Toll-like receptor 4 (TLR4) is a key mediator of innate immune signaling and has been implicated in alcohol responses in animal models and human alcoholics. Members of the Integrative Neuroscience Initiative on Alcoholism (INIA-Neuroimmune) consortium participated in the first comprehensive study across multiple laboratories to test the hypothesis that TLR4 regulates excessive alcohol consumption in different species and different models of chronic, dependence-driven, and binge-like drinking. Although TLR4 was not a critical determinant of excessive drinking, it was important in the acute sedative effects of alcohol. Current research efforts are directed at determining which neuroimmune pathways mediate excessive alcohol drinking and these findings will help to prioritize relevant pathways and potential therapeutic targets.

**Key words:** (+)-naloxone; chronic intermittent ethanol vapor; drinking-in-the-dark; lipopolysaccharide; operant self-administration; Toll-like receptor 4 knock-out

**Significance Statement**
Toll-like receptor 4 (TLR4) is a key mediator of innate immune signaling and has been implicated in alcohol responses in animal models and human alcoholics. Members of the Integrative Neuroscience Initiative on Alcoholism (INIA-Neuroimmune) consortium participated in the first comprehensive study across multiple laboratories to test the hypothesis that TLR4 regulates excessive alcohol consumption in different species and different models of chronic, dependence-driven, and binge-like drinking. Although TLR4 was not a critical determinant of excessive drinking, it was important in the acute sedative effects of alcohol. Current research efforts are directed at determining which neuroimmune pathways mediate excessive alcohol drinking and these findings will help to prioritize relevant pathways and potential therapeutic targets.

**Introduction**
Gene expression studies have shown strong representation of immune- and inflammatory-related genes in brains from human alcoholics and rodents after chronic alcohol treatment (Liu et al., 2006; Robinson et al., 2014; Crews and Vetrano, 2016). Deletion of immune genes reduced alcohol consumption in mice and pro-
vided corroborating behavioral validation for several immune mediators that were predicted by the genomic studies (Blednov et al., 2012; Mayfield et al., 2013). In addition, alcohol craving and consumption were positively correlated with elevated plasma levels of inflammatory cytokines in human alcoholicos (Leclercq et al., 2012; Leclercq et al., 2014) and immune marker expression in human postmortem brain was correlated with total lifetime alcohol consumption and age of drinking onset (Crews and Vetro, 2016).

Current research efforts are focused on determining which neuroimmune/inflammatory pathways are important for mediating excessive alcohol drinking. The family of Toll-like receptors (TLRs), in particular TLR4, is a major component of innate immune signaling and has been hypothesized to mediate inflammatory effects of alcohol in the brain (Alfonso-Loeches et al., 2010; Szabo and Lippi, 2014; Alfonso-Loeches et al., 2016). TLR4 activation is triggered through pathogen-derived ligands, such as the bacterial endotoxin lipopolysaccharide (LPS) and endogenous ligands including high mobility group box 1 protein (Vezzani et al., 2011). Acute ethanol exposure activates TLR4 signaling in brain astrocytes and microglia (Blanco et al., 2005; Fernandez-Lizarbe et al., 2013) and chronic ethanol also triggers TLR4-mediated neuroimmune responses (Lippi et al., 2013; Pascual et al., 2015; Alfonso-Loeches et al., 2016).

Some studies have suggested a role for TLR4 in alcohol drinking, although this has not been observed consistently. Systemic injection of LPS (a primary TLR4 ligand) increased voluntary ethanol consumption in mice (Blednov et al., 2011) and elevated plasma levels of LPS, along with increased intestinal permeability, were measured in human alcoholics (Leclercq et al., 2012). Selective knockdown of TLR4 in the CeA or the ventral tegmental area (VTA) (but not the ventral pallidum, VP) decreased binge drinking in alcohol-prefering (P) rats (Riu et al., 2011; June et al., 2015). Although TLR4 deficiency protected against ethanol-induced gial activation and induction of inflammatory mediators, Tlr4 knock-out (KO) mice did not differ from wild-type (WT) mice in chronic ethanol consumption (Alfonso-Loeches et al., 2010; Pascual et al., 2011). The TLR4 antagonist T342126 decreased drinking and the density of a microglial protein in the CeA in ethanol-dependent and nondependent mice, but the decreased drinking was apparently due to nonspecific effects (Bajo et al., 2016). Overall, there is strong evidence for activation of TLR4 and other neuroimmune mediators in chronic ethanol exposure, but a direct causal link between TLR4 and alcohol drinking behavior has not been shown.

Members of the Integrative Neuroscience Initiative on Alcoholism (INIA-Neuroimmune) consortium (http://www.inia-west.org/) participated in extensive multidisciplinary studies to test the hypothesis that TLR4 is directly involved in ethanol drinking and other ethanol-related responses. TLR4 signaling was inhibited using the following: (1) null mutant rats lacking the Tlr4 gene, (2) selective knockdown of Tlr4 mRNA in the nucleus accumbens (NAC) of mice using Cre recombinase-mediated Tlr4 gene excision, and (3) injection of the selective TLR4 antagonist (+)-naxalone in C57BL/6j mice. None of these approaches provided consistent evidence that TLR4 regulates ethanol intake in any drinking test in mice or rats. However, there was evidence that TLR4 mediates the acute sedative effects of ethanol. Electrophysiological studies revealed that genetic deletion of Tlr4 did not alter spontaneous GABAergic release in rat CeA neurons, but altered the kinetics of GABA<sub>A</sub> receptor-mediated IPSCs.

Materials and Methods

Animals

Rats. A rat line on a Wistar background harboring a nonfunctional Tlr4 gene was produced using transcription activator-like effector nucleosome mediated mutagenesis as described previously (Ferguson et al., 2013). This line carries a 13-base pair frame shift mutation in Tlr4 exon 1 and has a dramatically attenuated response to LPS challenge (Ferguson et al., 2013). Heterozygous breeding pairs were produced at the University of Pittsburgh (Pittsburgh, PA) and shipped to The Scripps Research Institute (La Jolla, CA) and the Indiana University School of Medicine (Indianapolis). WT control and homzygous KO (Tlr4<sup>−/−</sup>) littermates produced from these heterozygous pairs were used at the respective institutions. Offspring were weaned at ∼21 d of age and genotyped as described previously (Ferguson et al., 2013).

Mice. Male 12-week-old C57BL/6j mice (The Jackson Laboratory) were used to investigate the effects of (+)-naxalone on ethanol drinking. Mice were housed in groups of four before the drinking tests. Lights were on a reverse 12 h light/12 h dark cycle (lights on at 8:00 P.M.). These studies were conducted at The Scripps Research Institute.

Adult male mice with a floxed Tlr4 gene (i.e., mice with Tlr4 flanked by LoxP sites, denoted as Tlr4<sup>fl/fl</sup>) were generated on a C57BL/6 background as described previously (Sodhi et al., 2012) and used for knockdown studies in the NAc. Original breeders were produced at the University of Pittsburgh and mating pairs were shipped to The University of Texas at Austin. Mice were group-housed 4–5 to a cage on a 12 h light/12 h dark cycle (lights on at 7:00 A.M.). Ethanol consumption tests began when the mice were at least 2 months old.

Rats and mice at the different institutions were housed in temperature- and humidity-controlled vivaria with water and food provided <i>ad libitum</i>. All vivaria are approved by the Institutional Animal Care and Use Committee at each site. Animals were provided by the Association for Assessment and Accreditation of Laboratory Animal Care. All procedures adhered to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committees at each site.

LPS injections

Ethanol-naive adult male Tlr4 KO and WT rats (<i>n</i> = 4 per genotype) weighing 479–604 g at the beginning of the experiment were housed individually on a 12 h light/12 h dark cycle (lights on at 6:00 A.M.). Baseline body weight and food and water consumption were measured 24 h before LPS injection. Rats were injected intraperitoneally with 1 mg/kg LPS (L3024, Escherichia coli 0111:B4; Sigma-Aldrich) dissolved in saline 30 min before the start of the dark cycle. Immediately before injection and subsequently every 12 h for 7 d, body weight and food and water intake were measured. Seven to 10 d after a single LPS injection, in <i>vitro</i> electrophysiological experiments were performed to assess the acute ethanol effects on CeA GABAergic transmission.

