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Toll-like receptor 7: A novel neuroimmune target to reduce excessive alcohol consumption

Ruth L. Allard [a,1], Jody Mayfield [a,1], Riccardo Barchiesi [a], Nihal A. Salem [a], R. Dayne Mayfield [a,b,*]

[a] Waggoner Center for Alcohol and Addiction Research and The University of Texas at Austin, Austin, TX, 78712, USA
[b] Department of Neuroscience, The University of Texas at Austin, Austin, TX, 78712, USA

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ABSTRACT

Toll-like receptors (TLRs) are a family of innate immune receptors that recognize molecular patterns in foreign pathogens and intrinsic danger/damage signals from cells. TLR7 is a nucleic acid sensing endosomal TLR that is activated by single-stranded RNAs from microbes or by small noncoding RNAs that act as endogenous ligands. TLR7 signals through the MyD88 adaptor protein and activates the transcription factor interferon regulatory factor 7 (IRF7). TLR7 is found throughout the brain and is highly expressed in microglia, the main immune cells of the brain that have also been implicated in alcohol drinking in mice. Upregulation of *TLR7* mRNA and protein has been identified in postmortem hippocampus and cortex from AUD subjects that correlated positively with lifetime consumption of alcohol. Similarly, *Tlr7* and downstream signaling genes were upregulated in rat hippocampal and cortical slice cultures after chronic alcohol exposure and in these regions after chronic binge-like alcohol treatment in mice. In addition, repeated administration of the synthetic TLR7 agonists imiquimod (R837) or resiquimod (R848) increased voluntary alcohol drinking in different rodent models and produced sustained upregulation of IRF7 in the brain. These findings suggest that chronic TLR7 activation may drive excessive alcohol drinking. In the brain, this could occur through increased levels of endogenous TLR7 activators, like microRNAs and Y RNAs. This review explores chronic TLR7 activation as a pathway of dysregulated neuroimmune signaling in AUD and the endogenous small RNA ligands in the brain that could perpetuate innate immune responses and escalate alcohol drinking.

1. Introduction

Alcohol use disorder (AUD) is an ongoing health and societal burden in the United States and globally ("Alcohol Use Disorder (AUD) in the United States: Age Groups and Demographic Characteristics," 2023; GBD 2019 Risk Factors Collaborators, 2020; Sohi et al., 2022). Yet, AUD remains under-treated with still only three FDA-approved drugs of modest efficacy available to patients. The initial discovery of upregulated immune-related genes in the postmortem cortex of AUD subjects opened a new area of research and new molecular targets to treat AUD. This research area was fueled by gene expression studies showing strong representation of immune- and inflammatory-related genes in postmortem brains from humans with AUD and rodents exposed to chronic alcohol (Liu et al., 2004; Liu et al., 2006; Mayfield et al., 2002; Robinson et al., 2014). There is now much corroborating and cross-species evidence that chronic alcohol consumption induces upregulation of proinflammatory and immune-related genes in the brain (Doremus-Fitzwater and Deak, 2022; Erickson et al., 2019b).

Toll-like receptors (TLRs) are critical for innate immune signaling and are upregulated in the brains of humans and rodents after chronic alcohol (Atkinson, 2023; Crews et al., 2017). For example, increased protein levels of TLRs 2, 3, and 4 in the postmortem orbitofrontal cortex of alcoholics compared to moderate drinking controls were positively correlated with lifetime consumption of alcohol (Crews et al., 2013). Chronic alcohol treatment in mice increased levels of TLRs 2, 3, and 4 in the orbitofrontal and entorhinal cortices (Crews et al., 2013). mRNA expression of TLRs 2, 3, and 4 also increased in mouse prefrontal cortex after voluntary alcohol consumption (McCarthy et al., 2018). An analysis of TLRs in the orbitofrontal cortex of AUD individuals showed increased mRNA expression of TLRs 2–9 (Vetreno et al., 2021).

* Corresponding author. Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin, 2500 Speedway, Stop A4800, Austin, TX, 78712, USA.
E-mail address: dayne.mayfield@austin.utexas.edu (R.D. Mayfield).
[1] contributed equally.

