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Role of TLR7 in voluntary alcohol consumption

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Abstract

Overactivation of neuroimmune signaling has been linked to excessive ethanol consumption. Toll-like receptors (TLRs) are a major component of innate immune signaling and initiate anti- and pro-inflammatory responses via intracellular signal transduction cascades. TLR7 is upregulated in post-mortem brain tissue from humans with alcohol use disorder (AUD) and animals with prior exposure to ethanol. Despite this evidence, the role of TLR7 in the regulation of voluntary ethanol consumption has not been studied. We test the hypothesis that TLR7 activation regulates voluntary ethanol drinking behavior by administering a TLR7 agonist (R848) during an intermittent access drinking procedure in mice. Acute activation of TLR7 reduced ethanol intake, preference, and total fluid intake due, at least in part, to an acute sickness response. However, chronic pre-treatment with R848 resulted in tolerance to the adverse effects of the drug and a subsequent increase in ethanol consumption. To determine the molecular machinery that mediates these behavioral changes, we evaluated gene expression after acute and chronic TLR7 activation. We found that acute TLR7 activation produces brain region specific changes in expression of immune pathway genes, whereas chronic TLR7 activation causes downregulation of TLRs and blunted cytokine induction, suggesting molecular tolerance. Our results demonstrate a novel role for TLR7 signaling in regulating voluntary ethanol consumption. Taken together, our findings suggest TLR7 may be a viable target for development of therapies to treat AUD.

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⁵Conflicts of Interest

The authors declare no conflicts of interest.

1. Introduction

The neuroimmune system is heavily implicated in the development and maintenance of alcohol use disorder (AUD) (for review see (Erickson et al. 2019)). Innate immune components are upregulated in postmortem alcoholic brain and are correlated with lifetime ethanol use (Lewohl et al. 2000; Mayfield et al. 2002; Vetreno & Crews 2014). Ethanol initiates a positive feedback loop in which neuroimmune activation by alcohol generates a proinflammatory response to promote excessive alcohol consumption (Leclercq et al. 2014). Consequently, it is critical to understand the relationship between neuroimmune signaling and ethanol-related behavior in order to identify novel targets for the treatment of AUD. Here we focus on the role of one innate immune pathway in the regulation of voluntary ethanol consumption, the toll-like receptor 7 (TLR7) and myeloid differentiation primary response 88 (MyD88)-dependent pathway.

The innate immune system in the central nervous system (CNS) consists of a heterogeneous population of cells that are important for normal brain function and also recognize and respond to threats in the neuronal microenvironment (Williamson & Bilbo 2013). Microglia, astrocytes, and neurons initiate pro- or anti-inflammatory cytokine, chemokine, and other neuromodulator signaling. TLRs play a key role in neuroimmune activation and in regulating ethanol behavioral phenotypes (Crews et al. 2017). For example, TLR4 responds to bacterial endotoxin lipopolysaccharide (LPS) and regulates specific subsets of genes depending on distinct combinations of adaptor proteins, such as MyD88 and TIR-domain-containing adapter-inducing interferon- β (TRIF) (Lu et al. 2008). Generally, TLR4 activation leads to nuclear translocation of the transcription factor, nuclear factor kappa light-chain-enhancer of activated B cells (NF- κ B), which leads to expression of pro-inflammatory cytokines. (Lu et al. 2008). Although activation of TLR4 by LPS can increase voluntary ethanol intake (Blednov et al. 2011), TLR4 is not necessary for LPS-induced escalation of ethanol intake (Lainiola & Linden 2017; Harris et al. 2017). Taken together, these results suggest that TLR4 alone is not critical for regulating drinking and likely works in concert with several other neuroimmune factors in the brain to drive behavior.

Here we focus on defining the role of TLR7 in voluntary ethanol consumption. TLR7 is expressed in endosomes, is highly enriched in microglia, and responds to viral single-stranded RNA (Du et al. 2000; Hemmi et al. 2002; Erickson et al. 2018; Michaelis et al. 2019). TLR7 shares activation pathways with TLR4 as it signals through the adaptor protein MyD88, leading to translocation of transcription factors NF- κ B and interferon regulatory factor 7 (IRF7). TLR7 activation is associated with a pro-inflammatory immune response, primarily through downstream production of interferon- α (IFN α), TNF α , IL-6 and IL1 β .