For LPS effects on operant ethanol self-administration, adult male Tlr4 KO and WT rats (<i>n</i> = 5 per genotype) weighing 350–375 g at the beginning of the experiment were housed in groups of two per cage in a temperature-controlled (22°C) vivarium on a 12 h light/12 h reversed dark-light cycle (lights on at 10:00 P.M.). After completing operant ethanol self-administration training (described below), the rats were housed individually, injected with LPS (1 mg/kg, i.p.), and body weight and food/water intake were recorded daily. Ethanol and water self-
Ethanol intake tests in rats

**Two-bottle choice (2BC).** Rats were double-housed by sex and genotype (Tlr4 KO vs WT). Lights were on a reverse 12 h light/12 h dark cycle (lights on at 10:30 P.M.). On postnatal day 85, male and female rats were transferred from their home cages and singly housed in hanging stainless steel wire-mesh cages for habituation to this environment. Male and female litters, offspring of each genotype were tested using the 2BC ethanol consumption test beginning on postnatal day 90. Rats were given *ad libitum* choice access to 10% ethanol (v/v) and water for 5 weeks. Ethanol (grams per kilogram body weight) and water (milliliters per kilogram body weight) consumption were assessed 5 d/week and body weight (kilograms) was measured 3 d/week. Ethanol intake was converted to grams of absolute ethanol consumed/kilogram of body weight/day. Due to daily fluctuations in ethanol consumption in all groups, the data for each day were averaged across each week of access. The weekly data were submitted to a 3 × 2 × 5 (genotype by sex by week) mixed-model ANOVA, with genotype and sex as the between-subjects factors and week as the within-subjects factor.

**Operant self-administration.** Adult male Tlr4 KO (n = 12) and WT (n = 13) rats weighing 250–350 g at the beginning of the experiment were housed in groups of two per cage in a temperature-controlled (22°C) room on a reverse 12 h light/12 h dark cycle and the behavioral tests were conducted during the dark phase of the circadian cycle. Self-administration sessions were conducted in standard operant conditioning chambers (Med Associates). Animals were first trained to self-administer 10% ethanol (w/v) and water solutions until stable responses were maintained. The rats were subjected to an overnight session in the operant chambers with access to 1 lever (right lever) that delivered water [*fixed ratio 1 (FR1) schedule of reinforcement, i.e., each operant reversion chamber with access to 1 lever (right lever) that delivered water were maintained.* The rats were subjected to an overnight session in the operation and herein was considered a measure of intake. Ethanol self-administration data were analyzed by two-way repeated-measures ANOVA with group (Tlr4 KO vs WT) as the between-subjects factor and session as the within-subjects factor. Because the same rats were used to measure predendependent versus dependent ethanol self-administration, the number of lever presses for ethanol (last six sessions before ethanol vapor exposure) were averaged and compared with the average lever presses across six self-administration sessions during ethanol vapor exposure. Data were analyzed using two-way mixed models ANOVA with group as the between-subjects factor and time (predependent vs dependent) as the within-subjects factor.

**Ethanol intake tests in mice**

**Drinking-in-the-dark (DID).** The DID procedure is a binge-like drinking model, which capitalizes on the circadian rhythm in drinking and uses a discrete time of exposure to ethanol to optimize pharmacological and significant ethanol drinking (Rhodes et al., 2005; Rhodes et al., 2007; Thiele et al., 2014). Three factors are important in this model: (1) providing access to ethanol a few hours into the dark cycle, (2) not allowing access to water when ethanol is offered, and (3) using genetically predisposed mice. BECs in C57BL/6J mice in this model are reliably > 100 mg% after the final drinking period and motor impairments are detected using the accelerating rotarod and balance beam tests (Rhodes et al., 2005, 2007). C57BL/6J mice were individually housed 1 week before testing. On day 1, starting 3 h after lights off (11:00 A.M.), water bottles were replaced with drinking tubes containing 20% ethanol in tap water for 2 h. On day 4, the ethanol bottles remained for 4 h, with intakes recorded after both 2 and 4 h. Immediately after removal of the ethanol bottles, tail blood was sampled to measure BEC. On day 4, mice were injected with (+)-naloxone (0, 30, or 60 mg/kg; n = 8 per group) 30 min before the drinking session began (10:30 A.M.) to investigate its acute effects. The following week, saline or 60 mg/kg (+)-naloxone (n = 8 per group) was injected on all 4 d of DID testing 30 min before the drinking sessions to examine subchronic effects. (+)-Naloxone was synthesized by E.R. and freshly dissolved in saline and administered intraperitonially at 0.1 ml/g body weight. The μ-opioid inactive stereoisomer, (+)-naloxone, is a selective TLR4 inhibitor (Hutchinson et al., 2008; Wang et al., 2016). Data were analyzed separately at the 2 and 4 h time points using one-way ANOVA with (+)-naloxone dose as the between-subjects factor.

**CIE-2BC.** In the CIE-2BC model, excessive ethanol consumption is observed after 2BC limited-access periods are cycled with chronic passive exposure to ethanol vapor in rats (Vendruscolo and Roberts, 2014) or C57BL/6J mice (Becker and Lopez, 2004; Finn et al., 2007; Griffin et al., 2009). This has been used to model the motivational aspects of alcohol dependence and the excessive drinking associated with the addicted state. For 15 d (5 per week for 3 weeks), 30 min before the lights turn off (7:30 A.M.), C57BL/6J mice were individually housed for 2 h with access to 2 drinking tubes containing either 15% ethanol or water. Ethanol and water consumption during these 2 h periods were recorded. After this baseline period, mice were divided, based on equal ethanol and water consumptions, into two balanced groups to be exposed to CIE or air in identical vapor chambers. After baseline 2BC drinking, the CIE group was injected with 1.75 g/kg ethanol plus 68 mg/kg pyrazole (alcohol dehydrogenase inhibitor) and placed in the chambers to receive intermittent vapor for 4 d (vapor on for 16 h and off for 8 h). After each 16 h period of vapor, mice were removed and, on the second and fourth days, tail blood was sampled to determine BEC. Target BECs were 175–250 mg%. After the fourth day of exposure, mice were allowed 72 h of undisturbed time and were then given 5 d of 2 h access to bottles containing 15% ethanol and water to measure ethanol drinking and preference after vapor exposure. The control group was injected with 68 mg/kg pyrazole in saline and placed in chambers delivering air for the same periods as the CIE group and received 2BC testing at the same time as the ethanol vapor group. The vapor/air exposure and 5 d of 2BC testing were repeated for a total of 4 rounds of vapor and 2BC testing before (+)-naloxone testing. For the first study, 30 mice (n = 15 per group) were administered (+)-
naloxone (0, 3, 10, 30, 60 mg/kg) in a within-subjects manner 30 min before 2BC testing (7:00 A.M.). Drug injections were given every 3–4 d. For the second study, 20 mice were used in a between-subject design (n = 4–6 in 4 groups: control-vehicle, control-60 mg/kg (+)-naloxone, CIE-vehicle, CIE-60 mg/kg (+)-naloxone). Mice were injected 30 min before the 2BC procedure across 4 d of testing. Data from the first study were analyzed by two-way repeated-measures ANOVA (group as the between factor; dose as the within factor) and data from the second study were analyzed by three-way repeated-measures ANOVA (group and dose as the between factors; day as the within factor). Significant effects were further investigated using Fisher’s protected (P)LSD tests.

