Review

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Pharmacogenomics

The neuroimmune transcriptome and alcohol dependence: potential for targeted therapies

Transcriptome profiling enables discovery of gene networks that are altered in alcoholic brains. This technique has revealed involvement of the brain's neuroimmune system in regulating alcohol abuse and dependence, and has provided potential therapeutic targets. In this review, we discuss Toll-like-receptor pathways, hypothesized to be key players in many stages of the alcohol addiction cycle. The growing appreciation of the neuroimmune system's involvement in alcoholism has also led to consideration of crucial roles for glial cells, including astrocytes and microglia, in the brain's response to alcohol abuse. We discuss current knowledge and hypotheses on the roles that specific neuroimmune cell types may play in addiction. Current strategies for repurposing US FDA-approved drugs for the treatment of alcohol use disorders are also discussed.

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Gene expression profiling is a powerful tool for defining key regulatory genes as well as molecular targets for potential drug development or repurposing. In particular, gene expression profiling has been instrumental in understanding how the neuroimmune system contributes to various aspects of alcohol dependence. Some of the first microarray analyses of cortical samples from human alcoholic brain identified differentially expressed genes associated with alcohol use disorder (AUD) [1–9]. Immune-related genes emerged as an unexpected category of abundantly changed genes in alcoholics [3]. This discovery catalyzed investigation into the role of immune signaling in alcohol dependence. Many of the immune-related genes were related to inflammatory responses such as production of cytokines, chemokines, glial activation and signaling components of the NF-κB inflammatory pathway [5]. Complementing the gene expression data, NF-κB and its p50 homodimer were shown to be upregulated in human alcoholic frontal cortex [10]. In addition, human alcoholics show positive correlations between alcohol craving and serum levels of lipopolysaccharides, peptidoglycans, cytokines and chemokines (e.g., IL-8, IL-1β), suggesting that activation of the innate immune system may regulate alcohol craving and consumption [11–13]. Knockout of some of the immune genes nominated by the gene expression studies (*B2m*, *Cd14*, *Il1rn*, *Il6*, *Ctss* and *Ctsf*) reduced ethanol consumption and preference in the 24-h two-bottle choice test in mice and provided behavioral validation for these candidate genes [14]. In contrast, the immune activator lipopolysaccharide (LPS) increased ethanol consumption in mice [15], indicating that enhancement of immune signaling is associated with increased drinking as suggested in humans. These preclinical and clinical evidences support the hypothesis that activation of inflammatory pathways is a key process in alcohol dependence and further implicate neuroimmune pathways as relevant targets to treat AUD.

In brain, alcohol induces neuroinflammatory and neurodegenerative changes partially mediated by innate immune activation [16,17].

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The TLR pathway, which activates NF-κB and leads to the secretion of pro-inflammatory cytokines and chemokines, has been suggested to be a key regulator of alcohol-induced innate immune activation. It remains unclear which cell types are mediating this response in the CNS. Neurons, astrocytes and microglia release and respond to neuroimmune signaling components. To determine if cell-type specific modulation of neuroimmune signaling is important in alcohol dependence, it is necessary to understand the transcriptional changes that occur in each cell type after ethanol exposure. Transcriptomics has already been used to identify potential drugs for treating AUD [18], and transcriptome profiling in different cell types after ethanol exposure will further define discrete cellular effects in the brain.

The mechanisms by which alcohol triggers inflammation in the brain are only partially understood, complicated in part by the interplay between peripheral and central immune activation. Alcohol consumption increases intestinal permeability and levels of LPS, resulting in peripheral and central immune activation [11,12,15,19]. Moreover, the extent to which stress plays a role in central immune activation is another complicating factor. Literature has shown that stress increases brain cytokines which potentially act as neuromodulators of alcohol consumption (for review see [16]). An additional consideration is that withdrawal from alcohol is an extremely stressful event, causing stress-induced increases in CRF as well as cytokines [17]. Because of the potential effects of stress on central immune activation, the drinking studies cited in this review focus on ethanol administration (e.g., alcohol fed diet, two-bottle choice tests, operant self-administration) under non-stressful conditions, unless otherwise noted. Additionally, levels of cytokines and other neuroimmune mediators measured during alcohol withdrawal are not discussed here.

We will discuss the TLR neuroimmune pathway and its validated genomic targets as well as the potential for identifying new drug targets that are TLR- and cell-type specific. We also review alcohol-induced transcriptional changes in astrocytes, microglia and neurons and their role as immune regulators and cellular targets for future drug development. Finally, we discuss using cutting-edge technology, such as the Library of Integrated Network-based Cellular Signatures (LINCS) program and other genomics strategies, to predict drugs for the treatment of alcohol dependence. Although these techniques are still in their infancy, they provide promising examples of the future of drug repurposing as a strategy for advancing therapeutic options for AUD.

Toll-like receptors

TLRs & immune responses

Innate immunity is the body's first defense against infection and is responsible for most peripheral and central inflammatory responses. Acute inflammatory stressors induce a large number of genomic/transcriptional responses, and unlike adaptive immunity, do not require genetic rearrangement to mount an inflammatory response [20]. Cells of the innate immune system rely on germ-line encoded receptors that recognize broadly defined molecular motifs known as pathogen-associated molecular patterns (PAMPs). Multiple receptor families are comprised of transmembrane pattern-recognition receptors (PRRs), which initiate inflammatory signaling in response to a wide variety of PAMPs. TLRs represent a class of PRRs that are critical for both innate immune reactions to pathogens and for initiating adaptive immunity [20]. Most TLRs rely on homodimerization to achieve specific agonist recognition [21]. In addition to detecting molecular motifs associated with microorganisms, TLRs recognize endogenous ligands referred to as damage- (or danger-) associated molecular patterns (DAMPs), including β-defensin, heat shock proteins 60 and 70, stathmin, HMGB1 and reactive oxygen species [22,23]. The recognition of specific PAMPs or DAMPs leads to receptor activation and initiation of multiple signaling cascades, eventually resulting in NF-κB pathway activation and immune-related gene expression changes.