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Pharmacological and genetic manipulations of individual TLRs in rodents have also been shown to modulate drinking behavior. For example, genetic knockout of *Tlr2* in mice reduced alcohol consumption in some drinking models (Blednov et al., 2017; Corrigan et al., 2015). In male mice, knockout of *Tlr3* reduced voluntary alcohol consumption in an every other day drinking (intermittent) procedure (Blednov et al., 2021), whereas chronic activation of TLR3 increased intermittent drinking (Warden et al., 2019). Chronic TLR3 activation also increased operant self-administration of alcohol in alcohol-dependent male mice (Gano et al., 2023) and in male and female rats (Lovelock et al., 2022b; Randall et al., 2019). TLR4 is perhaps the most well-studied member of the TLR family. It plays a major role in alcohol-induced neurodegeneration (Moya et al., 2022; Pascual et al., 2021), but a comprehensive study in adult rodents showed that TLR4 does not directly regulate alcohol consumption (Harris et al., 2017).

The role of TLR7 signaling in AUD warrants further study considering that TLR7 and downstream innate immune genes are upregulated in human and rodent brains after chronic alcohol drinking and that repeated TLR7 stimulation increases drinking in rodent models. In the following sections, we discuss the effects of chronic alcohol on TLR7 expression and signaling responses in the brain, effects of repeated TLR7 activation on immune transcripts and alcohol consumption, and some of the endogenous sncRNA ligands that may activate TLR7 in the brain. Based on the current literature reviewed here, we propose that TLR7 is a novel neuroimmune target to reduce excessive alcohol drinking.

2. TLR7 activation and signaling

TLRs are pattern recognition receptors that detect pathogen-associated molecular patterns (PAMPs) of microorganisms as well as intrinsic danger or damage-associated molecular patterns (DAMPs). For TLR7, these include GU-rich single-stranded RNAs (ssRNAs) from viruses and endogenously derived (self) RNAs, such as microRNAs or other small noncoding RNAs (sncRNAs) (Chen et al., 2019; Petes et al., 2017). TLR7 is also activated by the synthetic RNA analogs imiquimod and resiquimod.

Transcription of *Tlr7* occurs in response to proinflammatory cytokine signaling, and TLR7 protein that is produced in the endoplasmic reticulum is trafficked via the Golgi to the endosome through a series of complex steps involving several chaperone and adaptor proteins (Petes et al., 2017). The endosomal localization of TLR7 is essential to detect viral nucleic acids that are endocytosed and to prevent inappropriate activation by self-ssRNAs (Diebold et al., 2004; Petes et al., 2017). Some endosomal TLRs have been shown to be mislocalized to the plasma membrane, however, which would increase access to extracellular ssRNAs (Atkinson, 2023). In addition to compartmentalization, there are other regulatory processes like ligand availability, receptor expression/trafficking, and signal transduction to help nucleic acid sensing TLRs discriminate between foreign and host nucleic acids (Lind et al., 2022).

TLR7, like all TLRs, has three distinct cellular domains, the first of which is an N-terminal ectodomain located inside the endosomal compartment that has two sites for binding ssRNAs and synthetic agonists like imiquimod and resiquimod (Ishida et al., 2021; Zhang et al., 2016; Zheng et al., 2023). The second domain is a single transmembrane hydrophobic helix that links the first domain in the endosomal compartment to the third domain in the cytoplasm. The cytoplasmic domain is the toll/interleukin-1 receptor domain that associates with the myeloid differentiation primary response 88 (MyD88) adaptor protein for signal transduction. Thus, upon binding ssRNA, TLR7 dimerizes and triggers MyD88-dependent signaling, leading to activation of the transcription factors interferon regulatory factor 7 (IRF7) or NF-κB and the production of type I interferons or other proinflammatory cytokines, such as TNFα, IL6, and IL1β (Petes et al., 2017).

3. TLR7 expression in the brain

TLR7 is widely expressed in the brain (Hawrylycz et al., 2012; Lein et al., 2007). Immunohistochemical findings showed strong neuronal expression of TLR7 (Lehmann et al., 2012a). However, single nucleus RNA sequencing (snRNA-seq) studies in rodents have shown that among the different CNS cell types, *Tlr7* is found predominantly in microglia and macrophages (Dilly et al., 2022; Michaelis et al., 2019; Salem et al., 2023). *In vitro* studies have also shown that microglia are highly sensitive to TLR7 stimulation. Compared to astrocytes, microglia had a higher abundance of *Tlr7* transcripts and were more sensitive to TLR7-mediated upregulation of innate immune genes (Madeddu et al., 2015; Michaelis et al., 2019; Zou et al., 2022). Brain microglia also demonstrated progressive morphological changes after chronic TLR7 activation *in vivo* (Michaelis et al., 2019). TLR7 microglial signaling is also implicated in neuronal cell death.