2BC (24 h continuous access). Four weeks after lentiviral Cre-recombinase injections in the NAc to knock down Tlr4 selectively (see below), a 2BC protocol was performed as described previously (Blednov et al., 2003). Tlr4FF male mice were allowed to acclimate for 1 week to individual housing. Two drinking bottles (containing water and ethanol) were available continuously to each mouse and bottles were weighed daily. Bottle positions were changed daily to control for position preferences. Mice were weighed every 4 d. After 4 d of water consumption (both bottles contained water), mice were offered 3% ethanol (v/v) versus water for 4 d. The amount of ethanol consumed (g/kg body weight per 24 h) was calculated for each mouse and these values were averaged for every concentration of ethanol. Immediately after 3% ethanol and water was offered for 2 d, followed by 8%, 10%, 12%, 14%, and finally 16% (v/v) ethanol (each concentration was offered for 4 d). Throughout the experiment, evaporation/spillage estimates were calculated daily using two bottles (containing water or the appropriate ethanol solution) placed in an empty cage. Data were analyzed by two-way repeated-measures ANOVA followed by Bonferroni post hoc tests.

2BC (24 h intermittent access). Intermittent (every other day) access to ethanol increases voluntary ethanol consumption in mice (Melendez, 2011). This drinking paradigm was tested in a different group of Tlr4FF male mice 4 weeks after lentivirus injections in the NAc. Mice were given access to a bottle of 15% (v/v) ethanol and a bottle of water during 24 h sessions every other day. The placement of the ethanol bottles was alternated with each drinking session to control for side preferences. The quantity of ethanol consumed was calculated as grams per kilogram body weight per 24 h. Evaporation/spillage estimates were calculated daily from two bottles (containing water or 15% ethanol solution) placed in an empty cage. Data were analyzed by two-way repeated-measures ANOVA followed by Bonferroni post hoc tests.

Acute behavioral effects

Ethanol-induced loss of righting reflex (LORR). Adult male Tlr4 KO and WT rats (n = 10 per genotype) weighing 350–400 g were housed in groups of two per cage. Each rat was handled 5 min/d for 1 week before beginning the experiment. On test day, the rats were injected with 4 g/kg ethanol (20% v/v in saline, i.p.). After injection, the rat was placed in a supine position on a V-shaped platform. The time to LORR (time between the injection and the moment in which the rat is not able to right itself) and the time of LORR (interval between the loss of ability to flip over onto all four limbs when placed in a supine position and the time when this reflex was successfully demonstrated three consecutive times within 60 s) were scored. Data were analyzed using an unpaired t test.

Selective knockdown of TLR4 in mouse NAc

Lentivirus-mediated knockdown of Tlr4. Tlr4FF male mice received bilateral injections into the NAc with either a vesicular stomatitis virus (VSV-G) pseudotyped lentivirus expressing Cre recombinase fused to an enhanced green fluorescent protein (EGFP) under the control of a cytomegalovirus (CMV) promoter (LV-Cre-EGFP) or an “empty” VSV-G pseudotyped lentivirus vector expressing only the EGFP transgene under a CMV promoter (LV-EGFP-Empty). Virus was produced as described previously (Lasek et al., 2007). Mice were anesthetized by isoflurane inhalation, placed in a model 1900 stereotaxic apparatus (Kopf Instruments), and administered preoperative analgesic (Rimadyl, 5 mg/kg). The skull was exposed and bregma and lambda visualized with a dissecting microscope. A digitizer attached to the micromanipulator of the stereotaxic apparatus was used to locate coordinates relative to bregma. Burr holes were drilled bilaterally above the injection sites in the skull using a drill equipped with a #75 carbide bit (Kopf Instruments). The injection sites targeted the NAc using the following coordinates relative to bregma: anteroposterior (AP) +1.49 mm, mediolateral (ML) ±0.9 mm, and dorsoventral (DV) −4.8 mm. Injections were performed using a Hamilton 10 μl microsyringe (model 1701) and a 30-gauge needle. The needle tip was lowered to the DV coordinate and retracted 0.2 mm. The

Figure 1. Tlr4 KO rats are resistant to LPS-induced toxicity. LPS produced transient reduction of food (A) and water (B) intake in male WT but not Tlr4 KO rats. The data are presented as mean ± SEM food and water intake (in grams) in 24 h periods. Day 0 represents intake measured during the 24 h before LPS (1 mg/kg, i.p.) injection. C, LPS induced a robust weight loss in WT but not Tlr4 KO rats. The data are presented as percentage change relative to preinjection baseline weight (error bars are obscured by the symbols). Data were analyzed by two-way repeated-measures ANOVA followed by Bonferroni post hoc tests. *p < 0.05, **p < 0.01, ***p < 0.001 compared with baseline; n = 4 per genotype. Arrows indicate injection of LPS (1 mg/kg, i.p.).
viral solution (1.0 μl with titer of $-3 \times 10^7$ pg gag antigen/ml in PBS, PBS) was injected into each site at a rate of 200 nl per min. After each injection, the syringe was left in place for 5 min before being retracted over a period of 3 min. Incisions were closed with tissue adhesive (Vetbond; 3M). Mice were housed individually after surgery and given a 2-week recovery period before starting the ethanol drinking tests. Separate groups of mice were used for each 2BC test.

**RNA isolation.** Approximately half of the treated **Tlr4** KO mice were killed within 24 h of completing the drinking tests. The brains were quickly removed, flash frozen in liquid nitrogen, and later frozen in optimal cutting temperature medium (VWR) in isopentane on dry ice and stored at −80°C. Brains were transferred to a cryostat set at −25°C for at least 1 h before sectioning. Sections (300 μm) were collected from +1.80 to +0.60 mm (AP) relative to bregma and transferred to glass slides that had been precooled on dry ice. Micropunch sampling was performed on a frozen stage at −25°C using a dual fluorescent protein flashlight (Nightsea) and the stereotaxic atlas of Paxinos and Franklin (2004) to confirm EGFP expression and the anatomical location of the injection site, respectively. Microdissection punches (Stoelting) with an inner diameter of 0.75 mm were used to obtain samples of NAc core and shell. This inner diameter corresponded to the viral spread around the injection site and minimized contamination from other tissue. Punches were taken bilaterally from four 300 μm sections and stored at −80°C until RNA extraction. All equipment and surfaces involved in the collection of tissue were treated with 100% ethanol and RNaseZap (Life Technologies) to prevent RNA degradation. Total RNA was extracted using the MagMAX-96 for Microarrays Total RNA Isolation Kit (Life Technologies) according to the manufacturer’s instructions. Yields and purity were assessed using a NanoDrop 8000 (Thermo Fisher Scientific), and quality of the total RNA preparation was determined using the Agilent 2200 TapeStation.

**Quantitative real-time PCR.** To verify **Tlr4** mRNA knockdown, single-stranded cDNA was synthesized from total RNA using the TaqMan High Capacity RNA-to-cDNA kit (Life Technologies). After reverse transcription, quantitative real-time PCR (qPCR) was performed in triplicate using TaqMan Gene Expression Assays together with the TaqMan Gene Expression Master Mix (Life Technologies) per the manufacturer’s instructions. The TaqMan Gene Expression assays used were **Tlr4** (Mm00445273_m1) and enhanced GFP (Mm04972299_m1). **Gapdh** (Mm99999915_g1) was used as a reference gene and relative mRNA levels were determined using the 2−ΔΔCT method (Schmittgen and Livak, 2008). **Gapdh** was used as the endogenous control because of its stable expression across samples. Reactions were performed in a CFX384 Real-Time PCR Detection System (Bio-Rad) and data collected using Bio-Rad CFX Manager.

**Target site verification.** The other half of the **Tlr4** KO mice were killed within 24 h of completing the drinking tests and transcardially perfused with PBS, pH 7.4, followed by 4% paraformaldehyde (PFA). Brains were harvested, postfixed for 24 h in 4% PFA at 4°C, and cryoprotected for 24 h in 20% sucrose in PBS at 4°C. Brains were placed in molds containing optimal cutting temperature compound (VWR) and frozen in isopentane on dry ice. The brains were equilibrated in a −12 to −14°C cryostat (Thermo Fisher Scientific) for at least 1 h and coronal 40 μm sections of the NAc were placed in sterile PBS. Serial sections (40 μm) of NAc (AP +2.00 to 0.00 mm) were mounted on slides with DAPII mounting medium (Vector Laboratories) and visualized using a Zeiss Axiocvert 200M Fluorescent Microscope equipped with a 10× objective to assess the location of the injection site. Quality of injection was scored quantitatively based on the strength of EGFP viral expression, injection location relative to target, and the spread of the virus. The injection was considered on target if the virus covered at least one-third of the NAc.