There are currently 11 known TLRs in humans and 13 in mice, and each receptor responds to specific classes of pathogens (Figure 1). TLRs 1–2, 4–6 and 11–13 are localized on the cell surface, whereas TLRs 3 and 7–9 are found on the endosome or lysosome compartments in the endoplasmic reticulum [24,25]. TLR2 recognizes lipoproteins, peptidoglycan (PGN) and dectin, and TLR2 dimerization with TLR1 or TLR6 allows for the discrimination between triacylated and diacylated lipoproteins, respectively. LPS primarily targets TLR4. TLR2 and TLR4 can also form oligomers, which recognize a broader range of microbial motifs [26]. TLR5 senses bacterial flagella and is commonly found in the intestine [27]. TLR11 responds to bacteria associated with urinary tract infections, such as uropathogenic *Escherichia coli* and a profilin-like protein from the parasite *Toxoplasma gondii* [28]. TLRs 3 and 7–9 recognize intracellular pathogen-derived nucleic acid motifs such as dsRNA, ssRNA and even mRNA from viruses, pathogens or infected cells [29]. TLR9 recognizes non-methylated CpG islands of bacterial and viral mRNAs. PAMPs for TLRs 10, 12 and 13 are not yet well established. Recent evidence suggests that TLR10 may act as a modulatory receptor with mainly inhibitory effects by forming a heterodimer with TLR2 [30]. TLR12 was

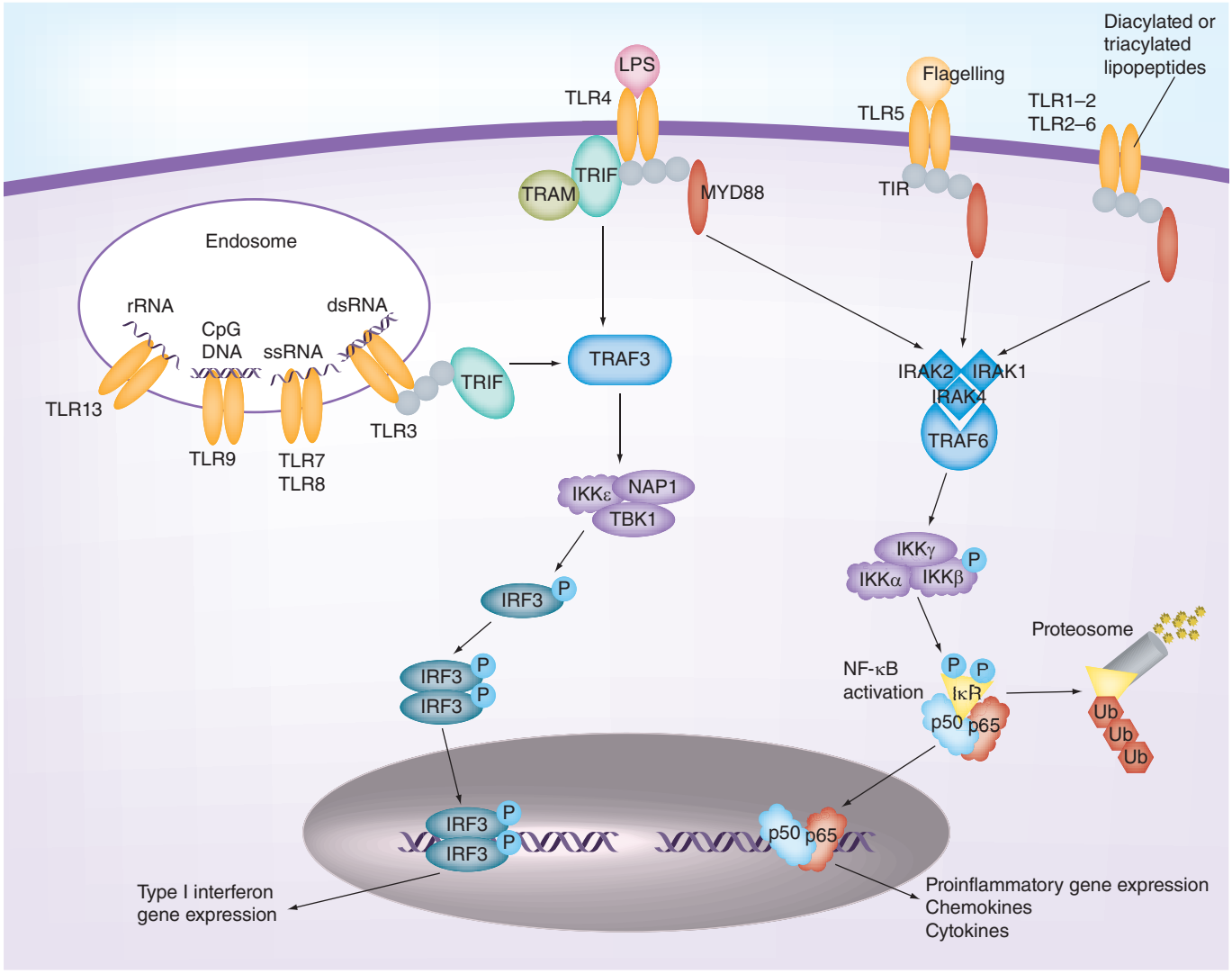

Figure 1. Toll-like receptors initiate inflammatory responses via two intracellular signaling transduction cascades.

hypothesized to form a heterodimer with TLR11 to recognize a profilin-like protein from *T. gondii* [31].