TLR7 is frequently grouped together with other endosomal TLRs 3 and 8. TLRs 3 and 7 are upregulated in brain tissue from post-mortem humans with AUD (McCarthy et al. 2017; Vetreno & Crews 2012). TLRs 3, 7, and 8 are differentially expressed after chronic ethanol exposure in rodents (Supplemental Fig. 1). Recent work demonstrates a critical role for TLR3 in the regulation of voluntary ethanol consumption in male and female rodents (Warden et al. 2019b; Warden et al. 2019a; Randall et al. 2019). The role of TLR7 in voluntary ethanol consumption remains uninvestigated. In this study, we tested whether

activation of TLR7-dependent signaling by the synthetic imidazoquinoline R848 alters ethanol consumption in male mice. We found that acute R848 administration decreases ethanol intake in mice, due in part, to a sickness response. However, repeated R848 pre-treatment increases ethanol consumption. Gene expression results indicate that these effects are driven by acute neuroimmune activation and molecular tolerance of innate immune signaling cascades, respectively. Our results also highlight the complex relationship between distinct TLR pathways and brain region specificity of neuroimmune signaling

2. Materials and methods

2.1 Mice

Male C57BL/6J mice were purchased from Jackson Laboratory at 6-8 weeks old. We restricted this study to males in part to replicate and expand on previous work on TLR7 expression in male mice. We are aware of sex differences in neuroimmune responses (Klein & Flanagan 2016; Kawai & Akira 2011), ethanol consumption (Middaugh et al. 1992; Hilderbrand & Lasek 2018) and in TLR7 expression (Souyris et al. 2018; Souyris et al. 2019). We will examine effects of TLR7 activation in females in a separate study as we did for TLR3 (Warden et al. 2019b). Mice were individually housed in standard polycarbonate shoebox cages on a 12-hr light/dark cycle at the University of Texas at Austin with food (TMH 1800 5LL2 chow) and water *ad libitum*. The rooms were maintained at an ambient temperature of 21 +/- 1 C, humidity (40-60%), and centrally controlled ventilation. All procedures were approved by the University of Texas Institutional Animal Care and Use Committee and adhered to NIH Guidelines (AAALAC accredited).

2.2 Drug administration

R848 (Cat # 144875-48-9, Invivogen, San Diego, CA) was dissolved in saline (0.9% NaCl) to 1 mg/ml and injected at a dose of 50 µg intraperitoneally (i.p.) across all experiments. This dose was chosen based on previous work in mice showing efficacy of the drug and robust immune response at 100 µg dose and work showing immune responses at a lower dose of 10 µg (Michaelis et al. 2019; Van et al. 2011). Volume matched saline (0.9% NaCl) was administered to control groups. Single use, sterile needles (27.5 gauge) were used to administer treatments. All injections were performed on an every other day schedule between 7am and 8am. R848 was chosen as a TLR7 agonist because of its greater potency compared to more commonly used imiquimod (Dowling et al. 2013). R848 is a TLR7 and 8 agonist in many species, however murine TLR8 is not responsive to imidazoquinolines such as R848 (Jurk et al. 2002).

2.3 Every other day two-bottle choice (EOD-2BC) procedure

Intermittent access to ethanol increases voluntary drinking in rodents (Simms et al. 2014; Wise 1973; Hwa et al. 2011; Rosenwasser et al. 2013). The two-bottle choice protocol was carried out as previously described (Blednov et al. 2011). Briefly, two 50 ml conical tube drinking bottles were available every other day to each mouse and weighed daily. One bottle always contained water. Food was available *ad libitum* and mice were weighed once per week unless otherwise noted. Mice were offered a choice between 15% ethanol (v/v) versus water for 24 hr sessions and water only was offered on off days. Aaper brand (Aaper Ethanol

and Chemical, Shelbyville, KY) 200-proof ethanol was used to mix solutions as v/v in tap water. Bottle positions were changed every day to control for position preferences. The quantity of ethanol consumed (g/kg body weight/24 hr) was calculated for each mouse, and these values were averaged across two drinking days for presentation. Throughout the experiment, evaporation/spillage estimates were calculated every day from two bottles placed in an empty cage, one containing water and the other containing the ethanol solution. Sample sizes for each experiment are specified in the results section but in general n=10-12 for drinking experiments.

2.4 Locomotor activity

Locomotor activity was measured in standard laboratory cages by Opto-Varimex-Mini (Columbus Instruments, Columbus, OH). Activity was monitored by six light beams placed along the width of the cage at 2.5-cm intervals, 1.5 cm above the floor. Each experimental cage had bedding and food and was covered by a heavy plastic lid with holes for ventilation and bottle of water. Motor activity (measured by beam crossings) was monitored in 10-min intervals and binned into 1-hr intervals for analysis.