**Whole-cell voltage-clamp electrophysiology.**

**Slice preparation.** Male WT and **Tlr4** KO rats (used previously for operant self-administration testing) were made ethanol dependent by CIE vapor...
Electrophysiological recording. Medial subdivision CeA neurons exposed to ethanol vapor exposure. The data were presented as mean ± SEM lever presses during 30 min sessions. Data were analyzed by two-way repeated-measures ANOVA with group as the between-subjects factor and session as the within-subjects factor.

B. Male WT and Tlr4 KO rats do not differ in ethanol (10%) self-administration after ethanol vapor exposure. The data were presented as mean ± SEM lever presses during 30 min sessions. Data were analyzed using two-way repeated-measures ANOVA with group as the between-subjects factor and session as the within-subjects factor.

Figure 4. There was no effect of genotype on ethanol self-administration in WT or Tlr4 KO rats before or after dependence. A, Male WT and Tlr4 KO rats do not differ in ethanol (10%) self-administration before ethanol vapor exposure. The data were presented as mean ± SEM lever presses during 30 min sessions. Data were analyzed by two-way repeated-measures ANOVA with group as the between-subjects factor and session as the within-subjects factor. B, Male WT and Tlr4 KO rats do not differ in ethanol (10%) self-administration after CIE exposure. Ethanol-dependent rats underwent cycles of 14 h on (BEC: 146.8 ± 3.6 mg/dl) and 10 h off. The same rats were used to measure predependent versus postdependent ethanol self-administration. For baseline, the number of lever presses for ethanol (last six sessions before ethanol vapor exposure) was averaged and compared with the average number of lever presses over six self-administration sessions during CIE exposure. The data are presented as mean ± SEM lever presses during 30 min sessions. Data were analyzed using two-way ANOVA with group as the between-subjects factor and time (predependent vs dependent) as the within-subjects factor (n = 8 for WT and n = 7 for KO, *p < 0.05 compared with baseline).

Results

Lack of LPS effects in Tlr4 KO rats

Tlr4 KO ethanol-naive rats are resistant to LPS-induced toxicity. We monitored reductions in food intake (anorexia), water intake (adipsia), and body weight as signs of LPS-induced “sickness-like” behavior (McCarthy et al., 1984; Cross-Mellor et al., 2000; van Heesch et al., 2013) in naive and ethanol self-administering Tlr4 KO and WT rats. Baseline measures of body weight or food and water consumption did not differ in KO and WT rats. In naive rats, LPS (1 mg/kg, i.p.) caused a transient reduction of food intake in WT, but not Tlr4 KO rats (Fig. 1A). There were significant main effects of LPS (F(1,42) = 60.7, p < 0.0001) and genotype (F(1,42) = 13.7, p < 0.001) on food intake and an interaction between the factors (F(1,42) = 30.2, p < 0.001). Profound anorexia occurred within 24 h after injection in WT rats, which persisted for at least 48 h (28.1 ± 2.0 g consumed 24 h before LPS vs 1.2 ± 0.8 g after LPS). WT rats gradually increased their food intake, reaching pre-LPS consumption levels by the sixth postinjection day (25.8 ± 3.4 g). In Tlr4 KO rats, LPS caused a transient reduction in food intake (from 27.6 ± 3.7 g to 22.9 ± 3.2 g) after 24 h, followed by increased food intake on the fourth postinjection day (34.2 ± 1.5 g).
LPS reduced water intake in WT rats only (Fig. 1B); this effect peaked on the second postinjection day, with drinking levels returning to baseline by the fourth postinjection day. There were main effects of LPS on water intake \((F_{(7,42)} = 7.1, p < 0.001)\) and a significant interaction between LPS and genotype \((F_{(7,42)} = 4.8, p < 0.001)\), but the main effect of genotype did not reach significance \((p = 0.05)\). Reductions in food and water intake in WT rats were accompanied by significant weight loss, a pattern not observed in KO rats. LPS produced weight loss in WT rats during the first 3 postinjection days, reaching a peak loss of 10% body weight \((F_{(7,42)} = 88.2, p < 0.001)\) and genotype \((F_{(7,42)} = 79.6, p < 0.001)\) and an interaction between the factors \((F_{(7,42)} = 73.6, p < 0.001)\). Body weight then stabilized and the resumption of food intake was associated with the reappearance of normal diurnal fluctuations in body weight when measured every 12 h, similar to Tlr4 KO rats (data not shown). Although a slight reduction in body weight was also observed in the 24–48 h after LPS injection in Tlr4 KO rats, its magnitude was <25% of that observed in WT \((-2.5%\) absolute weight loss). The resistance of Tlr4 KO rats to LPS-induced “sickness-like behavior” provides behavioral and functional validation that the Tlr4 gene had been deleted.

**Tlr4 KO rats trained to self-administer ethanol are resistant to LPS-induced toxicity**

Similarly to naive rats, there were no significant differences in baseline body weight or water and food intake in the WT and Tlr4 KO rats trained to self-administer 10% ethanol. LPS injections (14 d apart) significantly \((p < 0.001)\) reduced food intake in WT but not KO ethanol-trained rats (Fig. 2A). Two-way repeated-measures ANOVA showed main effects of LPS \((F_{(4,32)} = 63.9, p < 0.001)\) and genotype \((F_{(1,32)} = 8.4, p < 0.05)\) on food intake, as well as an interaction between the factors \((F_{(4,32)} = 43.8, p < 0.001)\). Although water intake was reduced after both LPS injections in WT rats, the intake over the 4 d period after LPS was reduced significantly only after the first injection (Fig. 2B), suggesting the development of tolerance to its effects. There were no effects of LPS injections on water intake in Tlr4 KO rats. There were main effects of LPS on water consumption \((F_{(4,32)} = 5.1, p < 0.001)\) and an interaction between LPS and genotype \((F_{(4,32)} = 4.0, p < 0.001)\) without a main effect of genotype.

In WT rats, two LPS injections induced a significant loss in body weight measured over a 4 d period after each injection (Fig. 2C). Two-way repeated-measures ANOVA showed main effects of LPS \((F_{(4,32)} = 35.8, p < 0.001)\) and genotype \((F_{(1,32)} = 68.4, p < 0.001)\) and an interaction between the factors \((F_{(4,36)} = 17.1, p < 0.001)\). LPS injections had no effect on the body weight of Tlr4 KO rats (Fig. 2C). Because there was not a full recovery in body weight after the first LPS injection in WT rats, we calculated LPS-induced loss in body weight by comparing the weight during days 1–4 after injection to the weight at the time of the most recent injection. The second LPS injection induced a significantly \((p < 0.05)\) less pronounced reduction of body weight \((-3.4\pm0.5%\) after the second LPS injection vs \(-7.1\pm0.7%\) after the first injection) and faster recovery in WT rats (Fig. 2D), suggesting the development of tolerance to its effects.

**LPS induces a transient reduction in ethanol self-administration in WT, but not Tlr4 KO rats**

At the end of self-administration training, the baseline lever presses for 10% ethanol were 19.8 ± 6.6 for WT and 13.3 ± 4.1 for KO rats. LPS (1 mg/kg, i.p.) was injected 30 min before the next session and rats were tested for ethanol and water self-administration in 30 min sessions for 14 consecutive days, followed by a second LPS injection and 14 more days of testing. Data after both LPS injections were averaged. A mixed-factorial ANOVA with genotype (Tlr4 KO vs WT) as the between-subjects factor and time point (baseline, 1–4 d post-LPS, and 5–14 d post-LPS) as the within-subjects factor showed a significant effect of time point \((F_{(1,8)} = 7.5; p < 0.01)\) and genotype × time point interaction \((F_{(2,8)} = 5.6; p < 0.05)\). A Newman–Keuls post hoc test showed that LPS treatment reduced operant responding for ethanol in the WT group \((p < 0.05)\) for 4 consecutive days after the injections (Fig. 3A). No genotype differences were found 5–14 d after LPS injections. A mixed-factorial ANOVA of the water self-administration data showed a significant effect of time point \((F_{(1,8)} = 7.5; p < 0.01)\), but no difference in the genotype × time point interaction (Fig. 3B).