TLRs initiate inflammatory responses via two intracellular signaling transduction cascades: MyD88-dependent pathway and MyD88-independent pathway. As shown in Figure 1, TLRs are activated by binding of PAMPs or DAMPs to initiate an innate immune response. After binding, all TLRs (except for TLR3) utilize the MyD88-dependent pathway, via recruitment of adaptor proteins in the cytoplasmic Toll/interleukin receptor (TIR) domain (MyD88, IRAK4 and IRAK) to activate NF-κB [32]. In contrast, TLR3 recruits TIR-domain-containing adaptor-inducing IFN-β (TRIF) and bifurcates to activate either type-I interferons or NF-κB [32]. Interestingly, TLR4 can also induce inflammatory responses via the MyD88-independent pathway by recruiting the TLR-adaptor proteins TRAM and TRIF [33].

TLRs & alcohol exposure

Differential expression of TLRs 1, 6 and 7, as well as genes downstream of NF-κB (e.g., *Casp8*, *Fadd*, *Ikbkb*, *Kbkg*, *Tradd*, *Map3k1*), were observed in mice predisposed to drink ethanol [34]. Microarray analysis of gene expression in *Drosophila* showed ethanol increases expression of genes in the Toll pathway [35,36], as seen in vertebrates. An increase in mRNA expression of *Tlr* 1, 2, 4 and 6–9 was detected in the liver of mice fed an ethanol-containing diet for 10 days [37]. Additionally, mice consuming ethanol for 5 weeks showed increased *Tlr* 2, 4 and 9 mRNA in the cerebellum [38]. Ten days of binge-like drinking increased brain mRNA and protein expression of TLRs 2–4 in rats [39]. Further support comes from study of human postmortem brain showing increased protein expression of TLRs 2–4, which correlated with lifetime alcohol consumption [39].

Despite the evidence that TLRs are key regulators of immune activity in response to alcohol, only a few studies have examined the role of TLRs in regulating behavioral responses to alcohol. Most research has focused on how ethanol changes TLR expression, in particular TLR4, due to its apparent ability to protect against ethanol-induced changes in immune gene expression. Knockout of TLR4 or its downstream signaling component, MYD88, as well as TLR4 antagonism by (+)-naloxone in mice decreased the duration of loss of righting reflex and motor impairment recovery time induced by ethanol [40]. A naturally TLR4-deficient mouse model C3H/HeJ showed a decrease in ethanol consumption compared with a control strain with a functional TLR4 receptor [41], although global knockout of TLR4 did not reduce ethanol consumption [42]. These studies highlight the potential role of TLR4 in ethanol's behavioral responses; however, it should be noted that these results cannot distinguish between peripheral and central TLR involvement. To determine if the immune targets in brain can directly modulate behavior, researchers injected siRNA into brain regions involved in alcohol abuse and monitored ethanol consumption. Injection of *Tlr4* siRNA into the central amygdala of alcohol-preferring rats reduced operant administration of ethanol but not sucrose [43]. Furthermore, infusion of amplicons for *Tlr4* or *Mcp1* siRNA into the central amygdala or ventral tegmental area inhibited target gene expression and altered binge-like drinking behavior [44,45]. Amplicons for scrambled siRNA did not inhibit TLR4 or MCP-1 expression or reduce binge drinking, suggesting that a specific neuronal TLR4/MCP-1 signal may regulate initiation of voluntary alcohol self-administration [44].

Other types of TLRs may also regulate inflammatory responses to alcohol. *In vitro* studies showed that acute ethanol treatment enhances pro-inflammatory cytokine production in response to a variety of TLR agonists. For example, acute ethanol enhanced Poly I:C (TLR3 ligand), PGN (TLR2 ligand) and microbial methylated DNA CpG (TLR9 ligand) stimulated TNF-α, IL-6 and IL-12 synthesis in murine macrophages [46,47]. In mouse models of alcoholic liver disease, activation of TLR3 attenuated alcoholic liver injury by stimulating Kupffer and stellate cells to produce the anti-inflammatory cytokine IL-10 [48]. However, ethanol-induced TLR3 activation can also have neurodegenerative effects. For example, chronic ethanol exposure combined with TLR3 stimulation via Poly I:C led to an increase in pro-inflammatory cytokines in rat brain (e.g. TNF-α, IL-6, IL-1β and MCP-1) [49,50]. It should be noted that in Qin *et al.* [50], ethanol was administered intragastrically which could cause stress-induced increases in cytokine responses (for review see [16]). Similar to TLR4, TLR2 may have a brain region-specific effect on alcohol-induced immune responses. Mice chronically treated with ethanol for 5 months showed increased levels of cytokines and chemokines in the striatum; however, there was no change in TLR2 knockout mice, suggesting that TLR2 also regulates ethanol-induced pro-inflammatory gene expression [51]. Elimination of TLR2 in the striatum also appeared to protect against abstinence-induced anxiogenic behavior, evaluated by elevated plus-maze and dark and light box tests [51]. These studies suggest that TLR signaling is critical for alcohol-induced cytokine production and represent potential targets for regulating behavioral responses to alcohol.

TLR cell-type specificity & alcohol modulation

The cellular specificity of TLRs is an important consideration for developing drug targets that control alcohol-induced inflammatory gene expression. As shown in Figure 2, the expression of TLRs and related signaling proteins has been demonstrated in all cell types, with the strongest expression in glia (microglia, astrocytes and oligodendrocytes) and more limited expression in neurons [52–55]. Consequently, the majority of studies have examined how ethanol activates TLRs in glia. Ethanol directly activates microglia and astrocytes, resulting in stimulation of NF-κB, MAPKs, MyD88-independent pathways and inflammatory responses [42,55–58]. However, neurons likely play a regulatory role in TLR responses to alcohol-induced inflammation, most likely by stimulating microglia. For example, neuronal stimulation causes release of HMGB1, an endogenous ligand for TLR2, TLR4 and RAGE that has been shown to increase NF-κB-mediated transcription of pro-inflammatory cytokines [39,59].