Our recent snRNA-seq and spatial transcriptomic study of mouse medial prefrontal cortex confirmed that *Tlr7* is predominantly expressed in microglia and macrophages (~10% of total microglia population expressed *Tlr7)* (Salem et al., 2023). The percentage of microglia expressing *Tlr7* was even higher in mouse amygdala (25.5%, R.D. Mayfield, unpublished data) and rat amygdala (Dilly et al., 2022). These results, together with high sensitivity to TLR7 stimulation, suggest that microglia play a predominant role in TLR7-mediated signaling. As the innate immune cells and first responders in the brain, microglial TLR7 signaling may be an important mechanism for regulating neuroinflammatory responses. Microglia depletion studies have demonstrated that microglia are required for dependence-induced escalations in alcohol consumption but do not alter drinking in non-dependent mice (Warden et al., 2020). A predominant role for glial cells in neuroinflammatory gene expression changes associated with alcohol dependence is supported by snRNA-seq findings in both human and mouse prefrontal cortex (Brenner et al., 2020; Salem et al., 2023). However, it should be noted that the role of glial cells in alcohol dependence is not restricted to neuroinflammatory signaling (Miguel-Hidalgo, 2018).

4. Chronic alcohol increases expression of TLR7 and related immune genes in the brain

Chronic alcohol exposure also induces activation of microglia and increases expression of TLR7 in human and rodent brains. *TLR7* mRNA and protein were upregulated in postmortem hippocampus from AUD individuals compared to control moderate drinkers, and *TLR7* mRNA correlated positively with lifetime alcohol consumption (Coleman et al., 2017). High-mobility group box 1 (HMGB1), a nuclear protein that extracellularly acts as a DAMP molecule to activate innate immune signaling, and a cell marker protein for microglia, were also upregulated in the hippocampus of AUD subjects. Similar results were found in rat hippocampal-entorhinal cortical slice cultures, where alcohol exposure for 48 h increased expression of *Tlr7* mRNA and protein and HMGB1 (Coleman et al., 2017). Alcohol also increased levels of the transcriptionally active phosphorylated NF-κB p65 subunit (pNF-κB-p65) and increased expression of *Myd88* and *Tnfa* that was inhibited by an siRNA against TLR7. Similar to findings in the hippocampus, *TLR7* mRNA and protein were upregulated in the orbitofrontal cortex of AUD individuals, and TLR7 protein expression correlated positively with lifetime consumption of alcohol (Qin et al., 2021). pNF-κB-p65 and IRF7 were also upregulated in the cortex of AUD cases. Intragastric administration of binge-like doses of alcohol (5 g/kg/day for 10 days) in mice also increased TLR7 protein in orbitofrontal and entorhinal cortices that colocalized with neuronal and microglial cell markers (Qin et al., 2021). As in the cortex of AUD cases, binge-like alcohol treatment in mice induced activation of pNF-κB-p65 and proinflammatory immune genes. These findings all show induction of TLR7 and downstream genes in hippocampal and cortical tissue from AUD subjects and rodents with a history of alcohol exposure.

TLR7/MyD88-dependent signaling leads to activation IRF7 (Petes et al., 2017; Zheng et al., 2019). Studies that did not specifically investigate TLR7 activation have shown that IRF7 expression in the brain is highly responsive to alcohol perturbation and is increased in human alcoholics (Kapoor et al., 2019) and in animal models after chronic intermittent ethanol (CIE) exposure (Erickson et al., 2019a), two-bottle choice chronic intermittent ethanol (2BC-CIE) with or without repeated stress exposure (Farris et al., 2020), or two-bottle choice every other day (2BC-EOD) ethanol consumption (Erickson et al., 2018). Cell-type enrichment studies indicated that CIE alters *Irf7* in both astrocytes and microglia (Erickson et al., 2019a), while expression changes induced by 2BC-EOD drinking are found only in astrocytes (Erickson et al., 2018). Table 1 summarizes the effects of ethanol on TLR7 and IRF7 expression levels in different species and brain regions.

Alcohol-induced increases in *Tlr7* expression may sensitize mice to the effects of TLR7 agonists. The agonists imiquimod (R837) and the more potent resiquimod (R848) activate TLR7 and TLR7/8, respectively. Due to species differences in the structure and abundance of murine vs. human TLR8, R848 does not activate TLR8 in rodents (Govindaraj et al., 2011). Both agonists have been used in rodent models to study TLR7-alcohol interactions. A single acute dose of imiquimod given after chronic treatment with binge-like doses of alcohol produced synergistic increases in the following responses compared to the effects of alcohol or imiquimod alone: a) ↑ mRNA expression of innate immune genes in whole brain, b) ↑ TLR7 and HMGB1 immunopositive cells in the entorhinal cortex, c) ↑ cell size of activated microglia in the entorhinal cortex, and d) ↑ neurodegeneration in orbitofrontal and entorhinal cortices (Qin et al., 2021). A low dose of imiquimod has also been shown to increase the neurotoxic effects of alcohol in rat hippocampal-entorhinal cortical slice cultures (Coleman et al., 2017).