2.5 Body temperature measurements

Mice for this experiment were placed on a reverse light-dark cycle to allow for body temperature measurements to be taken during the animals' active period. Body temperature was measured with a rectal thermometer 1, 2, 3, 4, 6, and 24-hr after drug administration.

2.6 Water intake and saccharin preference

For water intake measurements, a single water bottle was weighed and then placed in a standard mouse cage for 24 hours before being measured again. Water intake was measured by change in bottle weight every other day. For saccharin preference, mice were given access to 0.0016% saccharin solution or water every other day for 24-h. After 6 days of access to this concentration (3 data points), the concentration was increased to 0.0033% saccharin. Intake and preference were calculated using the change in bottle weight, normalized by animal weight, and averaged across two days to control for position preferences (n=10/group). In both experiments, mice were injected with R848 (50 µg, i.p.) on drinking days immediately before bottles were made available.

2.7 Brain collection and brain region isolation

Brains were removed after cervical dislocation and flash frozen in liquid nitrogen. Flash frozen brains were mounted in optimum cutting temperature compound (OCT, VWR) and quickly frozen in isopentane on dry ice. Micropunches were obtained from coronal sections (300 µm) of prefrontal cortex, nucleus accumbens, hippocampus, amygdala, and ventral tegmental area as described previously (Osterndorff-Kahanek et al. 2015). Coordinates (relative to bregma): prefrontal cortex (+2.1 mm to +1.8 mm), nucleus accumbens (+1.8 mm to +0.6 mm), hippocampus (00 mm to 00 mm), amygdala (−0.9 mm to −1.8 mm), ventral tegmental area (−2.80mm to −3.4mm).

2.8 Quantitative RT-PCR

Total RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Life Technologies, Grand Island, NY). Total RNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Grand Island, NY). Reverse transcription was performed using the Applied Biosystems High Capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). PCR amplification was performed using TaqMan Universal PCR Master Mix and primer pairs and probes (Thermo Fisher Scientific, Grand Island, NY). All primers used were Applied Biosystems TaqMan® Gene Expression Assays. Reactions were performed using TaqMan® Universal PCR Master Mix in 10- μ L reactions containing 10 ng of cDNA. All samples were normalized to *Gusb* or *Gapdh*. The relative quantification of mRNA levels was determined using BIORAD software as previously described (Osterndorff-Kahanek et al. 2015; Osterndorff-Kahanek et al. 2013).

2.9 Statistical analysis

Data are reported as mean $\pm$ SEM values, unless otherwise noted. The statistics software program GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used to perform 2-way ANOVA, Mixed-effects analysis (REML), Pearson correlations, and Student's *t*-tests. Drinking and other behavioral data were analyzed by 2-way repeated measures ANOVA or mixed-effects analysis followed by Bonferroni post-hoc tests. Mixed-effects analyses were used in cases where sample sizes were different between treatment conditions. In both cases, the Geisser-Greenhouse correction was used to correct for sphericity. Grubbs test ($\alpha = 0.05$) was used to detect potential outliers but no outliers were noted or excluded. Student's *t*-test (two-tailed) with Holm-Sidak correction for multiple comparisons was used to analyze raw qRT-PCR data and immunohistochemical data. Complete reporting of all statistical tests, sample sizes, and adjusted *p*-values can be found in Supplemental Table 2.

3. Results

3.1 TLR7 activation during drinking reduces ethanol consumption, preference, and total fluid intake

Ethanol exposure upregulates *Tlr7* mRNA across various models and procedures, suggesting it may play a role in alcohol-related behaviors (Lawrimore & Crews 2017; Coleman et al. 2017; McCarthy et al. 2018). To determine if TLR7 activation regulates ethanol consumption, we administered R848 (50 μ g, i.p.), or saline (0.9% NaCl) on ethanol-drinking days during EOD drinking (Fig. 1A). R848 decreases ethanol intake, preference, and total fluid intake (Fig. 1B). A reduction in total fluid is indicative of a sickness response, though there were no differences in body weights over the course of the 4-week experiment (Supplemental Table 1). When R848 was discontinued and mice were allowed to continue drinking ethanol in the EOD-2BC procedure, drug-treated mice continued to drink less ethanol despite comparable levels of total fluid intake. R848-treated mice normalized to control levels of ethanol intake and preference over the course of 6-8 Ethanol-drinking days. These results show that activation of TLR7 decreases ethanol consumption and preference, but this effect may be at least partially driven by a sickness response.