Chronic alcohol abuse also activates microglia in the orbital frontal cortex of alcoholics and in the cortex of ethanol-treated mice [49]. In microglial cultures, ethanol upregulates TLR2 and TLR4 expression, causing increased production of inflammatory mediators [60]. Moreover, TLR4 expression and TLR2/4 association in cultured microglia appear to be necessary for alcohol-induced microglia activation and production of inflammatory mediators [38,57,60]. Facci *et al.* (2014) demonstrated that TLR 2-4 endogenous ligands prime microglia (but not astrocytes) across the central nervous system for ATP-dependent IL-1β release [61]. Microglia-specific priming may be linked to the increased IL-1β that is observed in ethanol-fed mice and human alcoholic frontal cortex [38], providing evidence that microglia are necessary mediators of alcohol-induced inflammatory responses.

Additionally, astrocytes may be important in TLR-mediated inflammatory signaling, although fewer

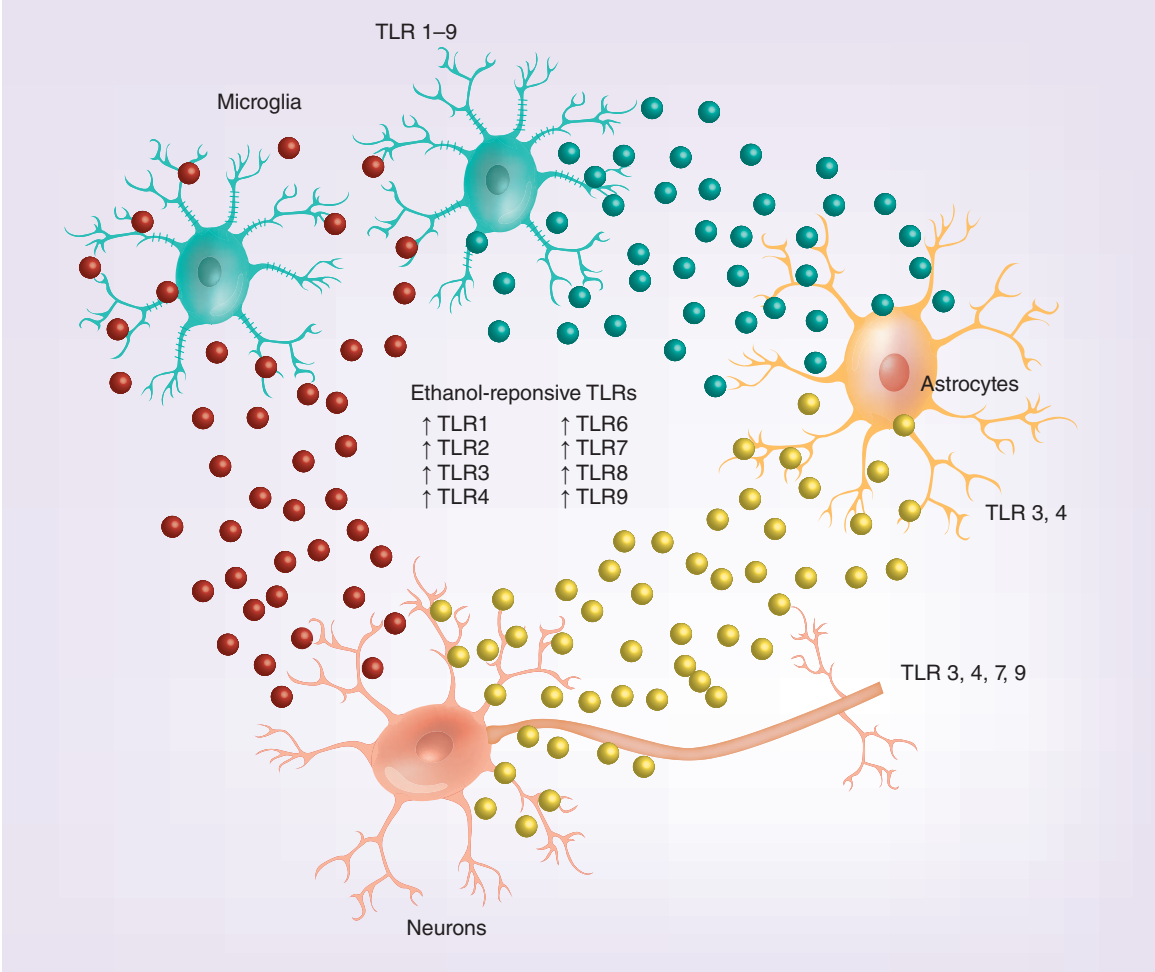

Figure 2. Alcohol-sensitive expression of Toll-like receptors and cell-type specificity.

studies to date have examined their specific role after chronic alcohol exposure. Ethanol treatment activated MAPK, NF-κB and AP-1 inflammatory pathways via a TLR4-dependent mechanism in cultured astrocytes [55]. Furthermore, ethanol-stimulated astrocytes produced IL-1R1 by activating the same inflammatory mediators and signaling cascades observed in the cerebral cortex of ethanol-fed animals [62]. Alfonso-Loeches *et al.* [63] linked astrocyte-mediated inflammatory responses to the TLR pathway by showing that TLR4 is critical for ethanol-induced inflammatory signaling. This group demonstrated that siRNA-mediated knockdown of TLR4 abolished alcohol activation of MAPK and NF-κB responses, which was also observed in TLR4-deficient cultures [63]. The studies above support the pivotal role of TLRs in astrocyte and microglia activation in ethanol-induced inflammation.