The *Tlr7* gene is located on the X chromosome (O'Leary et al., 2016). It has been suggested that partial or escaped inactivation of the X chromosome in immune and other cells could cause TLR7 to be more highly expressed in females (Souyris et al., 2019). Increases in expression and activation of TLR7 could possibly sensitize females to the effects of alcohol and contribute to some of the differential responses to alcohol that have been observed between the sexes. Additional work is required to characterize possible sex differences in alcohol sensitivity to TLR7 pathway activation.

Table 1
Ethanol-induced upregulation of TLR7 and IRF7 in human and rodent brain.

	Upregulation of TLR7	
Human AUD	Hippocampus	Coleman et al. (2017)
Human AUD	Cortex, orbitofrontal	Qin et al. (2021)
Mouse binge ethanol	Hippocampal-entorhinal cortex slice culture	Qin et al. (2021)
Mouse 2BC-EOD	Cortex, prefrontal	Erickson et al. (2018)
Rat in vitro ethanol	Hippocampal-entorhinal cortex slice culture	Coleman et al. (2017)
	Upregulation of IRF7	
Human AUD	Cortex, orbitofrontal	Qin et al. (2021)
Human AUD	Cortex, dorsolateral	Kapoor et al. (2019)
Mouse 2BC-EOD	Cortex, prefrontal (astrocytes)	Erickson et al. (2018)
Mouse CIE	Cortex, prefrontal (astrocytes)	Erickson et al., 2019a
Mouse CIE	Cortex, prefrontal (microglia)	Erickson et al., 2019b
Mouse 2BC-CIE-Stress	Cortex, prefrontal	Farris et al. (2020)
Mouse binge ethanol	Cortex, orbitofrontal and entorhinal	Qin et al. (2021)

5. Chronic TLR7 activation produces behavioral and molecular tolerance

R837 and R848 induce acute sickness responses in mice and rats, characterized by decreased food, water, or saccharin intake, weight loss, and decreased voluntary locomotor activity (Damm et al., 2012; Grantham et al., 2020; Lovelock et al., 2022a; Michaelis et al., 2019). The effects of R848 were prevented in mice with genetic knockout of either TLR7 or MyD88 (Michaelis et al., 2019), indicating classical TLR7 signaling in the sickness response. A single intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) injection of R848 was able to produce the sickness response in mice (Michaelis et al., 2019). Thus, stimulation of TLR7 in the brain directly mediates an immune response without peripheral involvement. Even low doses that do not produce the sickness response when given i.p., did so when given i.c.v. (Michaelis et al., 2019). After repeated i.p. injections of R848 or R837, however, tolerance develops to the behavioral effects (Grantham et al., 2020; Lovelock et al., 2022a; Michaelis et al., 2019).

The behavioral tolerance was associated with development of molecular tolerance. *Tlr7* and proinflammatory genes were initially upregulated in the hypothalamus of mice following a single injection of R848 (10 μg, i.p.) but returned to baseline after 12 daily doses (Michaelis et al., 2019). Another study measured expression of both MyD88- (*Tlr7, Tlr4, Myd88, Irf7, Il1b*) and TRIF-dependent (*Tlr3, Trif, Ikke, Irf3*) pathway genes after a single injection of R848 (50 μg, i.p.) and found upregulated genes from both pathways at 8 h post-injection in mouse prefrontal cortex, nucleus accumbens, hippocampus, and ventral tegmental area, while there was downregulation in the amygdala (Grantham et al., 2020). However, after 24 h, only *Irf7* remained upregulated across all brain regions, whereas *Irf3* was downregulated in several regions. Chronic administration of R848 produced downregulation of *Tlr7* and *Tlr3* in mouse prefrontal cortex, while there was persistent upregulation of *Irf7* (Grantham et al., 2020).