**Lack of evidence for a role of TLR4 in ethanol consumption**

No genotype differences in ethanol self-administration by WT or Tlr4 KO rats before or after dependence

Although there was no significant genotype effect on baseline responses in the ethanol self-administration test above, we compared operant responding before and after ethanol dependence specifically...
in both genotypes. Figure 4A shows ethanol self-administration in WT and Tlr4 KO rats before ethanol vapor exposure (precondition). Operant self-administration of 10% ethanol varied over time (session effect: \( F_{(1,14)} = 3.3, p < 0.0005 \), but there were no genotypic or genotype \( \times \) time differences. After CIE vapor exposure (after dependence), there were also no differences in self-administration, with both WT and Tlr4 KO rats showing similar increases in intake (\( F_{(1,13)} = 12.3, p < 0.005; \) Fig. 4B).

2BC ethanol intake in male and female WT vs Tlr4 KO rats

We then compared sexes and genotypes in a 2BC chronic drinking paradigm (WT: males, \( n = 23 \); females, \( n = 16 \); Tlr4 KO: males, \( n = 15 \); females, \( n = 11 \)). The omnibus mixed ANOVA of the ethanol-drinking data revealed a marginally significant 3-way (genotype \( \times \) sex \( \times \) week) interaction for 10% ethanol (v/v) intake (\( F_{(4,244)} = 2.4, p = 0.05 \)). There was a significant two-way (sex \( \times \) week) interaction (\( F_{(4,244)} = 3.3, p = 0.01 \) and a marginally significant main effect of sex (\( F_{(1,61)} = 4.0, p = 0.05 \)). As seen in Figure 5A, female rats drank more ethanol initially than male rats. This effect was observed for the Tlr4 KO rats only during the first week of ethanol access (\( F_{(1,25)} = 11.7, p < 0.01 \), whereas the WT rats displayed this effect across the first 3 weeks of ethanol access (\( F_{(1,38)} > 4.5, p < 0.05 \)). There were also changes in ethanol intake across weeks: specifically, WT male rats displayed significant (\( p < 0.05 \)) increases between week 1 versus week 4 and week 1 versus week 5, whereas Tlr4 KO male rats displayed significant (\( p < 0.05 \)) increases between week 1 versus week 2 and week 1 versus week 5. There was a significant decrease in ethanol intake by female Tlr4 KO rats between weeks 1 and 2. The omnibus mixed ANOVA of the water-drinking data revealed a significant two-way (sex \( \times \) week) interaction (\( F_{(4,244)} = 8.5, p < 0.001 \)) and a significant main effect of week (\( F_{(2,242)} = 6.7, p < 0.001 \)). As shown in Figure 5B, male rats initially drank more water than females, with the effect in drinking significance (\( p < 0.05 \)) in the WT, but not Tlr4 KO rats during week 1. In addition, a modest but significant (\( p < 0.05 \)) increase across weeks was observed in both the female Tlr4 KO (week 1 vs week 4 and week 1 vs week 5) and WT (week 1 vs week 3, week 1 vs week 4, and week 1 vs week 5) rats. The omnibus mixed ANOVA of the ethanol preference data revealed a significant two-way (sex \( \times \) week) interaction (\( F_{(4,244)} = 12.6, p < 0.001 \)) and significant main effects of week (\( F_{(4,244)} = 2.9, p < 0.05 \)) and sex (\( F_{(1,61)} = 22.4, p < 0.001 \)). However, the main effect of genotype regarding ethanol preference did not reach significance (\( p = 0.06 \)). Female WT and KO rats displayed decreased ethanol preference across weeks 4–5 and 3–5, respectively, compared with the first week (Fig. 5C). The omnibus mixed ANOVA of the body weight data revealed a significant two-way (sex \( \times \) week) interaction (\( F_{(4,244)} = 8.5, p < 0.001 \), along with significant main effects of week (\( F_{(4,244)} = 6.7, p < 0.001 \)) and sex (\( F_{(1,61)} = 505.5, p < 0.001 \)). As seen in Figure 5D, female rats weighed significantly less than male rats, with no differences observed for genotype.

No significant effect of (+)-naloxone on DID in C57BL/6J mice

We examined the effect of pharmacological inhibition of TLR4 signaling in mice using a binge-like drinking model. (+)-Naloxone treatment did not alter ethanol (20%) intake (Fig. 6A) or BEC measured on drinking day 4 of the DID test. There was no overall effect of (+)-naloxone at 2 h (\( F_{(2,21)} = 1.4, p = 0.3 \)) or 4 h (\( F_{(2,21)} = 0.1, p = 0.9 \)), nor was there an effect on BEC measured at 4 h (\( F_{(2,21)} = 0.4, p = 0.7 \)). BECs for vehicle-treated, 30 mg/kg (+)-naloxone-treated, and 60 mg/kg (+)-naloxone-treated mice were 73.6 \( \pm \) 13.7, 80.8 \( \pm \) 19.8, and 58.2 \( \pm \) 19.7 mg/dl, respectively. Administration of 60 mg/kg (+)-naloxone over 4 d also had no significant effect on DID (Fig. 6B) or on BEC. There was a trend toward reduction of drinking by (+)-naloxone at 2 h (\( F_{(1,14)} = 3.1, p = 0.1 \), but not at 4 h (\( F_{(1,14)} = 1.5, p = 0.2 \)). BECs measured at 4 h for vehicle- and 60 mg/kg (+)-naloxone-treated mice were 71.9 \( \pm \) 21.2 and 41.9 \( \pm \) 5.6 mg/dl, respectively, but the effect was not significant (\( F_{(1,14)} = 1.9, p = 0.2 \)).

Effect of (+)-naloxone on 2BC and CIE-2BC drinking in C57BL/6J mice

We then tested the effect of individual injections of (+)-naloxone (3–60 mg/kg) in mice under conditions of 2BC excessive drinking after CIE exposure. BECs were 186.6 \( \pm \) 7.2, 149.6 \( \pm \) 10.7,
174.8 ± 9.8, and 176.6 ± 9.0 mg/dl across the four cycles of vapor exposure. Increased ethanol (15%) drinking over 2 h was observed in the CIE-2BC group compared with the control 2BC group (Fig. 7A). An overall within-subjects ANOVA revealed a significant effect of group \( (F(1,28) = 30.5, p < 0.0001) \) and a group \( \times \) dose interaction \( (F(4,112) = 3.2, p < 0.05) \). Further analysis revealed that this interaction was due to the decrease in ethanol intake by 60 mg/kg (+)-naloxone compared with vehicle \( (p < 0.01) \) and compared with 3 mg/kg \( (p < 0.05) \), 10 mg/kg \( (p < 0.01) \), and 30 mg/kg \( (p < 0.01) \) (+)-naloxone in the CIE group. There was no effect of (+)-naloxone in the control 2BC group. To further explore the high dose, we treated separate groups of control 2BC and CIE-2BC mice with 60 mg/kg (+)-naloxone for 4 d (Fig. 7B). There was a significant difference between 2BC and CIE-2BC mice in baseline drinking \( (F(1,16) = 23.2, p < 0.001) \) after vapor exposure. These groups were each

Figure 8. Effect of Tlr4 knockdown in NAc on ethanol intake in the continuous and intermittent access 2BC tests in Tlr4<sup>F/F</sup> male mice. A–C, Ethanol intake (g/kg/24 h) (A), preference for ethanol (B), and total fluid intake (g/kg/24 h) (C) in the 2BC continuous access test in untreated control Tlr4<sup>F/F</sup> mice (n = 10) and Tlr4<sup>F/F</sup> mice injected with LV-Cre-EGFP (n = 20) or LV-Cre-Empty (n = 10). D–F, Ethanol (15%) intake (g/kg/24 h) (D), preference for ethanol (E), and total fluid intake (g/kg/24 h) (F) in the 2BC intermittent access test in untreated control Tlr4<sup>F/F</sup> mice (n = 10) and Tlr4<sup>F/F</sup> mice injected with LV-Cre-EGFP (n = 20) or LV-Cre-Empty (n = 11). Each point is the average of 2 d of drinking. The data are presented as mean ± SEM. Data were analyzed by two-way repeated-measures ANOVA.