TLR-specific drugs alter alcohol consumption

Because alcohol activates astrocytes and microglia via TLR4 signaling, drug targets may require cell-type as well as TLR-specificity. One potential drug already being investigated is (+)-naloxone, which inhibits TLR4-mediated microglial activation and decreases proinflammatory cytokine expression [63]. Another microglia-related drug is minocycline, which reduces microglial and neuroimmune activation [64]. Minocycline also decreases ethanol consumption, albeit by an unknown mechanism [65]. It is hypothesized that the reduced drinking occurs via neuroimmune signaling because minocycline reduces mRNA expression of *Il1*β, *Il6* and other cytokines linked to alcohol abuse in mouse cortex and hippocampus [66]. However, it remains unknown if minocycline could reduce cytokine expression via a TLR-dependent pathway. Phosphodiesterase-4 (PDE4) inhibition is also of interest because PDE4 inhibitors reduce alcohol consumption [67–69]. In addition, cAMP-specific PDE4 regulates LPS-TLR4 induced inflammatory cytokine expression in mouse models of liver fibrosis, a condition commonly attributed to alcohol abuse [70]. Finally, PDE4 inhibitors have been shown to reduce NF-κB binding, leading to decreased inflammatory cytokine production in macrophages [71]. A promis-

ing preliminary study by Avila *et al.* [72] showed that alcohol-induced activation of astrocytes and microglia in mouse brain was attenuated in PDE4b knockout mice and by pharmacological inhibition using a PDE4 inhibitor (rolipram, 5 mg/kg). A remaining question is if cell-specific TLR responses are critical to behaviors related to alcohol consumption. Further investigation of TLR- and cell-type specificity will be needed to determine if these are relevant molecular targets with therapeutic potential.

Cell-type specificity

Astrocytes

Alcohol-induced molecular changes in astrocytes

Astrocytes have diverse roles in the CNS, many of which are still being elucidated. As discussed earlier for TLR signaling, astrocytes play a substantial role in immune responses by producing and secreting inflammatory modulators. In addition, they are highly involved in neurotransmitter uptake and recycling. Astrocytes can also communicate with each other and influence neuronal functioning through Ca^{2+} signaling.

Chronic alcohol causes molecular changes in astrocytes that could affect their function. Some studies suggest alcohol dependence is related to a hyperexcitable state caused by excess extracellular glutamate [73]. Expression changes in astrocyte glutamate transporters are observed in alcoholics and alcohol-exposed rodents [74,75]. Upregulation of glutamate transporters in postmortem alcoholic brain could be a sign of compensation for increased levels of extracellular glutamate. In cultured astrocytes, ethanol exposure alters the distribution of the glutamate transporter GLAST, shifting GLAST expression from the cytoplasm to the plasma membrane [76]. Ethanol-treated rats display lower levels of the astrocyte glutamate transporter GLT-1 [77]. Modulating glutamate transporter levels or activity alters drinking behavior. Blocking astrocytic glutamate uptake via GLT-1 attenuates binge-drinking behavior in mice [78]. Adenosine-mediated glutamate signaling has also been implicated in alcohol abuse. Deletion or knockdown of ENT1, an adenosine transporter, leads to reduced expression of GLT-1 and an astrocyte-specific water channel, AQP4. Treatment with the β-lactam antibiotic ceftriaxone, a potent inducer of glutamate uptake in astrocytes, increases GLT-1 expression and decreases ethanol drinking in mice, an effect dependent on AQP4 expression [79]. Ceftriaxone blocks glutamate increases in the extracellular space and increases glutamine synthetase activity in the nucleus accumbens [77]. It is effective in reducing relapse drinking in alcohol-preferring rats after long-term ethanol dependence [80] and in attenuating withdrawal [81]. These studies all point to a central role for astrocyte glutamate uptake in drinking behavior. Ceftriaxone, or similar compounds that target GLT-1, could potentially be used to treat alcohol withdrawal and other AUD-related behaviors. Baclofen, which has anticraving effects in patients with alcohol dependence [82], also affects glutamate transporter expression, as it prevented changes in subcellular localization of GLAST in cultured alcohol-treated astrocytes. This indicates a possible link between astrocytic glutamate transport and baclofen's anticraving effects [76]. However, other non-glutamatergic responses in astrocytes could also be modulated by long-term alcohol exposure and play a role in alcohol dependence. Astrocytes thus appear to be a viable cellular target for some alcohol-related drugs, supporting our earlier point of the relevance of examining cell-type specificity in alcohol-neuroimmune signaling.

Alcohol induces reactive astrocytes

When astrocytes respond to a pathological condition in the brain, they exhibit an evolutionarily conserved phenotype known as astrocyte reactivity. This response is characterized by upregulation of the intermediate filament and astrocyte-specific marker, GFAP, as well as cellular hypertrophy and elongated processes. Astrocyte reactivity has been observed in multiple brain regions, including the frontal cortex, hippocampus and cerebellum of ethanol-exposed mice and rats [49,83–88]. Differences in GFAP-positive astrocyte distributions in human brains have also been shown [89]. Alcohol-fed mice show increased *Gfap* mRNA [38]. Vimentin, another intermediate filament and indicator of astrocyte reactivity, was highly upregulated in a binge model of alcohol drinking in rats and was associated with alcohol-induced cell death [1]. Although the molecular, functional and behavioral consequences of alcohol-induced astrocyte reactivity are unknown, evidence suggests that this reactivity is commonly occurring in specific brain regions following alcohol exposure.

Astrocyte reactivity involves transcriptional induction triggered by signaling cascades that can result in long-lasting functional adaptations [90], which may be either beneficial or detrimental to neuronal functioning. For example, in the event of a traumatic brain injury, reactive astrocytes play both helpful and harmful roles. They can prevent infections and spread of cellular damage by the generation of a barrier across the injured area known as a glial scar. However, they can also block neuronal regeneration by secreting growth-inhibitory molecules to prevent axonal extensions. Reactive astrocytes can alter metabolism, disrupt the blood–brain barrier and alter ion and neurotransmitter uptake. They have the capacity to release various molecules that affect nearby cells in many ways. Many subtypes of reactive astrocytes exist, all precisely altering

their function to adjust to their unique pathological environment. For example, acute brain injury induces a response distinct from that of astrocytes affected by slowly progressing neurodegenerative disorders; however, both types of conditions upregulate GFAP and exhibit hypertrophic cell bodies and processes [90]. Unique groups of genes are altered depending on the different models of astrocyte reactivity. In a study comparing gene expression changes in isolated reactive astrocytes induced by two distinct models (LPS injection and experimentally-induced stroke), 50% of the differential expression was specific to the injury model [91]. Many of the top upregulated genes in LPS-treated astrocytes also show differential expression in existing gene expression datasets from human alcoholics and alcohol-exposed mice from our laboratory (unpublished), indicating that alcohol-induced reactivity may overlap with functional changes in astrocytes induced by immune perturbation.