3.2 TLR7 activation produces an acute sickness response

Sickness response consists of a number of measurable behavioral reactions including fluid intake, food intake, locomotor activity, and body temperature (Kent et al. 1992; Dantzer 2001; Damm et al. 2012). Acute activation of TLR7 using R848 produces a sickness response that is dependent on TLR7 (Michaelis et al. 2019). To determine whether the reduction in total fluid intake observed in the previous experiment was the result of a sickness response, we measured saccharin preference, water intake with no ethanol available, locomotor activity, and body temperature in response to R848 or saline. Throughout all behavioral tests, mice received R848 or saline on an injection schedule that matched the previous ethanol drinking experiment (50 µg, i.p., every other day). R848 decreased saccharin intake and total fluid (Fig. 2A) but caused no change in saccharin preference or body weight over the course of the experiment (Supplemental Table 1). R848 decreased water intake when no ethanol was present (Fig. 2B) and R848-treated mice failed to gain weight over time compared to saline-treated controls (Supplemental Table 1). To measure locomotor activity and body temperature, mice were injected and then immediately placed in activity chambers or measured for body temperature. R848 decreased locomotor activity and body temperature across 24 hr (Fig. 2C-D). The reduction in locomotor activity was observed across both the light and dark cycle and the reduction in body temperature was only observed during the dark phase 2-4 hr after injection. Taken together, these results suggest that reductions in alcohol consumption and total fluid are at least partially driven by a sickness response facilitated by TLR7 activation.

3.3 Repeated TLR7 activation produces behavioral tolerance measured by sickness response

Previous work shows that repeated R848 administration leads to behavioral tachyphylaxis, meaning animals no longer show a sickness response after recurring doses of R848 (Michaelis et al. 2019). We replicated these findings with the dose of R848 and time course used in the initial ethanol drinking experiment (Fig. 3A). Briefly, mice were injected with R848 (50 µg, i.p.) every other day for 20 days (10 total injections). On the final injection day, mice were placed in activity monitor cages or measured for body temperature across 24 hours. After 10 repeated injections of R848, locomotor activity in drug-treated mice increased compared to their initial reading and was not different from control mice injected with saline (Fig. 3B-C). Similarly, body temperature was not different between R848-injected versus saline-injected mice, though there was a slight increase in body temperature at the 2-hr timepoint after injection compared to saline controls (Fig. 3D-E). These results are consistent with previous work showing repeated R848 administration causes behavioral tolerance measured by locomotor activity, food intake, and body weight (Michaelis et al. 2019) and suggest that mice may not have been experiencing sickness at later timepoints of the ethanol drinking experiment.

3.4 TLR7 activation before drinking increases ethanol consumption and preference

Pre-exposure to LPS one to four weeks before drinking increases ethanol intake (Blednov et al. 2011). Repeated pre-treatment with LPS also leads to behavioral tolerance measured by locomotor activity, body weight, and food intake (Kubera et al. 2013; Elgarf et al. 2014;

Musaelyan et al. 2018). We hypothesized that R848 functions similarly to LPS because of their similar behavioral profiles and induction of overlapping immune pathways. To test this hypothesis, mice were repeatedly injected with R848 or saline and given two weeks to recover from injections before EOD drinking (Fig. 4A). R848 pre-treatment increased ethanol intake and preference, with no effect on total fluid intake (Fig. 4B). Drug-treated mice also showed weight gain that was similar to controls over the course of the experiment (Supplemental Table 1). These results suggest that the timing of innate immune induction plays an important role in regulating behavior.