Figure 9. Verification of injection site after Tlr4 mRNA knockdown in NAc. A, Composite microscope image of a coronal section of the NAc after lentivirus injection using fluorescent microscopy (left) to show EGFP marker signal (green) and bright-field (right) to demonstrate neuroanatomy. B, Coronal brain atlas diagram of the NAc injection site with blue circles showing the NAc and green ovals illustrating the typical lentivirus location and spread.
divided into two subgroups to receive (+)-naloxone or vehicle based on equal drinking averages and equal BECs in the CIE-2BC groups. Vapor chamber BECs for the vehicle-treated mice were 165.7 ± 12.5, 176.6 ± 3.2, 176.6 ± 16.8, and 214.9 ± 20.0 mg/dl and BECs for the 60 mg/kg (+)-naloxone-treated mice were 170.7 ± 14.1, 187.9 ± 9.6, 191.3 ± 9.1, and 214.1 ± 14.4 mg/dl across the 4 cycles of exposure. Although the CIE effect was significant ($F_{(1, 16)} = 30.2, p < 0.001$), there was no significant treatment effect ($F_{(1, 16)} = 0.0005, p = 1.0$) or group × treatment interaction ($F_{(1, 16)} = 0.2, p = 0.6$). Within-dose comparisons across injections also revealed no overall significant effects, but if each treatment day is compared with baseline, there was a significant effect of 60 mg/kg (+)-naloxone on day 3 ($p = 0.03$). However, drinking did not differ from vehicle-treated mice on this (or any other) day. These results support a modest but inconsistent effect of 60 mg/kg (+)-naloxone on ethanol drinking in dependent mice.

**Effect of Tlr4 mRNA knockdown in mouse NAc on 2BC drinking**

Selective knockdown of TLR4 signaling in the CeA or VTA (but not the VP) decreased binge-like drinking in P rats (Liu et al., 2011; June et al., 2015). In the current study, we examined Tlr4 knockdown in mouse NAc, another important brain region in binge drinking. However, knockdown in the NAc did not alter ethanol intake, preference for ethanol, or total fluid intake in the 24 h continuous access 2BC test in which Tlr4$^{EGFP}$ treated and untreated mice could drink either water or a series of increasing ethanol (3–16%) concentrations (Fig. 8A–C).

In the 2BC test with intermittent access to 15% ethanol, comparison of Tlr4$^{EGFP}$ treated (LV-EGFP-Cre; LV-EGFP-Empty) and control (untreated) mice revealed a significant treatment × day interaction for ethanol consumption ($F_{(6, 172)} = 2.3; p < 0.05$), but not for ethanol preference or total fluid intake (Fig. 8D–F). Two-way repeated-measures ANOVA showed no main effect of treatment in any test. Post hoc analyses for each day when corrected for multiple comparisons also showed no significant differences between LV-EGFP-Cre versus LV-EGFP-Empty treated mice or between LV-EGFP-Cre treated versus control untreated mice. Although there was a trend toward increased alcohol consumption in the intermittent test after Tlr4 mRNA knockdown in NAc, this was not statistically significant.

**Verification of Tlr4 knockdown in NAc**

Verification of successful knockdown of Tlr4 mRNA in the NAc involved a two-step process: (1) confirmation of needle placement and (2) quantitation of Tlr4 mRNA. Half of the treated (LV-EGFP-Cre, LV-EGFP-Empty) Tlr4$^{EGFP}$ mice were used to verify injection coordinates. Figure 9A is a representative image of a coronal section of the NAc (AP +1.49 mm) from a lentivirus-treated Tlr4$^{EGFP}$ mouse. The left side is a fluorescent image that shows the EGFP signal (surrogate marker for lentivirus transduction) in green and DAPI (a stain that visualizes the nuclei of all cells) in blue. The right side of the image is a bright-field image used to better visualize the neuroanatomical landmarks. Figure 9B is a brain atlas image of a coronal section (AP +1.49 mm) showing the desired target coordinates, with the blue circles highlighting the NAc and the green ovals demonstrating the location of the LV-EGFP-Cre and LV-EGFP-Empty infection. All samples obtained from mice in both drinking experiments were on target and demonstrated an EGFP signal that covered at least one-third of the NAc.

Micropunches of the injection site were obtained from the other half of the treated (LV-EGFP-Cre, LV-EGFP-Empty)
**TLR4** KO rats. Ethanol (44 mM) significantly (p < 0.01–0.05) increased s/mIPSC frequencies in CeA neurons from both genotypes; ethanol increased the mean sIPSC frequency by 37.6 ± 11.4% in WT and 45.7 ± 14.2% in **TLR4** KO rats (Fig. 12A, B), and increased the mIPSC frequency by 26.4 ± 7.1% in WT and 43.6 ± 13.5% in **TLR4** KO rats (Fig. 13C, D). Acute ethanol did not alter s/mIPSC amplitudes or kinetics in CeA neurons from either genotype (Fig. 12). There was no significant difference in the acute ethanol-induced increases of CeA GABA release in neurons from naive WT and **TLR4** KO rats.

**CIE vapor exposure**

In CeA neurons from CIE-treated WT and **TLR4** KO rats, acute ethanol (44 mM) application also increased s/mIPSC frequencies across the different treatment groups using two-way ANOVA. There was a significant main effect of treatment (F(2,46) = 6.7, p < 0.01), but not genotype (F(2,46) = 1.3, p > 0.05), on sIPSC frequencies and a significant interaction between treatment and genotype (F(4,92) = 7.3, p < 0.01). Bonferroni post hoc tests showed significant differences in ethanol effects in the LPS-treated rats compared with naive and CIE-treated WT rats, whereas there were no significant differences among the individual treatment groups of **TLR4** KO rats. There were no main effects of treatment or genotype on acute ethanol-induced potentiation of sIPSC frequencies between naive and CIE-treated rats.

**Discussion**

INIA-Neuroimmune investigators used extensive behavioral and electrophysiological tests to examine the role of TLR4 in acute and chronic ethanol responses in rats and mice (summarized in Table 2). Different genetic and pharmacologic approaches were used to inhibit TLR4-mediated signaling and different

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**Table 1. Baseline spontaneous and miniature IPSCs in CeA neurons from naive, CIE-treated, and LPS-treated WT and **TLR4** KO rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>Amplitude</th>
<th>Rise time**</th>
<th>Decay time</th>
<th>Frequency</th>
<th>Amplitude</th>
<th>Rise time**#</th>
<th>Decay time</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Naive</td>
<td>1.6 ± 0.4</td>
<td>79.6 ± 6.1</td>
<td>2.4 ± 0.1</td>
<td>8.7 ± 0.9</td>
<td>5.0 ± 0.1</td>
<td>52.7 ± 2.2</td>
<td>3.2 ± 0.2</td>
<td>7.1 ± 1.9</td>
</tr>
<tr>
<td>KO Naive</td>
<td>1.2 ± 0.4</td>
<td>78.9 ± 11.3</td>
<td>3.1 ± 0.2</td>
<td>9.1 ± 0.9</td>
<td>0.3 ± 0.2</td>
<td>56.8 ± 3.1</td>
<td>2.5 ± 0.1</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>WT CIE</td>
<td>1.6 ± 0.4</td>
<td>65.2 ± 6.1</td>
<td>2.7 ± 0.1</td>
<td>8.5 ± 0.7</td>
<td>0.3 ± 0.1</td>
<td>46.6 ± 0.7</td>
<td>2.7 ± 0.2</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>KO CIE</td>
<td>1.0 ± 0.4</td>
<td>80.2 ± 13.6</td>
<td>2.4 ± 0.2</td>
<td>8.7 ± 1.1</td>
<td>0.4 ± 0.4</td>
<td>58.8 ± 5.4</td>
<td>2.2 ± 0.1</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>WT LPS</td>
<td>0.3 ± 0.3</td>
<td>98.9 ± 19.1</td>
<td>2.5 ± 0.1</td>
<td>10.6 ± 0.8</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>KO LPS</td>
<td>1.5 ± 0.6</td>
<td>70.7 ± 5.7</td>
<td>2.4 ± 0.1</td>
<td>8.6 ± 1.7</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