Alcohol-exposed astrocytes as immune regulators

Astrocyte reactivity and accompanying changes in expression of immune-related genes can cause release of pro- or anti-inflammatory mediators, depending on the injury or disease context [92]. Some studies have identified the specific inflammatory mediators released in response to ethanol. For example, acute ethanol treatment of cultured astrocytes causes upregulation of iNOS and COX-2 [56], and chronic ethanol activates the NLRP3 inflammasome in astrocytes from cerebral cortex and in primary cultures of astrocytes [93]. Ethanol also promotes TLR4 signaling in astrocytes and microglia, which leads to upregulation of inflammatory cytokines, including IL-1β, TNF-α, IL-6, iNOS and COX-2 [63]. Knockout of TLR4 partially prevents the upregulation of GFAP observed after chronic ethanol-stimulated inflammation, indicating that TLR4 plays a role but is not solely responsible for inducing astrocyte reactivity [63].

It is unclear how astrocyte-specific immune signaling regulates drinking and the development of alcohol dependence. Expression of *Ccl2*, an inflammatory chemokine, is increased in alcohol-exposed tissue [84]. Astrocytes are a prime source of CCL2, and immune stimulation can cause striking upregulation of *Ccl2* in astrocytes [94]. Astrocyte-specific *Ccl2* overexpression in the hippocampus reduces ethanol-induced depression of long-term potentiation [95]. Another study shows that *Ccl2* and other inflammatory cytokines are increased in specific brain regions of adult, but not adolescent mice, after ethanol gavage treatment [84]. Immune-related changes that depend upon sex, including sexually dimorphic cytokine signaling, have been reported in astrocytes after alcohol exposure [96]. Thus, several factors may regulate distinct inflammatory mediators that influence the role of astrocytes in alcohol-neuroimmune responses.

Astrocyte Ca^{2+} signaling in drinking behavior

Secretion of neurotransmitters and other factors can activate astrocytic G-protein coupled receptors (GPCR), leading to release of intracellular Ca^{2+}. Through Ca^{2+} waves, astrocytes can communicate with each other and influence neuronal activity. Astrocyte-neuronal Ca^{2+} signaling may be an important mechanism that is modulated by alcohol exposure. Studies have already shown that astrocyte activity can affect motivation for alcohol. The use of designer receptors activated by designer drugs (DREADDs) in nucleus accumbens core astrocytes led to the discovery that increased Ca^{2+} activity through DREADD activation reduces motivation for ethanol after a 3-week period of abstinence [88]. Blocking gap-junction hemichannels, through which astrocytes can propagate Ca^{2+} waves and release other molecules important for cell-to-cell communication [97], leads to increased motivation to self-administer ethanol after 3 weeks of abstinence. This indicates that modulating the activity of discrete populations of astrocytes could alter synaptic plasticity and influence drug-seeking behavior. Alcohol may also cause specific GPCR expression changes, altering Ca^{2+} signaling and astrocyte responses. Inflammatory mediators such as LPS, TGF-β1 and IFN-γ induce notable expression changes in several GPCRs, effector proteins and downstream signal transduction pathways in cultured astrocytes [98]. Considering that the effects of neuroimmune activation are still relatively unexplored and neuroimmune mediators affect many pathways and cellular processes, it may be useful to consider targeting more discrete immune signaling processes. For example, if alcohol-induced alterations in astrocyte Ca^{2+} signaling regulate alcohol consumption, then targeting astrocyte-specific GPCRs could be a useful therapeutic tool.

Alcohol-responsive transcriptome in astrocytes

There are dynamic gene expression and transcriptional changes in reactive astrocytes in the face of immune modulation and alcohol exposure, and knowledge of the networks and pathways could reveal targeted cellular therapies for alcohol dependence. Microarray studies of postmortem frontal cortex from alcoholic patients showed altered expression of astrocyte-specific genes [9,99] and genes generally associated with glial function [3]. Astrocytes express many of the same genes as other cell types, and many innate immune genes are expressed in both microglia and astrocytes [100]. Most gene expression studies in brain have not determined which immune genes are altered in the different cell

types. Astrocytes and microglia are often grouped and discussed together as the immune cells of the brain. However, considering the diverse nature and functions of reactive astrocytes, it is feasible that molecular alterations in astrocytes are very different from those in microglia. Figure 3 summarizes some of the possible alcohol-induced functional changes discussed in this review that could result from astrocyte transcriptional adaptations. Dissecting the alcohol-induced transcriptome changes in astrocytes will help define alcohol's discrete effects in brain and identify potential cellular pathways and targets.

Microarray profiling of gene expression changes after acute ethanol exposure in cultured cortical astrocytes identified several immune and glial-specific genes, including *Gfap* [101]. *Hsf1*, a heat shock transcription factor, was activated in astrocytes, and expression profiling of heat-shock stressed astrocytes revealed overlapping changes between ethanol exposure and heat stress, indicating that the heat shock pathway is important in ethanol-induced gene expression changes. There was also a striking resemblance between expression changes in astrocytes and cultured hepatocytes after alcohol exposure, suggesting common signaling mechanisms between the two types of cells.

Studies utilizing longer ethanol exposures will provide more insight into the long-term molecular adaptations of astrocytes. There are also significant differences in gene expression between astrocytes *in vitro* and astrocytes isolated from mature brains [91]. Cultured astrocytes appear to exhibit signs of reactivity without any stimulation, while astrocytes co-cultured with neurons show resting morphology, suggesting that neuronal interactions are important for maintaining nonreactive states. Thus, *in vivo* transcriptome analysis in astrocytes isolated from chronic ethanol-treated animals is an important research direction.