3.5 Acute TLR7 activation produces brain region specific changes in innate immune transcript abundance

Transcriptional regulation of neuroimmune components plays an important role in regulating behavior. To identify molecular changes that mediate behavior in response to R848, we profiled gene expression of signaling components downstream of TLR7 and associated pathways using quantitative RT-PCR. Previous work indicates that acute TLR7 activation leads to downstream upregulation of MyD88-dependent signaling and there is cross-talk between MyD88- and TRIF-dependent pathways (Y.-F. Flung et al. 2018; B. Liu et al. 2016). Thus, we evaluated expression of MyD88- (*Tlr7*, *Tlr4*, *Myd88*, *Irf7*, *Il1b*) and TRIF-dependent (*Tlr3*, *Trif*, *Ikkε*, *Irf3*) signaling components 8 and 24 hours after R848 (50 µg, i.p.) or saline across brain regions previously evaluated for neuroimmune gene expression (Fig. 5A). These time points were chosen based on the half-life of R848 (4-6 hours) and the time-point when ethanol drinking data were acquired (24 hours). At the 8-hour timepoint, MyD88- and TRIF-dependent signaling were upregulated in PFC, NAC, HIP, and VTA, whereas MyD88-dependent signaling was downregulated in AMY (Fig. 5B; Supplemental Fig. 2). These results show that R848 activated neuroimmune signaling by up-regulating both MyD88- and TRIF-dependent pathways, except in the amygdala where they were downregulated. At the 24-hour timepoint, only *Irf7* was upregulated across all brain regions, whereas *Irf3* was downregulated in several brain regions (Fig. 5C; Supplemental Fig. 3). These results demonstrate that TLR7 activation causes rapid transcriptional changes on the receptor level that are normalized by 24 hr post-injection, while downstream transcriptional changes are persistent.

3.6 Repeated TLR7 activation produces molecular tolerance measured by innate immune gene transcript abundance

Repeated activation of TLR7 leads to molecular tolerance measured by pro-inflammatory cytokine gene expression, meaning pro-inflammatory cytokine transcript abundance diminishes in response to R848 over time (Michaelis et al. 2019). We hypothesized that this effect is driven by downregulation of *Tlr7* mRNA in response to repeated activation. To test this hypothesis, we measured *Tlr7* and downstream component transcript abundances after repeated R848 injections (Fig. 6A). We observed the most robust changes in the PFC (Fig. 5B), which is consistently identified as a critical brain region for the regulation of neuroimmune gene expression and ethanol-related behavior. Thus, we focused our transcript abundance studies on the PFC. Repeated R848 pre-exposure caused downregulation of *Tlr7* and *Tlr3* and no change in cytokine (*Il1b* and *Il6*) transcripts in the PFC, suggesting molecular tolerance (Fig. 6B). Interestingly, *Irf7* transcript abundance remained upregulated

despite its upstream receptor being downregulated. It may be the case that transcript abundances in this case do not reflect functional changes in protein considering IRF7 requires phosphorylation in order to become activated and initiate transcription.

4. Discussion

Despite evidence that TLR7 gene and protein expression are changed by ethanol consumption, the role of TLR7 in regulating behavior is not established. Our results reveal a novel role for TLR7 in the regulation of ethanol consumption. Here we show that chronic, repeated ethanol exposure alters endosomal TLR gene expression across brain regions. Acute activation of TLR7 during drinking decreases ethanol intake, but repeated pre-exposure increases ethanol consumption. Acute activation of TLR7 causes a sickness response as measured by water intake, body temperature, and locomotor activity. Repeated activation of TLR7 diminishes sickness response, driven by molecular tolerance of TLR7 signaling pathways. These results highlight the relationship between innate immune function and maladaptive behaviors like high ethanol consumption. Importantly, these results add to the growing body of literature indicating that the innate immune system is an important regulator of and potential therapeutic target for alcohol use disorder.

MyD88 and TRIF signaling pathways appear to have opposing effects on voluntary ethanol intake, respectively. Deletion of MyD88 increases drinking, whereas inhibition of TRIF-dependent signaling decreases drinking (Blednov et al. 2017; McCarthy et al. 2018). Increased drinking in MyD88 KO mice was unexpected given that LPS, which activates both MyD88- and TRIF-dependent signaling through TLR4, also increases drinking. These results might be explained by the fact that deletion of MyD88 led to increases in *Tlr3*, *Trif*, and *Irf3* expression in the PFC, suggesting MyD88 knockout removed the negative regulation of TRIF-dependent signaling (Blednov et al. 2017). Additionally, global gene knockout removes the ability to probe timing-dependent changes in neuroimmune signaling with behavior. In the context of our findings, it appears that activation of both MyD88- and TRIF-dependent signaling at the same time with TLR7 results in a sickness response that causes reductions in ethanol and total fluid intake. When the two pathways are activated prior to ethanol access, ethanol intake increases, similar to the effects of LPS (Blednov et al. 2011).