Collectively, these studies showed rapid upregulation of *Tlr7* and proinflammatory genes across different mouse brain regions after acute R848 exposure, followed by normalization or downregulation after chronic exposure, indicating development of molecular tolerance. However, chronic TLR7 activation produced sustained upregulation of *Irf7* (Grantham et al., 2020) and progressive changes to microglia indicative of increased microglial surveillance (Michaelis et al., 2019), suggesting mechanisms for persistent immune activation. These findings further demonstrate that activation of TLR7 that begins in the periphery impacts innate immune signaling in the brain, as reported previously (Damm et al., 2012). This study also showed that acute TLR7 activation using R837 (s.c. or i.p.) in rats induced distinct inflammatory markers in the spleen and liver compared to the hypothalamus, such as peripheral expression of interferons and strong brain activation of the transcription factor NF-IL6 (Damm et al., 2012).

6. Chronic TLR7 activation increases voluntary alcohol consumption in rodents

The acute sickness response following stimulation of TLR7 is associated with decreased alcohol consumption in rodent drinking models (Grantham et al., 2020; Lovelock et al., 2022a). However, repeated administration of R837 or R848 has been shown to increase alcohol consumption in these models. Pretreatment with R848 (50 μg, i.p.) administered every other day (10 total doses), followed by a 2-week recovery period, significantly increased voluntary alcohol consumption in a 2BC-EOD drinking procedure in male mice (Grantham et al., 2020). We have since replicated these findings in two cohorts of male mice (unpublished data, R.D. Mayfield). The molecular effects were measured separately 24 h after a similar treatment protocol. Chronic every other day injection (10 doses) of R848 resulted in downregulation of *Tlr7* in the prefrontal cortex, while *Irf7* remained upregulated, as it did after acute activation (Grantham et al., 2020). Repeated activation

of TLR7 using a different agonist and treatment protocol has also been shown to increase operant self-administration of alcohol in male and female rats (Lovelock et al., 2022a). In rats expressing stable levels of alcohol self-administration, R837 (10 mg/kg, i.p.) was administered once every 15 days, while the animals had continuous access to operant drinking sessions. The day after the third R837 injection, both male and female rats significantly increased self-administration of alcohol during a 30-min session (Lovelock et al., 2022a). Gene expression was measured 24 h after the fourth dose of R837, revealing upregulation of *Irf7* in rat nucleus accumbens core and anterior insula in both sexes. This is consistent with previous findings in the prefrontal cortex from male mice (Grantham et al., 2020). As discussed earlier, IRF7 expression in the brain is also increased in AUD cases and in alcohol-dependent mice. While persistent upregulation of certain immune transcripts may drive alcohol consumption, the role of IRF7 in drinking behavior is not known. Identifying a molecular mechanism will also require better understanding of the endogenous activators of TLR7 signaling in the brain.

The rodent models allude to the possibility that chronic TLR7 activation could be involved in drinking escalations in AUD. In the human brain, this could occur through repeated activation of TLR7 by endogenous ssRNAs, as we discuss in the following sections.

7. Endogenous TLR7 ligands in the brain

In addition to sensing nucleic acids from pathogens and synthetic small molecule agonists, TLR7 detects endogenously produced sncRNAs, such as microRNAs and Y RNAs. ssRNAs that are found in the brain may originate from the periphery or from within the brain. TLR7 stimulation (Zou et al., 2022) and chronic alcohol exposure (Vore and Deak, 2022) can both increase permeability of the blood-brain barrier (BBB). The combined inflammatory effects of chronic alcohol and TLR7 activation on the BBB could increase brain levels of extracellular ssRNAs that enter the endosome and bind TLR7, further propagating neuroimmune signaling.

ssRNAs are released from cells in various forms – as free RNA, bound to ribonucleotide protein complexes, or inside extracellular vesicles (EVs) (Gulìa et al., 2020). EVs are small lipid bilayer-enclosed vesicles containing proteins and nucleic acids that are released by virtually all cells (Driedonks and Nolte-'t Hoen, 2018). EVs can cross the BBB through various mechanisms (Banks et al., 2020), and cellular communication through EVs is critical for maintaining homeostasis. EVs have been shown to contain many sncRNAs and may be an important delivery mechanism for endogenous RNA species (Di Liegro et al., 2017; Driedonks and Nolte-'t Hoen, 2018).

8. MicroRNA TLR7 ligands and chronic alcohol exposure in the brain

MicroRNAs (miRNAs) are highly conserved sncRNAs that regulate post-transcriptional gene expression. Beyond this conventional role, miRNAs act as endogenous signals for receptor activation, including activation of TLRs.