Microglia

Alcohol activation of microglial genes

As previously noted, initial interest in alcohol-neuroimmune responses came from microarray studies that

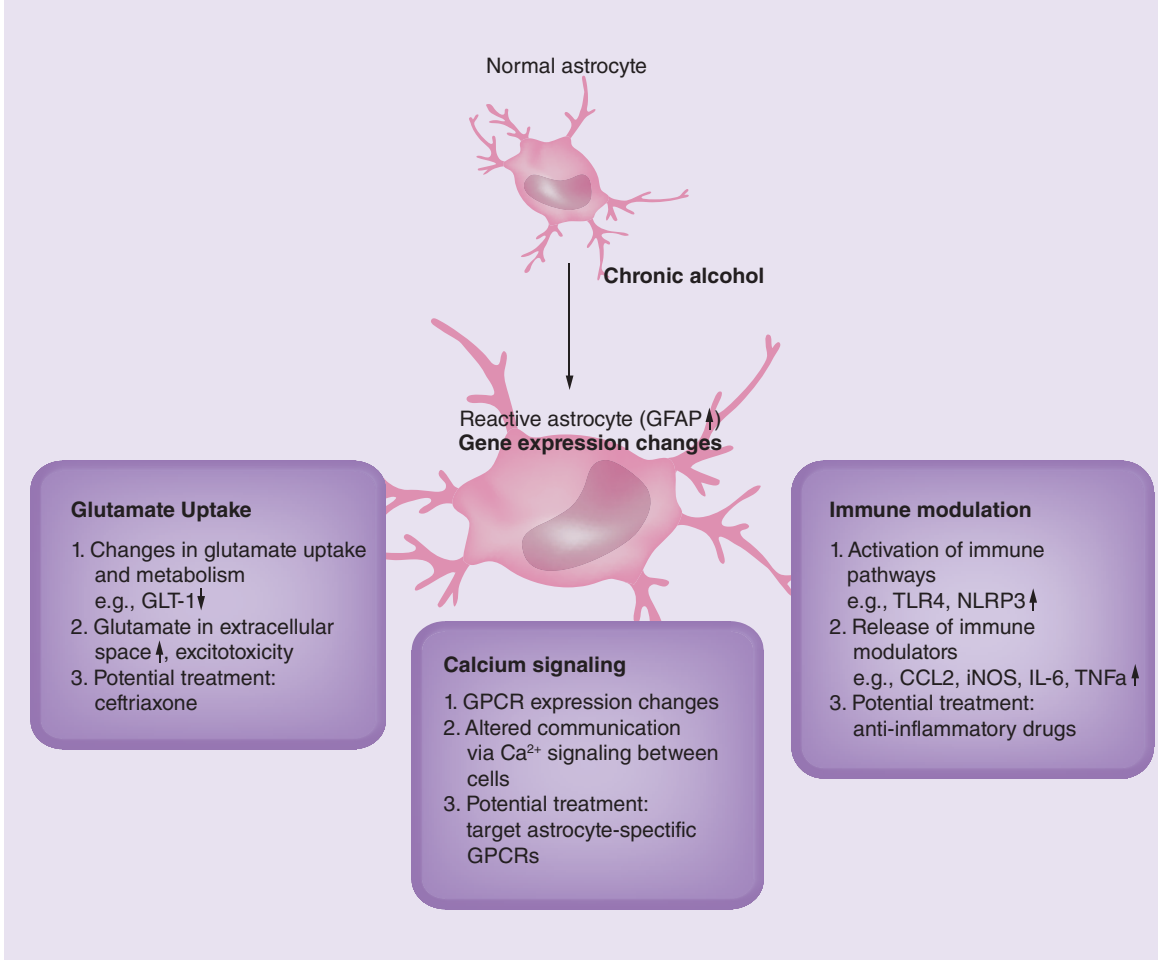

Figure 3. Possible alcohol-induced functional changes that potentially result from astrocyte transcriptional adaptations.

identified immune and inflammatory transcripts and pathways in alcohol-exposed tissue from mice, rats and human tissue [3–5,34,102–104]. Several immune genes found in these microarray studies were validated in rodent ethanol consumption paradigms, further corroborating a role for neuroimmune signaling in drinking behavior [14,105]. Microglia are the resident immune cells in the brain and many of the validated transcripts are expressed in or secreted from microglia (*Cd14*, *Ctsf*, *Ctss*, *Il1rn*, *Il6)*, prompting interest in the role of this cell type in ethanol consumption [14,106]. Since then, many studies have found that *in vitro* ethanol treatment of microglia leads to activation and the release of pro-inflammatory cytokines, while chronic ethanol consumption results in microglial activation in rodents and human postmortem tissue [57,63,107–110].

Gene expression in isolated microglia

The alcohol-neuroimmune field is progressing rapidly, and there is increasing interest in cell-specific changes. Cell-type specific databases provide one tool to identify the cellular basis of the transcriptome changes by identifying the gene co-expression modules that are enriched in different cells. This strategy has also helped to identify discrete cells that are targeted depending on the ethanol drinking paradigms, time points and brain regions studied [111–114]. Because microglia make up only 5–15% of cells in the brain, it is likely that key microglial gene expression changes are missed when examining whole tissue [115], making it necessary to perform transcriptome studies in isolated microglia. It will also become important to identify subtypes of microglia in gene expression studies, as microglia can be both pro- and anti-inflammatory as well as play a role in neurogenesis and synaptic remodeling [116].

Drug identification & the transcriptome

Transcriptome studies have been helpful in identifying drugs that could potentially be used in the treatment of AUD. An *insilico* bioinformatics study examined gene expression data collected from adolescent and adult mice exposed to 20% ethanol using a 4 h/day, 4-day drinking-in-dark paradigm [18]. Several neuroimmune pathways related to microglial action were altered in the adult but not the adolescent mice. The study also showed that minocycline, a drug that modulates neuroimmune signaling and microglial activity, could be given intraperitoneally and cross the blood–brain barrier. Treatment with minocycline led to a significant reduction in ethanol consumption in the adult mice. This study highlights how transcriptome data can be successfully used to identify pathways that are altered by ethanol consumption and ultimately identify drugs that target these pathways.