TLR7 activation during drinking reduces ethanol consumption and this effect is likely driven by a sickness response, but the persistent decreases in ethanol consumption require more nuanced analysis. We show that mice no longer experience sickness-inducing effects of the drug after repeated R848 administration, suggesting that mice theoretically no longer experienced sickness at later timepoints of the ethanol-drinking experiment. Additionally, reduced ethanol intake persists up to six days beyond the time that drug injections stop whereas total fluid intake matches control levels almost immediately after drug injections stop. Continued reductions in ethanol intake could be driven by conditioned taste aversion, where mice associate ethanol with the early sickness effects of the drug and it takes a period of time to extinguish this association once the injections stop. Mice in the ethanol-drinking and saccharin preference experiments also showed no difference in body weight, despite receiving R848 injections for the same duration as the water-only experiment and

consuming slightly less total fluid than controls. Whether or not R848 prevented weight gain could be due to variability between cohorts of mice or could be suggestive of a protective effect of another reward (ethanol or saccharin) on the sickness-inducing effects of the drug. Interestingly, TLR7 activation caused no difference in saccharin preference despite lower overall levels of total fluid intake, meaning mice still chose saccharin over water more frequently. Mice were able to detect sweet taste because when the concentration of saccharin increased, their preference also increased. In light of our findings that both MyD88 and TRIF pathways are upregulated across brain regions in response to R848, the reduction in sickness response is surprising considering evidence that MyD88 + TRIF activation shows synergism through enhanced cytokine production (Bagchi et al. 2007). We might expect TLR7 activation + ethanol to exacerbate signs of sickness and perhaps that is why R848-treated mice drank less, though our results suggest otherwise. Another possibility is that antagonism between TLR pathways or between ethanol and TLR pathways drives differences in behavior. Indeed, acute ethanol exposure blunts peripheral cytokine production caused by TLR7 activation (Pruett et al. 2004). It would be informative to determine whether this is true of central cytokine induction and how this protective effect plays a role in behavior.

Peak neuroimmune response is critical to whether ethanol drinking behavior increases, decreases, or remains unchanged (Warden et al. 2019a; Warden et al. 2019b). Peak responses decrease ethanol intake, but ethanol intake generally increases when ethanol is available hours to days after immune activation, (Blednov et al. 2011; Randall et al. 2019). In our studies, TLR7 activation by R848 occurs immediately before ethanol access (between 7-8 AM), but maximum ethanol consumption occurs during the dark phase meaning ethanol consumption started ~12 hr after injection. Given the half-life of R848 and neuroimmune transcript abundances measured at 8 and 24 hr, the peak immune response likely occurred immediately prior to or concomitantly with ethanol consumption. At 8 hr post-injection, both MyD88- and TRIF-dependent pathways were up-regulated across most brain regions. At 24 hr post-injection, only *Irf3* and *Irf7* expression were altered. *Irf3* was down-regulated, while *Irf7* was up-regulated across brain regions 24 hr after injection. These results highlight potential homeostatic interactions between the two major neuroimmune pathways. IRF3 is generally thought to be an output transcription factor of the TRIF-dependent pathway and IRF7 is an output of MyD88-dependent signaling. *Irf3* may be downregulated to compensate for the upregulation of *Irf7*, leading to a net neutral output of cytokine transcripts. Indeed, there is evidence to suggest that TRIF is a negative regulator of MyD88-dependent signaling in peripheral immune cells (Seregin et al. 2011), but additional work is required to demonstrate this effect centrally. We administered R848 peripherally, meaning peripheral immune activation is likely an important contributor to behavioral responses, especially sickness behavior. Peripheral R848 administration elevates circulating IL6 and TNF α and increases CD8+ T-cell infiltration (Baenziger et al. 2009; Alam et al. 2018; Yin et al. 2015; Van et al. 2011). It is important to consider that these circulating peripheral immune factors can influence behavior and brain gene expression by directly crossing the BBB or by acting on receptors at the interface of the BBB.

We observed no changes in innate immune transcript abundance after mice were pre-exposed to R848 followed by EOD-2BC (Supplemental Fig. 4). This result supports our

hypothesis that gene expression changes caused by R848 drive changes in behavior because by the end of the experiment, mice showed no differences in ethanol consumption or body weight. We found that immediately after chronic R848 exposure, *Tlr7* was downregulated, *Irf7* was upregulated, and there were no differences in cytokine transcript abundance. These findings are consistent with previous reports and support the hypothesis that repeated activation of TLR7 leads to molecular tolerance through downregulation of the receptor, leading to no difference in cytokine production. The transcription factor, IRF7, and the genes it regulates, are notable potential targets for mediating ethanol drinking behavior because *Irf7* is the only gene upregulated 24 hr after a single dose of R848 and after repeated R848 administration.

