) B6.B10ScN mice, $n = 10$ [$F_{(1,22)} = 37.4$, $P < 0.001$, effect of genotype; $F_{(9,198)} = 206$, $P < 0.001$, effect of time; $F_{(9,198)} = 12.3$, $P < 0.001$, genotype $\times$ time interaction]. (D) MyD88 KO mice, $n = 8$ [$F_{(1,20)} = 52.2$, $P < 0.001$, effect of genotype; $F_{(9,180)} = 229$, $P < 0.001$, effect of time; $F_{(9,180)} = 17.6$, $P < 0.001$, genotype $\times$ time interaction]. Values represent mean $\pm$ S.E.M. Data were analyzed by repeated measures two-way ANOVA and Bonferroni post hoc tests (** $P < 0.01$, *** $P < 0.001$ compared to control).

Figure 3. Recovery from ethanol-induced motor incoordination in female mice lacking CD14, TLR2, TLR4, or MyD88

Data represent time (s) on the rotarod after injection of ethanol (2 g/kg, i.p.). The same group of control mice is represented in A–D (C57BL/6J mice, $n = 26$). (A) CD14 KO mice, $n = 6$ [$F_{(1,30)} = 0.01$, $P > 0.05$, effect of genotype; $F_{(8,240)} = 95.6$, $P < 0.001$, effect of time; $F_{(8,240)} = 0.2$, $P > 0.05$, genotype $\times$ time interaction]. (B) TLR2 KO mice, $n = 8$ [$F_{(1,32)} = 2.8$, $P > 0.05$, effect of genotype; $F_{(8,256)} = 127$, $P < 0.001$, effect of time; $F_{(8,256)} = 1.15$, $P > 0.05$, genotype $\times$ time interaction]. (C) B6.B10ScN mice, $n = 12$ [$F_{(1,36)} = 18.5$, $P < 0.001$, effect of genotype; $F_{(8,288)} = 129$, $P < 0.001$, effect of time; $F_{(8,288)} = 5.8$, $P < 0.001$, genotype $\times$ time interaction]. (D) MyD88 KO mice, $n = 6$ [$F_{(1,30)} = 66.9$, $P < 0.001$, effect of genotype; $F_{(8,240)} = 69.9$, $P < 0.001$, effect of time; $F_{(8,240)} = 15.8$, $P < 0.001$, genotype $\times$ time interaction]. Values represent mean $\pm$ S.E.M. Data were analyzed by repeated measures two-way ANOVA and Bonferroni post hoc tests (** $P < 0.01$, *** $P < 0.001$ compared to control).

Figure 4. Recovery from diazepam-induced motor incoordination in male mice lacking CD14, TLR2, TLR4, or MyD88

Data represent time (s) on the rotarod after injection of diazepam (5 mg/kg, i.p.). The same group of control mice is represented in A–D (C57BL/6J mice, $n = 13$). (A) CD14 KO mice, $n = 12$ [$F_{(1,23)} = 33$, $P < 0.001$, effect of genotype; $F_{(6,138)} = 206$, $P < 0.001$, effect of time; $F_{(6,138)} = 16.6$, $P < 0.001$, genotype $\times$ time interaction]. (B) TLR2 KO mice, $n = 10$ [$F_{(1,21)} = 2.2$, $P > 0.05$, effect of genotype; $F_{(6,126)} = 217$, $P < 0.001$, effect of time; $F_{(6,126)} = 2.3$, $P < 0.05$, genotype $\times$ time interaction]. (C) B6.B10ScN mice, $n = 13$ [$F_{(1,24)} = 17.6$, $P < 0.001$, effect of genotype; $F_{(6,144)} = 249$, $P < 0.001$, effect of time; $F_{(6,144)} = 8.3$, $P < 0.001$, genotype $\times$ time interaction]. (D) MyD88 KO mice, $n = 9$ [$F_{(1,20)} = 80.8$, $P < 0.001$, effect of genotype; $F_{(6,120)} = 71.4$, $P < 0.001$, effect of time; $F_{(6,120)} = 24$, $P < 0.001$, genotype $\times$ time interaction]. Values represent mean $\pm$ S.E.M. Data were analyzed by repeated measures two-way ANOVA with Bonferroni post hoc tests (*** $P < 0.001$ compared to control).

Figure 5. Recovery from diazepam-induced motor incoordination in female mice lacking CD14, TLR2, TLR4, or MyD88

Data represent time (s) on the rotarod after injection of diazepam (5 mg/kg, i.p.). The same group of control mice is represented in A–D (C57BL/6J mice, $n = 11$). (A) CD14 KO mice, $n = 9$ [$F_{(1,18)} = 5.7$, $P < 0.05$, effect of genotype; $F_{(7,126)} = 112$, $P < 0.001$, effect of time; $F_{(7,126)} = 3.0$, $P < 0.01$, genotype $\times$ time interaction]. (B) TLR2 KO mice, $n = 10$ [$F_{(1,19)} = 1.2$, $P > 0.05$, effect of genotype; $F_{(7,133)} = 134$, $P < 0.001$, effect of time; $F_{(7,133)} = 0.7$, $P > 0.05$, genotype $\times$ time interaction]. (C) B6.B10ScN mice, $n = 10$ [$F_{(1,19)} = 3.8$, $P > 0.05$, effect of genotype; $F_{(7,133)} = 154$, $P < 0.001$, effect of time; $F_{(7,133)} = 2.8$, $P < 0.05$, genotype $\times$ time interaction]. (D) MyD88 KO mice, $n = 8$ [$F_{(1,17)} = 9.8$, $P < 0.01$, effect of genotype; $F_{(7,119)} = 90.1$, $P < 0.001$, effect of time; $F_{(7,119)} = 4.1$, $P < 0.001$, genotype $\times$ time interaction]. Values represent mean $\pm$ S.E.M. Data were analyzed by repeated measures two-way ANOVA and Bonferroni post hoc tests (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control).