IPSCs in CeA neurons from naive rats (WT: n = 12; KO: n = 12), CIE-exposed rats (WT: n = 8; KO: n = 10), and ethanol-naive rats (WT: n = 8; KO: n = 8) injected with LPS (2 mg/kg, i.p.). Two-way ANOVA revealed no main effects of treatment, genotype, or the interaction of baseline frequencies, amplitudes, and decay times of the spontaneous IPSCs. There was a main effect of treatment (F(2,46) = 7.3; p < 0.01) on the rise time, but no main effect of genotype or interaction between genotype and treatment. Likewise, there were no main effects of treatment or genotype and no interaction between treatment and genotype on the frequency, amplitude, and decay time of baseline miniature IPSCs from naive (WT: n = 5; KO: n = 9) and CIE-exposed (WT: n = 5; KO: n = 6) rats. However, there were significant main effects of treatment (F(1,23) = 7.4, p < 0.05) and genotype (F(1,31) = 11.8, p < 0.01) on baseline miniature IPSC rise time, but no interaction between genotype and treatment. Values are mean ± SEM.

*p < 0.05, **p < 0.01, effect of treatment, #p < 0.01, effect of genotype.

N/A, Not applicable.
TLR4 KO rats were resistant to these effects as reported previously in Tlr4 KO mice (Qureshi et al., 1999; Kalis et al., 2003; von Meyenburg et al., 2004; Yang et al., 2014). We further demonstrated that WT ethanol-trained rats developed tolerance to LPS-induced toxicity in agreement with studies in ethanol-naive rats (McCarthy et al., 1984; Cross-Mellor et al., 2000). Female B6 mice also showed evidence of tolerance to decreased body weight after a second LPS injection (Blednov et al., 2011).

LPS increased ethanol consumption in mice (Blednov et al., 2011) and elevated serum levels of LPS were positively correlated with alcohol craving in humans (Leclercq et al., 2012; Leclercq et al., 2014). In WT rats, LPS transiently reduced ethanol self-administration, but had no long-term effects on drinking in either genotype. The initial reduction in ethanol responding (1–4 d after LPS) may be due to induction of “sickness-like” behavior that affects the motivation for ethanol similar to effects on food/water intake. We found no differences in ethanol self-administration in WT and Tlr4 KO rats before or after dependence and no consistent genotype differences in 2BC drinking over 5 weeks. The inconsistent decrease in dependence-driven drinking by 60 mg/kg (+)-naloxone may be due to nonspecific/aversive effects of this high dose, as reported in rats (Tanda et al., 2016).

TLR4 knockdown in the CeA or VTA decreased binge drinking in P rats (Liu et al., 2011; June et al., 2015). Given the prominent role of the NAc within the mesocorticolimbic reward circuit in alcohol abuse and dependence, we further studied the effects of TLR4 in this brain region. However, Tlr4 mRNA knockdown in mouse NAc did not reduce 2BC continuous or limited access drinking. In addition to brain region, there are other key differences between the studies. We examined male C57BL/6j mice using the 2BC continuous and intermittent models, whereas the previous work was in P rats (the sex was not reported) using a binge-drinking model. The P rats had increased levels of GABA<sub>A</sub> receptor <i>α2</i> subunits and TLR4 in the CeA and VTA, and <i>α2</i> subunit-specific knockdown in the CeA inhibited TLR4 function and decreased drinking (Liu et al., 2011). Furthermore, <i>Tlr4</i> knockdown in the CeA decreased <i>α2</i> subunit expression and also reduced drinking (Liu et al., 2011). Therefore, the TLR4-related effects on binge drinking in P rats may be due to altered GABAergic (<i>α2</i> subunit-specific) responses.

Although TLR4 signaling did not regulate ethanol consumption consistently in our rodent models or in other studies and genotype, acute ethanol did not have significant effects on sIPSC amplitudes (WT: 111.3 ± 9.4% of control; KO: 115.8 ± 5.1% of control) or kinetics (rise time: WT, 97.0 ± 3.3% of control; KO, 106.6 ± 3.7% of control; decay time: WT, 112.5 ± 7.5% of control; KO, 117.6 ± 9.7 of control). A Representative sIPSCs recorded from CeA neurons of naive WT and Tlr4 KO rats. B Acute ethanol increased the mean sIPSC frequency in CeA neurons by 37.6 ± 11.4% in WT (from 1.4 ± 0.5 to 1.6 ± 0.6 Hz, n = 9) and by 45.7 ± 14.2% in KO (from 0.9 ± 0.2 to 1.2 ± 0.3 Hz, n = 10) rats. There was a significant main effect of ethanol (F<sub>1,12</sub> = 11.0, p < 0.01), but no main effect of genotype or interaction between ethanol and genotype. Acute ethanol did not have significant effects on mIPSC amplitudes (WT: 111.3 ± 9.4% of control; KO: 115.8 ± 5.1% of control) or kinetics (rise time: WT, 97.0 ± 3.3% of control; KO, 106.6 ± 3.7% of control; decay time: WT, 112.5 ± 7.5% of control; KO, 117.6 ± 9.7 of control). C Representative mIPSCs recorded from CeA neurons of naive WT and Tlr4 KO rats showing an increase in mIPSC frequencies after acute ethanol application. D Acute ethanol increased mIPSC frequencies in CeA WT and Tlr4 KO neurons. There was a significant main effect of ethanol (F<sub>1,12</sub> = 11.8, p < 0.05) on mIPSC frequencies in WT (26.4 ± 7.1%; from 0.5 ± 0.1 to 0.6 ± 0.1 Hz, n = 5) and KO neurons (43.6 ± 13.5%; from 0.3 ± 0.1 to 0.4 ± 0.1 Hz, n = 9), but no significant main effects of genotype or the interaction between ethanol and genotype. Ethanol had no effects on mIPSC amplitudes (WT: 103.3 ± 2.9% of control; KO: 93.6 ± 3.6% of control) or kinetics (rise time: WT, 96.7 ± 2.8% of control; KO, 99.9 ± 4.9% of control; decay time: WT, 102.9 ± 2.3% of control; KO, 96.7 ± 11.6% of control) in neurons from WT and Tlr4 KO rats. Statistical significance was calculated by two-way repeated-measures ANOVA (Ap < 0.05). The data are presented as mean ± SEM.
Figure 13. Acute ethanol enhances GABA release in CeA neurons from WT and Tlr4 KO rats differently after CIE exposure. A, Traces of sIPSC recordings in CeA neurons from WT and Tlr4 KO male rats exposed to CIE vapor. B, Bath application of 44 mM ethanol facilitated sIPSC frequencies in only 6 of 10 KO neurons (46.5 ± 23.1% of control; from 1.2 ± 0.7 to 1.7 ± 0.8 Hz, n = 6) and decreased (< 85% of control, n = 3) or had no effect (85–115% of control, n = 1) on sIPSC frequencies in the remainder (85.5 ± 9.3% of control; from 0.7 ± 0.1 to 0.5 ± 0.04 Hz) of KO neurons. Two-way repeated-measures ANOVA of the peak ethanol effects from all of the tested cells showed a significant main effect of ethanol (F(1,16) = 9.3, p < 0.05), but no effect of genotype or interaction between ethanol and genotype. Acute ethanol effects on sIPSC frequencies were transient in the neurons from KO compared with WT rats.