An important aspect of our work is that we attempt to link gene expression changes across brain regions to changes in behavior. TLR7 is primarily microglial and changes in microglial abundance and morphology can have impacts on neuronal function, such as changes in neuroplasticity. TLR7 activation is associated with microglial activation and reactive astrocyte expression in the hippocampus and neuronal degeneration *in vitro* (Rizzo et al. 2019; Coleman et al. 2017). In agreement with other studies (Michaelis et al. 2019), glial mRNA was upregulated in response to the R848 24 hr after administration (data not shown). *In vitro* work suggests that TLR7 agonists induce apoptosis and restrict dendritic arborization (H.-Y. Liu et al. 2013; Coleman et al. 2017). The TLR7 agonist Imiquimod enhances contextual fear memory and depressive-like behavior in mice and this is associated with prevention of long-term potentiation in the hippocampus (Schaffer-CA1 pathway) (Kubo et al. 2013; Kubo et al. 2012). Taken together, these previous findings demonstrate that changes in TLR7-dependent neuroimmune expression can influence neuronal function and circuitry. This raises the possibility that TLR7 activation alters synaptic plasticity in critical cortico-limbic circuits required for alcohol consumption.

We show that chronic, repeated ethanol exposure generally increases expression of TLRs consistent with other reports, though this is brain region dependent. Similarly, we observed brain-region-specific changes in neuroimmune gene expression in response to TLR7 activation. The amygdala shows a unique signature of downregulated neuroimmune expression compared to all other brain regions 8 hr after R848. We previously observed downregulated neuroimmune signatures in the amygdala after ethanol (McCarthy et al. 2018) and PolyI:C (unpublished observation). These findings might be explained by brain region dependent changes in expression of neuroimmune and microglial markers in human alcoholic brain (He & Crews 2008) and brain region specific density and expression profiles of microglia (Grabert et al. 2016; De Biase et al. 2017). We note that the unique amygdala signature appears to be transient: 24 hr after R848 injection, the direction of change of mRNA levels was not different across brain regions. An understudied area of research appears to be determining baseline brain region differences in innate immune gene expression compared to ethanol- and drug-induced changes. This information would help to identify the causes and functional consequences of brain-region-specific neuroimmune signatures.

TLR7 ligands, such as imiquimod and R848, are used as topical agents for the treatment of psoriasis and warts or as adjuvants with vaccines (I. F.-N. Hung et al. 2016; Weldon et al.

2012). Imiquimod is FDA-approved for the treatment of basal cell carcinoma (Bath-Hextall et al. 2014). TLR7 ligands, imiquimod and R848, have garnered recent interest as possible treatments for cancer because of their interferon induction capabilities. Pre-clinical studies showed promise for the use of imiquimod as an antiviral and antitumor treatment (Pilch et al. 2018; Yin et al. 2015; Cheadle et al. 2017; Dovedi et al. 2013; Bourquin et al. 2011). Our results show that acute and chronic activation of TLR7 leads to changes in expression of TLRs 3 and 4. These results have implications for the chronic use of this drug in humans as it suggests the innate immune system may not respond to potential threats appropriately, which is of particular concern to already immunocompromised patients. It will be important to determine if there is cross-tolerance to other ligands, like viral RNA and DNA.

Considering the current use of R848 as an adjuvant with vaccines (Weldon et al. 2012; Gupta et al. 2020; Sauder et al. 2003) and its use in several ongoing clinical trials (Link to clinical trials), it may be valuable to collect data on ethanol consumption in these studies to determine if there is a relationship between prolonged R848 administration and human ethanol consumption.

In summary, we demonstrate a novel role for TLR7 signaling in the regulation of voluntary ethanol consumption in mice and identify possible crosstalk between distinct TLR-dependent pathways. Prolonged activation of TLR7 leads to increased ethanol consumption driven by changes in neuroimmune gene expression. We conclude that TLR7 may be a viable target for the development of immune-based therapies, though careful attention should be paid to sickness response, immune tolerance, and the timing of drug administration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Acute activation of TLR7 by the agonist R848 during drinking decreases alcohol consumption partially due to sickness response
- Repeated activation of TLR7 prior to drinking increases alcohol consumption
- Repeated activation of TLR7 causes behavioral and molecular tolerance
- MyD88- and TRIF-dependent pathways are upregulated across most brain regions in response to TLR7 activation, except for the amygdala where they are downregulated

Fig. 1.