Let-7. Members of the lethal-7 (*let-7*) family (*let-7a-7i*, *miR-98*) are abundantly expressed in the brain with high cross-species sequence conservation (Ma et al., 2021; Roush and Slack, 2008). *Let-7* miRNAs regulate cell differentiation and proliferation and tumor growth (Ma et al., 2021). The sequence GUUGUGU found in *let-7b* is a TLR7 recognition motif first identified in ssRNA40 derived from HIV (Forsbach et al., 2008; Heil et al., 2004). In addition to the GU-rich sequence, TLR7 and MyD88 were required for synthetic *let-7b* induction of cytokine release in microglia and induction of apoptosis in mouse cortical and hippocampal neurons (Lehmann et al., 2012a). These results translated to findings *in vivo*, where intrathecal injection of *let-7b* into the cerebrospinal fluid induced neurodegeneration in wild-type but not in TLR7 knockout mice (Lehmann et al., 2012a). Neuronal susceptibility to *let-7b* in the knockout mice was restored by *in utero* transfection of TLR7. Synthetic oligoribonucleotides corresponding to *let-7a*, *let-7c*, and *let-7g* were also shown to induce a cytokine response through TLR7 activation (Lehmann et al., 2012a). An analysis of the *let-7* family identified several other members that induce cytokine release and other responses in microglia in a TLR7-dependent manner (Buonfiglioli et al., 2019). The UUGU sequence (present in *let-7a-g*, *let-7i*, *miR-98*) was identified as the minimum required motif for activating TLR7 (Buonfiglioli et al., 2019). Thus, *let-7b* and other family members are potent endogenous activators of TLR7 in brain microglia.

The *let-7* family is also implicated in AUD and alcohol-induced neurotoxicity. We showed that this family of miRNAs was upregulated in the postmortem prefrontal cortex from AUD subjects (Lewohl et al., 2011) and in mouse cortex after voluntary alcohol consumption (Nunez et al., 2013). Chronic binge-like alcohol treatment in mice increased expression of TLR7 protein and *let-7b* and proinflammatory genes in cortical regions and increased sensitivity to TLR7-induced neurodegeneration that was blocked by a TLR7 inhibitor (Qin et al., 2021). A *let-7b*-TLR7 mechanism was confirmed in organotypic brain slice cultures from mice, where an antagomir to *let-7b* blocked alcohol-induced neurodegeneration (Qin et al., 2021). In hippocampal-entorhinal cortex slice cultures from rats, alcohol also increased expression of *Tlr7* and *let-7b* and release of *let-7b*/HMGB1 complexes in EVs from microglia, and increased the neurotoxic effects of *let-7b* and TLR7 activation (Coleman et al., 2017). Collectively, these findings demonstrate that *let-7b* is a highly sensitive TLR7 ligand in the brain that plays a role in alcohol-induced neuroinflammation.

MiR-21. Neurotoxicity induced by *miR-21* also depends on TLR7 activation. Increased levels of *miR-21* in EVs were isolated from the brains of rhesus macaques with simian immunodeficiency virus encephalitis (SIVE) (Yelamanchili et al., 2015). Oligonucleotides of wild type *miR-21* encased in synthetic EVs induced neurotoxicity in hippocampal neuronal cultures, but a mutation in the TLR7 binding motif of *miR-21* eliminated the neurotoxic effects (Yelamanchili et al., 2015). EVs were a critical delivery mechanism as neuronal cultures inoculated with naked ssRNAs did not show neurotoxic effects. Furthermore, EVs from macaques with SIVE also produced neurotoxicity in hippocampal neurons and activated TLR7 signaling in HEK cells expressing TLR7.

Both *miR-21* and *miR-14a* are regulated in a sex-dependent manner by binge drinking (Ibáñez et al., 2020), and are discussed together in the following section.

MiR-146a. *miR-146a* is a highly upregulated miRNA in the plasma of mice and humans during sepsis, and when tested alone, was sufficient to induce glial activation *in vitro* and *in vivo* (Zou et al., 2022). Treatment of cultured microglia and astrocytes with *miR-146a-5p* or i.c.v. injection of *miR-146a-5p* in mice increased production of cytokine and chemokine genes (Zou et al., 2022). The microglial responses were much more robust compared to astrocytes, consistent with higher *Tlr7* expression in microglia. There were also increased numbers of brain microglia after i.c.v injection of *miR-146a-5p*. Knockout of TLR7 inhibited the proinflammatory responses of *miR-146a-5p* in cultured microglia and in the cortex and hippocampus. Furthermore, TLR7 knockout septic mice showed preserved BBB integrity, reduced expression of cytokine genes in the cortex and hippocampus, and reduced brain microglia expansion. These results indicate that *miR-146a* induces proinflammatory innate immune activation through TLR7-dependent signaling in the brain.