Drug repurposing

Drug repurposing is the process of identifying a new therapeutic indication for an existing drug or drug candidate. The concept of US FDA-approved drug repurposing as a strategy for therapeutic development has received increased support because it can resurrect a failed drug or expand the number of clinical uses for a successful one [117–123]. The primary advantage is that the time to clinical trial can be dramatically reduced. There are numerous examples of successfully repurposed drugs that are beyond the scope of the current review and are only briefly highlighted here. A notable example of drug repurposing is buprenorphine which was used to treat moderate pain, then subsequently used for treating heroin and other opioid addictions [124]. Buspirone was initially marketed as an anxiolytic, but more recently has been identified as a potential candidate to treat drug addiction and compulsive behavior disorders based on its high affinity for D3 receptors (as well as 5-HT1A and D2 receptors) [125]. In addition, a study of nonhuman primates indicates that buspirone attenuates drug-taking relapse in abstinent animals [126]. Other general examples of repurposed drugs include sildenafil for erectile dysfunction (developed as an anti-hypertensive) [127], thalidomide (original indication: nonbarbiturate sedative-hypnotic), finasteride (original indication: prostatic hyperplasia) and chlorpromazine (original indication: antihistamine) that are also effective treatments for erectile dysfunction, leprosy, hair loss and psychosis, respectively [128–130]. There are a limited number of repositioned drugs that have been identified as potential AUD therapeutics. These include topiramate, which was originally used as an anticonvulsant drug used to treat epilepsy [131,132], naltrexone, an opiate receptor antagonist [133] and aripiprazole, an atypical antipsychotic, with potential for treating alcohol dependence [134]. And, relevant to the current review and the role of the neuroimmune system in AUD, there is evidence that ibudilast, a nonselective phosphodiesterase inhibitor, decreases alcohol consumption in animal models [135].

There is growing support for computational approaches to drug repurposing as outlined in recent reviews [136–139]. The NIH is currently funding multiple research institutions to refine and extend a database of human cellular responses. This funding supports the LINCS program, and includes funding to the Broad Institute which originally established the Connectivity Map (CMAP) database [140,141], cataloging the biological responses of approximately 1 million gene expression profiles using L1000 technology. This approach has been shown to identify existing drugs and

elucidate modes of action for novel chemicals based on findings from expression studies [142]. L1000 technology is a high-throughput method to estimate genome-wide mRNA expression based on unique perturbations by drugs, ligands and other small molecules applied at different time points and doses in cell culture. Recently, the NeuroLINCS Center (University of California, CA, USA) was funded to include neuronal and glial signatures from normal and diseased-induced pluripotent stem cells, thus expanding the LINCS database to noncancerous cell lines.

In a successful application of this approach, data from the Connectivity Map (CMAP, [143]) were queried with the gene expression signatures of Alzheimer's disease to identify compounds putatively beneficial for the disease [144]. A somewhat different approach used SNPs related to aversive memory combined with Ingenuity Pathway Analysis to predict drugs that might modulate memory, and the effectiveness of one of these drugs was verified in human studies [145]. The use of genomics to predict drugs for brain diseases is in its infancy, but these examples are quite promising for future therapeutic development. For example, these computational approaches, which integrate gene expression signatures of drugs and small molecules with disease, have been successful in identifying potential therapeutics for complex disorders such as obesity [146] and inflammatory bowel disease [147]. In addition, gabapentin, which has been shown to be effective in treating alcohol dependence and preventing relapse in clinical trials [148] was identified as a potential therapeutic target based on RNA-sequencing of human postmortem brain [149]. A key question is whether consistent changes in gene expression profiles and networks found in human alcoholics and animal models can be used to predict drugs that will normalize the targeted gene networks and provide new treatments for AUD. This research direction provides an important avenue for discovering novel compounds that target immune signaling in alcohol dependence.

Conclusion and future perspective

There is increasing evidence for neuroimmune system involvement in AUD. This work is also leading to a better appreciation for the roles of glial cells, including astrocytes and microglia, in the brain's response to alcohol. Strategies for drug repurposing based upon transcriptional profiles for the treatment of AUD, particularly relevant to the neuroimmune system, represent an important area of emerging research.

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Executive summary

Toll-like receptors and alcohol

- TLRs are key regulators of alcohol-induced neuroimmune activation.
- Alcohol exposure leads to changes in expression of many TLR pathway components.
- Genetic modulation of TLR signaling components can change alcohol behaviors.
- Evidence supports roles for neurons, astrocytes and microglia in TLR-mediated alcohol responses.
- Drugs targeting TLR pathways can modulate alcohol consumption.

Cell-type specific changes

- **Astrocytes**
  - Alcohol exposure causes molecular changes in astrocytes that can affect crucial functions such as glutamate uptake.
  - Alcohol can induce astrocyte reactivity, a conserved astrocyte phenotype for responding to stress and disease.
  - Astrocytes may play key roles in immune modulation after alcohol exposure.
  - Astrocyte Ca^{2+} signaling could play a role in modulating alcohol-related behaviors.
  - Alcohol-induced transcriptional and functional changes in astrocytes are largely unknown.
- **Microglia**
  - Alcohol induces expression of immune genes that are expressed in or secreted from microglia.
  - Microglial gene expression changes can be used to predict drugs that reduce alcohol consumption.
  - Alcohol-mediated changes in isolated microglia can elucidate their specific role in alcohol-immune responses.

Drug repurposing

- Global transcription-based analyses identifying drug candidates for the treatment alcohol use disorders could lead to improved therapeutic strategies.

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•• **Work demonstrates novel convergent evidence for biological networks related to excessive alcohol consumption, which may prove fundamentally important in the development of pharmacotherapies for alcohol dependence.**