R848 decreases ethanol intake, preference, and total fluid intake. (A) C57BL/6J male mice were injected on every ethanol-drinking day during every other day, two-bottle choice (EOD-2BC) drinking. After 11 injections, R848 administration ceased and mice continued EOD drinking (gray). (B) R848 decreased ethanol intake (g/kg/day), preference for ethanol, and total fluid intake. When no drug was administered, ethanol intake and preference slowly normalized to control levels while total fluid rapidly normalized after injections ceased; (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 10-12$ /group)

Fig. 2.

R848 decreases saccharin intake, water intake, body temperature, and locomotor activity. (A) C57BL/6J male mice were injected with R848 on every saccharin-drinking day during EOD drinking (0.0066% saccharin). After 6 days, saccharin concentration was increased to 0.0165% saccharin. R848 decreased saccharin intake and total fluid intake, with no effect on saccharin preference; (n = 7-10/group) (B) Mice were injected with R848 every other day while given access to a single water bottle. Water intake was measured only on injection days. R848 significantly decreased water intake. When injections stopped (gray), water intake normalized to control levels. (n = 7-9/group) (C) Mice received a single injection of R848 immediately prior to lights-off (0 hr) and were measured 1, 2, 3, 4, 6, and 24 hr after injection. R848 decreased body temperature 2 and 3 hr after injection during the dark phase. (n = 8/group) (D) Mice received a single injection of R848 immediately prior to lights-off (0 hr) and then placed in activity monitor cages with access to food and water *ad libitum*. R848 decreased locomotor activity during both the dark and light phase; (*p < 0.05, **p < 0.01, ***p < 0.001, n = 10/group)

Fig. 3.

Repeated R848 administration causes behavioral tolerance. (A) Mice received 10 repeated injections of R848 every other day. On the final injection day, mice were either placed in activity monitors or returned to home cages and measured for body temperature across 24 hr. (B) Repeated R848 results in no difference in locomotor activity. (n = 10/group) (C) Locomotor activity returned to control levels after repeated R848 treatment. (D) Body temperature was not changed by repeated R848 treatment. Mice increased body temperature to match controls after repeated R848 administration at the 3 hr timepoint. (*p < 0.05, **p < 0.01, ***p < 0.001, n = 5-6/group).

Fig. 4.

Repeated R848 pre-exposure increases ethanol intake and preference. (A) C57BL/6J mice were injected on the 10-day EOD injection schedule and then allowed to recover for two weeks. After recovery, mice underwent EOD-2BC drinking. (B) Repeated R848 pre-exposure increased ethanol intake and preference, with no effects on total fluid intake; (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 10$ /group).

Fig. 5.

Brain region specific innate immune activation by single injection of R848 in alcohol-naïve mice. (A) Simplified diagram of TLR pathways measured. All TLRs, including TLR7 and except TLR3, signal through the MyD88-dependent pathway, which activates interferon regulatory factor 7 (IRF7) and NFκB transcription factors and leads to the downstream transcription of Type I interferons and pro- and anti-inflammatory cytokines. TRIF-dependent signaling leads to translocation of IRF3 and downstream transcription of Type I interferons like IFNβ. Genes measured are shown in bold text. (B) At the 8 hr timepoint, R848 upregulates both MyD88- and TRIF-dependent pathway components across brain regions, except the amygdala, where they are downregulated; (n = 4-6/group). (C) At the 24 hr timepoint, R848 upregulates only *Irf7* across all brain regions, while it downregulates *Irf3* in the nucleus accumbens and hippocampus. Transcripts levels are represented as relative expression (Ct), normalized to endogenous housekeeping genes (*p < 0.05, **p < 0.01, ***p < 0.001, n = 4-7/group).

Fig. 6.

Repeated R848 pre-exposure produces molecular innate immune tolerance in alcohol-naïve mice. (A) Mice underwent the 10-day EOD injection schedule of R848. PFC was dissected 24 hr after the final injection and used for RT-qPCR. (B) Repeated R848 exposure decreased *Tlr7* and *Tlr3*, increased *Irf7*, and caused no change in *Il1b* and *Il6* transcript abundances. The levels of transcripts are represented as relative expression (Ct), normalized to endogenous housekeeping genes. The white and green colors indicate low and high transcript abundance, respectively; (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 7-8/\text{group}$).