MiR-146a is also regulated by alcohol exposure. Cultured mouse cortical astrocytes exposed to alcohol showed increased release of EVs containing alcohol-sensitive miRNAs, such as *miR-146a* (Ibáñez et al., 2019). Administering these EVs to cultured neurons induced expression of proinflammatory genes and apoptosis. *In vivo* findings showed that binge drinking reduced levels of anti-inflammatory *miR-146a-5p* and *miR-21* in plasma EVs from human and mouse female adolescents but increased levels from intoxicated male adolescents (Ibáñez et al., 2020). The sex-dependent differences were evaluated further in the mouse cortex after alcohol withdrawal. After a 2-week withdrawal period, *miR-146a-5p* was significantly increased in cortex from adolescent male

mice, while both *miR-146a-5p* and *miR-21* were significantly decreased and their proinflammatory target genes were increased in cortex from adolescent females.

Collectively, these findings indicate that alcohol-induced changes in extracellular levels of *miRNAs* that bind TLR7 could regulate neuroinflammatory responses in the brain and that EV-containing miRNAs could be useful biomarkers of disease.

9. Y RNA TLR7 ligands

There are other non-canonical sncRNAs derived from longer precursor RNAs called Y RNAs (yRNAs) that activate TLR signaling. yRNAs have been detected in cell lines, retroviruses, and in the blood and other body fluids from multiple species (Driedonks and Nolte-'t Hoen, 2018; Gulìa et al., 2020). We have identified multiple yRNA transcripts in human and mouse brain (R.D. Mayfield, unpublished data), but their function is unknown. The nomenclature for the derivative sncRNAs is varied in the literature. Here, we use yRNA-derived small RNA (ysRNA) (Shi et al., 2022). The size of most parent yRNAs are ~83–110 nucleotides, while most ysRNA fragments are ~25–35 nucleotides (Driedonks and Nolte-'t Hoen, 2018; Guglas et al., 2020). The enzyme RNAseL cleaves yRNAs in response to UV damage or innate immune activation. ysRNAs are similar in size and structure to miRNAs and were originally classified as such. However, yRNAs are transcribed by RNA polymerase III and processed independently of DICER and thus represent a separate class of non-canonical RNAs (Gulìa et al., 2020). yRNAs are implicated in DNA replication, RNA quality control, immune signaling, and inflammatory diseases (Driedonks and Nolte-'t Hoen, 2018). yRNAs and ysRNAs can be bound and stabilized by various RNA binding proteins, such as Ro60, which is often targeted by the immune system in autoimmune diseases (Boccitto and Wolin, 2019). They are also one of the most prominent RNA species in EVs (Driedonks and Nolte-'t Hoen, 2018).

There are four known genes that code for yRNAs in humans (*RNY1*, *RNY3*, *RNY4*, and *RNY5*) and two in mice (*Rny1* and *Rny3*). Human transcripts (Y1, Y3, Y4, Y5) are not limited to four genes, however, and also include an abundance of pseudogenes (Gulìa et al., 2020). yRNAs have been shown to regulate TLR signaling, including activation of TLR7 (Driedonks et al., 2020). For example, human (Y1, Y3, Y4, Y5) and mouse Y1 transcripts were shown to activate TLR7 in HEK 293 cell lines transfected with TLR7, though hY5 produced weak activation (Greidinger et al., 2007). Both human and mouse Y3 activated TLR3 in 293 cells expressing TLR3. ysRNAs (but not precursor yRNAs) associated with Ro60 induced cell death and inflammation in cultured human monocytes and mouse macrophages, which were blocked by antisense oligonucleotides to ysRNAs (Hizir et al., 2017). In addition, intracellular and extracellular ysRNA/Ro60 induction of inflammation and apoptosis in monocytes/macrophages was blocked by TLR7 inhibition.

As TLR7 and immune activators, ysRNAs are ligands of interest for their possible role in immune signaling in the brain and in AUD. This is an unexplored area of research that could discover novel neuroimmune activators and signaling mechanisms. After detecting yRNAs in both human and mouse brain, our future work will focus on identifying ysRNAs that may act as endogenous regulators of TLR7 function.