Figure 6. Clearance of blood ethanol in mice lacking CD14, TLR2, TLR4, or MyD88

Data represent the clearance of blood ethanol over 4 h in (A) male and (B) female mice after injection of ethanol (4 g/kg, i.p.). Control C57BL/6J mice (n= 8 males; n= 14 females); CD14 KO (n= 4 males; n= 9 females); TLR2 KO (n= 4 males; n= 9 females); B6.B10ScN (n= 11 males and females); and MyD88 KO mice (n= 7 females). Values represent mean $\pm$ S.E.M. Data were analyzed by one-way ANOVA. BEC, blood ethanol concentration.

Table 1

Recovery from ethanol-induced hypothermia in male and female control and mutant mice

		Time (min)							1/Slope
Sex	Controls/Mutants	0	60	120	180	240	300	360	
Males	C57BL/6J n=6	37.4±0.1	34.5±0.26	34.1±0.4	34.7±0.41	34.5±0.62	35.7±0.32	36.0±0.29	228.4±66.7
	CD14 n=6	37.3±0.1	34.4±0.26	34.6±0.26	34.9±0.23	35.6±0.23	36.2±0.28	36.5±0.37	147.9±29.1
	TLR2 n=6	37.3±0.38	34.9±0.26	34.4±0.34	35.2±0.31	35.8±0.24	36.2±0.13	36.4±0.16	169.6±24.9
	TLR4 n=6	37.7±0.1	34.8±0.12	34.5±0.3	35.3±0.19	35.6±0.2	36.0±0.32	36.3±0.3	209.6±54.2
	MyD88 n=6	37.9±0.14	34.9±0.41	34.9±0.33	35.2±0.4	35.9±0.15*	36.7±0.18	37.3±0.24*	155.3±36.5
Females	C57BL/6J n=7	37.2±0.3	34.9±0.22	35.4±0.17	36.0±0.21	36.6±0.13	36.8±0.14	36.9±0.07	149.6±14.1
	CD14 n=7	37.5±0.38	34.5±0.30	34.9±0.13	35.8±0.11	37.0±0.13	37.3±0.19	37.4±0.13	102.5±11.8
	TLR2 n=7	38.3±0.05**	34.5±0.33	34.7±0.19	35.5±0.21	36.4±0.24	37.2±0.25	37.4±0.08	96.4±7.6
	TLR4 n=7	37.9±0.13	34.3±0.28	35.5±0.17	35.9±0.19	36.4±0.18	37.1±0.20	37.4±0.17	112.7±16.6
	MyD88 n=8	38.4±0.17***	35.2±0.22	35.1±0.15	36.0±0.24	36.5±0.26	37.4±0.22	37.6±0.14	114.8±10.9

Data represent the mean body temperature $\pm$ S.E.M. ($^{\circ}$ C) before (time 0) and after injection of ethanol (3.6 g/kg, i.p.), measured every 30 min over 360 min. Data were analyzed by two-way ANOVA. There was a significant effect of genotype in male and female MyD88 KO mice ($P < 0.05$) and significant genotype $\times$ time interaction effects in female TLR2, TLR4, and MyD88 mutant mice ($P < 0.001$). Post hoc comparisons are indicated as follows:

* $P < 0.05$;

** $P < 0.01$; and

*** $P < 0.001$; compared to the control (C57BL/6J) time point of the corresponding sex. Data in the last column represent the mean slope (expressed as 1/slope) of the curves $\pm$ S.E.M during recovery from ethanol-induced hypothermia (from 60 to 360 min); one-way ANOVA revealed no significant genotype effects on slope in male or female mice.

Table 2

Summary of the acute effects of ethanol and other sedatives in mice lacking CD14, TLR2, TLR4, or MyD88.

Mutants	EtOH (3.6 g/kg)		Gaboxadol (55 mg/kg)		Pentobarb (50 mg/kg)		EtOH (2 g/kg)		Diazepam (5 g/kg)		EtOH (3.6 g/kg)		Rate of Clearance	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
CD14	=	=	↓	↓	=	=	=	=	↑	↑	=	=	=	=
TLR2	=	=	↓	↓	=	=	=	=	=	=	=	=	=	=
TLR4	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	=	=	=	=
MyD88	↓	↓	↓	↓	=	=	↑	↑	↑	↑	↑	↑	=	=

Duration of loss of righting reflex, recovery from motor incoordination and hypothermia, and blood ethanol clearance in male (M) and female (F) mutant vs. control C57BL/6J mice injected with ethanol or other sedative drugs. ↓ indicates reduced duration of LORR compared with corresponding control mice; ↑ indicates increased (faster) recovery from the motor incoordination or hypothermic effects of ethanol compared with control mice; = indicates no significant difference from control mice. EtOH, ethanol; LORR, loss of righting reflex; pentobarb, pentobarbital.