C

Figure 14. LPS injection alters GABA responses to acute ethanol in CeA neurons from WT but not Tlr4 KO rats. A, Representative recordings of sIPSCs from LPS-treated WT and Tlr4 KO male rats. B, Seven to 10 d after a single LPS injection (1 mg/kg, i.p.), acute 44 mM ethanol application reduced sIPSC frequencies by 41.4 ± 4.0% (from 1.3 ± 0.3 to 0.7 ± 0.2 Hz, n = 8) in CeA neurons from LPS-treated WT rats. Ethanol’s effects on sIPSC frequencies in LPS-treated KO rats (an overall increase of 29.9 ± 21.6%, n = 7) were more varied, with ethanol-induced potentiation (> 115% of control, n = 3) or no effect (85–115% of control, n = 4) occurring in a cell-specific manner. There was a significant main effect of ethanol treatment (F(1,13) = 5.9, p < 0.05), but not genotype, and no interaction between genotype and ethanol. There were no significant effects of acute ethanol on the amplitudes (WT: 93.6 ± 12.9%; KO: 112.3 ± 11.1% of control) or kinetics (rise time: WT, 109.9 ± 4.4%; KO, 97.8 ± 2.2%; decay time: WT, 90.2 ± 14.7%; KO, 115.2 ± 10.1% of control) in LPS-treated WT and Tlr4 KO rats. The data are presented as mean ± SEM. Statistical significance was calculated by two-way repeated-measures ANOVA (p < 0.05).

D

(Alfonso-Loeches et al., 2010; Pascual et al., 2011; Bajo et al., 2016; Blednov et al., 2017a), Tlr4 KO rats displayed lower sensitivity than WT to the acute sedative effects of ethanol. This is consistent with results in mice lacking Tlr4 or WT mice treated with (-)-naloxone (Wu et al., 2012b; Corrigan et al., 2015; Blednov et al., 2017a). ethanol had no significant effects on the mean mIPSC amplitudes (WT: 93.6 ± 12.9%; KO: 112.3 ± 11.1% of control) or kinetics (rise time: WT, 109.9 ± 4.4%; KO, 97.8 ± 2.2%; decay time: WT, 90.2 ± 14.7%; KO, 115.2 ± 10.1% of control) in LPS-treated WT and Tlr4 KO rats. The data are presented as mean ± SEM. Statistical significance was calculated by two-way repeated-measures ANOVA (p < 0.05).
and tonic currents were altered in TLR4 pathway components. However, frequencies of sIPSCs properties of GABA
Previous work in mice showed that LPS reduced firing of VTA especially in naive rats, agrees with our study suggesting that CD14 ethanol effects on spontaneous GABAergic transmission, espe-
TLRs and associated pathways may regulate some of ethanol’s acute responses synergistically.
We examined GABAergic transmission in CeA neurons because TLR4 pathway components were involved in ethanol’s acute effects on GABAergic IPSPs in these synapses (Bajo et al., 2014) and because Tlr4 knockdown in the CeA of P rats potentially reduced drinking via a GABAergic mechanism (Liu et al., 2011). Deletion of Tlr4 did not affect baseline spontaneous GABA release in CeA neurons, but altered the kinetics of GABA_A receptor-mediated IPSCs, indicating a role for TLR4 in GABA_A receptor function (Otsi et al., 1994). Generally, the kinetic properties of GABA_A-mediated currents are determined by subunit composition, posttranslational modifications, trafficking, synap-
tic localization, and clustering (Jacob et al., 2008; Wu et al., 2012a; Dixon et al., 2014) and we cannot exclude potential contributions from these factors.
Deletion of CD14, an accessory protein for LPS-mediated activation of TLR4, had no effects on CeA GABAergic transmission (Bajo et al., 2014), indicating different synaptic effects for different TLR4 pathway components. However, frequencies of sIPSCs and tonic currents were altered in Il1rn KO mice (Bajo et al., 2015). Collectively, our findings support distinct roles for neuroimmune regulators in basal GABAergic transmission.
Acute ethanol increased action potential-dependent and -independent GABA release in CeA neurons from naive and CIE-treated WT and Tlr4 KO rats in agreement with previous studies (Roberto et al., 2003; Roberto et al., 2004; Gilpin et al., 2011; Cruz et al., 2013; Herman et al., 2013). The lack of differences in acute ethanol effects on spontaneous GABAergic transmission, especially in naive rats, agrees with our study suggesting that CD14 (rather than TLR4) is critical for ethanol effects in CeA neurons (Bajo et al., 2014). After LPS exposure, acute ethanol reduced CeA GABAergic transmission in WT but not Tlr4 KO neurons. Previous work in mice showed that LPS reduced firing of VTA neurons and increased ethanol drinking (Blednov et al., 2011). Despite the synaptic changes in WT neurons observed 7–10 d after LPS exposure, LPS did not alter ethanol self-administration in WT or Tlr4 KO rats at this time point. Although our results support the involvement of the TLR4 pathway in regulating CeA GABA transmission, it is likely that other immune mediators released during LPS-induced peripheral and central immune act-
We used different methods to target TLR4 and different models of ethanol intake, but there was little evidence that TLR4 regulates drinking directly. In microglia, (+)-naloxone and (+)-
naltrexone may block TLR4 downstream signaling preferentially via the TRIF-IRF3 pathway (Wang et al., 2016), indicating that they may not block all arms of TLR4 signaling. Given that there were no consistent differences in 2BC drinking and operant self-administration of ethanol between WT and Tlr4 KO rats, it is unlikely that the lack of (DID and 2BC) or inconsistent (CIE-2BC) effect of (+)-naloxone is due to incomplete TLR4 inhibition, particularly at the 60 mg/kg dose. Collectively, our results in mice and rats are in agreement with studies showing that Tlr4 KO and WT mice did not differ in ethanol consumption (Alfonso-Loeches et al., 2010; Pascual et al., 2011; Blednov et al., 2017a). Our (+)-naloxone results in mice are consistent with another TLR4 inhibitor that also decreased ethanol consumption nonspecifically in mice (Bajo et al., 2016). To date, only knockdown of Tlr4 mRNA in the CeA or VTA of P rats decreased binge drinking and this was associated with α2 subunit-specific GABAergic signal-
ing. However, there is now consistent evidence across species for a role of TLR4 in mediating the acute sedative effects of alcohol, which could be an indicator of overall sensitivity to alcohol and susceptibility to develop dependence.
TLR4 mediates ethanol-induced increases in brain cytokines/chemokines and neurodegeneration and inhibition of TLR4 pre-
vents or reverses ethanol-induced neuroimmune responses (Fernandez-Lizarbe et al., 2009; Alfonso-Loeches et al., 2010; Zou
and Crews, 2014; Bajo et al., 2016). Chronic ethanol exposure may also disrupt the integrity of the blood–brain barrier via TLR4-dependent signaling (Rubio-Araiz et al., 2016). TLR4 was associated with cognitive impairment and anxiety-related behavior during ethanol withdrawal (Pascual et al., 2011; Pascual et al., 2015) and with synaptic remodeling and anxiety induced by binge drinking in adolescent mice (Montesinos et al., 2016). However, our comprehensive study using different species and sexes, different methods to inhibit TLR4, and different tests of chronic, dependence-driven, and binge-like consumption indicates that TLR4 is not a critical gene for regulating ethanol drinking and inhibition of TLR4 signaling alone would not be an effective therapeutic strategy. Ethanol promotes physical interactions between TLR4 and TLR2 (Fernandez-Lizarbe et al., 2013) and TLR4/TRL2 interactions were implicated in ethanol-induced sedation (Corrigan et al., 2015), which warrants consideration that TLR4 and other neuroimmune mediators may regulate ethanol responses synergistically. Analyses of the conserved cross-species gene networks in alcoholics and animal models exposed to chronic alcohol will help to prioritize immune/inflammatory pathways (and candidate drugs) with improved therapeutic potential for alcohol use disorders.

References