10. TLR7 antagonists as potential treatments for AUD

Agonists of TLR7 and other nucleic acid sensing TLRs are being investigated as vaccine adjuvants for infectious diseases and as immune boosting therapies in cancers, while small molecule TLR7 antagonists have been developed that are being studied in autoimmune diseases (Lind et al., 2022) and in treating severe COVID-19 (Khalifa and Ghoneim, 2021; Liu et al., 2022). TLR7-specific therapeutic options could also hold promise for AUD treatment strategies. Some of these antagonists are able to cross the BBB and would be interesting candidates to test in animal drinking models and ultimately in human trials. For example, CMPD2, a small molecule TLR7 antagonist has been shown to block TLR7-mediated neurodegeneration in response to ethanol (Qin et al., 2021). For AUD and other neuroinflammatory diseases, small molecule TLR7 antagonists that reach the brain could potentially target the overactivated neuroimmune signaling that is associated with disease progression.

11. Summary and perspectives

Innate immune pathways disrupt homeostasis when chronically activated, leading to the overproduction of proinflammatory cytokines and chemokines. Targeting the molecular sources of chronic neuroimmune signaling has taken precedence in the treatment of many different neurodegenerative and psychiatric diseases, including AUD (Adebambo and Ojoh, 2024; Grantham et al., 2023; Namba et al., 2021). As we discuss here, TLR7 stimulation in the brain may be a source of persistent immune signaling in AUD that contributes to excessive alcohol consumption. For example, repeated TLR7 activation using R848 or R837 increased voluntary alcohol consumption in different rodent drinking models and caused persistent upregulation of *Irf7* in the brain (Grantham et al., 2020; Lovelock et al., 2022a). Upregulation of IRF7 in the brain has also been found in AUD subjects and in different animal models after chronic drinking or binge-like alcohol treatment (Erickson et al., 2018; Erickson et al., 2019a; Farris et al., 2020; Kapoor et al., 2019; Qin et al., 2021). Thus, there is corroborating evidence implicating this transcription factor as a downstream target to reduce excessive alcohol drinking.

Both TLR7 stimulation (Zou et al., 2022) and chronic alcohol exposure (Vore and Deak, 2022) increase permeability of the BBB, which may increase availability of extracellular ssRNAs in the brain, leading to persistent activation of TLR7 and exacerbation of alcohol drinking. Some of the RNAs could be biomarkers of disease or potential targets to prevent chronic TLR7 activation in the brain. sncRNAs are often found in EVs that can cross the BBB into the circulatory system, thus facilitating isolation and detection of biomarkers circulating in the blood. However, given the various classes of sncRNAs, identifying which class to investigate is in itself a challenge. For example, yRNAs have emerged as important regulators of immune and inflammatory diseases and have been shown to activate TLR signaling (Driedonks et al., 2020). We have detected yRNAs in mouse and human brains and plan to investigate the ysRNA derivatives as potential endogenous regulators of neuroimmune signaling in AUD.

Tlr7 is highly expressed in microglia (Dilly et al., 2022; Michaelis et al., 2019; Salem et al., 2023). Microglia are highly sensitive to TLR7 signaling and mediate neurodegeneration through TLR7-dependent mechanisms (Lehmann et al., 2012a, 2012b). Microglia have also been shown to regulate alcohol drinking behavior. For example, depletion of microglia prevented escalation of drinking in alcohol-dependent mice (Warden et al., 2020) and also prevented escalation of drinking caused by chronic activation of TLR3 (Warden et al., 2021). Thus, microglial signaling may promote alcohol consumption under conditions of sustained immune activation. Upregulation of alcohol-responsive reactive astrocyte genes has also been reported after microglia depletion (Warden et al., 2021). These results are supported by an snRNA-seq analysis in human and mouse prefrontal cortex that showed most alcohol-responsive genes are in glial cells (Brenner et al., 2020; Warden et al., 2020). Furthermore, an snRNA-seq and spatial transcriptomics study found that neuroinflammatory gene expression changes in prefrontal cortex from alcohol-dependent mice were specifically enriched in microglia (Salem et al., 2023). The integration of single cell and spatial transcriptomics could provide unprecedented insights into neuroimmune mechanisms by mapping cell-type and brain regional gene expression changes in response to alcohol and other stressors. Manipulating TLR7 signaling in individual cell types will be an important next step to identify cellular mechanisms of increased alcohol drinking, as well as identifying the predominant blood biomarkers that activate

immune responses.

CRediT authorship contribution statement

Ruth L. Allard: Writing – original draft, Conceptualization. **Jody Mayfield:** Writing – review & editing, Writing – original draft. **Riccardo Barchiesi:** Writing – original draft. **Nihal A. Salem:** Writing – original draft. **R. Dayne Mayfield:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors report no competing interests.

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