

## Ethanol intake enhances inflammatory mediators in brain: role of glial cells and TLR4/IL-1RI receptors

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### 1. ABSTRACT

The brain is one of the major target organs of ethanol actions, and its chronic and acute intoxication results in significant alterations in brain structure and function, and in some cases to neurodegeneration. Glial cells and Toll-like receptors (TLRs) are vital players in CNS immune response; dysregulation of this response plays an important role in brain damage and neurodegeneration. Ethanol has immunomodulatory effects and induces specific alterations in the TLRs response in many tissues. These actions depend on the cell type, ethanol dose and treatment duration, as well as the concomitant presence of pathogens and their characteristics. Recent findings indicate that low concentrations of ethanol (10 mM) promote inflammatory processes in brain and in glial cells by up-regulating cytokines and inflammatory mediators (iNOS, NO, COX-2), and by activating signaling pathways (IKK, MAPKs) and transcriptional factors (NF-kappaB, AP-1) implicated in inflammatory injury. TLR4/IL-1RI receptors may be involved in ethanol-mediated inflammatory signaling, since blocking these receptors abolishes the production of ethanol-induced inflammatory mediators and cell death. We propose that at low physiologically relevant concentrations, ethanol facilitates TLR4/IL-1RI recruitment into lipid rafts microdomains, leading to the activation and signaling of these receptors. In summary, current results suggest that TLR4/IL-1RI are important targets of ethanol-induced inflammatory brain damage.

### 2. INTRODUCTION

Research and technological advances over the past two decades have indicated that brain disruption and damage play crucial roles in the consequences of drug abuse and addiction. Alcohol is one of the most common drugs and overdoses can lead to brain damage, even to death. The World Health Organization examined mental disorders in primary care offices and found that alcohol dependence or harmful use was present in 6% of patients. In Britain, 1 in 3 patients in community-based primary health care practices presented at-risk drinking behavior. Heavy binge drinking is also becoming increasingly frequent in women and high school students from different countries (1), and the prevalence of both alcohol-related problems and neurological deficits is more frequently observed among adolescents (2).

It is well established that the brain is a major target for alcohol actions, and heavy alcohol consumption has long been associated with brain damage. Even uncomplicated alcoholics, with no specific neurological or hepatic problems, show signs of regional brain damage and cognitive dysfunction (3). Studies clearly indicate that alcohol is neurotoxic with direct effects on the nerve cells. Chronic ethanol abuse and alcoholism are associated with multiple primary and secondary disease states, which, typically start with experimentation and gradually progress to addiction over the course of several years. Frequently, high-quantity alcohol consumption by alcoholics may

damage the CNS in ways that contribute to the progression of addiction. Human alcoholics are known to suffer from a loss of brain mass and function (4, 5), and experimental studies have demonstrated that heavy binge drinking can cause neurodegeneration (6). Although the mechanisms involved in ethanol-induced brain injury are not clearly understood, recent evidences suggest the involvement of inflammatory brain damage.

Inflammation and glial activation are associated with brain injury, and are involved in the pathogenesis of neurodegenerative diseases (7, 8). Emerging data also indicate the role of innate immunity, the Toll-like receptors (TLRs) in particular, as vital players in this orchestrated immune response in the CNS. Furthermore, the dysregulation of this immunological response against CNS-associated antigens could play a significant role in brain damage and neurodegeneration (9). Glial cells express numerous TLRs, which allow for the recognition of different pathogens and potentially endogenous TLR agonists (10, 11). The immediate activation of resident glia via TLR likely serves as an amplification pathway to maximize proinflammatory response within the CNS compartment. In the present article we will briefly review the evidences that demonstrate the role of glial cells and TLRs in the immune response in the CNS. We will also describe the current findings which indicate that ethanol intake can induce inflammatory mediators in brain, and discuss the potential role of the activation of both glial cells and the innate immune system in ethanol-induced brain damage.

### 2.1. Glial cells as a hallmark of brain inflammation

It is increasingly recognized that the most important CNS disorders are not merely defined by the enigmatic emergence of dysfunctional neurons, but are in fact largely orchestrated by glial-cell-controlled inflammatory processes. In multiple sclerosis, the archetypical inflammatory CNS disease, and in Alzheimer's disease, stroke and Parkinson's disease, inflammation has been implicated in the disease process and the glial-cell involvement has been clearly shown. Until recently, glial cells in the CNS have been considered as elements that contributed only to feed and support neurons. However, evidence is rapidly accumulating to suggest that glial cells indeed play very important roles in CNS development, repair (12) and neurotransmission (13). They are also central functional elements in CNS vascularization, inflammation, neuroprotection and in the innate immune responses (14-17). In fact, glial cells respond to various injuries and insults (18), and are capable of proliferating throughout life, acting as neural stem cells with the potentiality of generating neurons even in the adult brain (19).

Among glial cells, microglia and astrocytes are responsible for the immune functions within the brain, and they play key roles in inflammatory response. Microglia, the macrophages of brain parenchyma, is in a down-regulated state in comparison with other tissue macrophages in the healthy brain. However, they are rapidly stimulated in response to injury or infection, and

their morphology changes and acquire an array of functions, including phagocytosis, up-regulation of cell-surface molecules, and the production and secretion of inflammatory mediators (17). Astrocytes are also important in the immune response, contributing to the establishment and maintenance of the blood brain barrier (BBB) (20), and modulating the migration of monocytes and lymphocytes across the BBB (21). These cells respond vigorously to brain injury and seem to play an important role in the fine tuning of brain inflammation (22). Indeed, injury to the CNS is inevitably accompanied by astrocytic hypertrophy, proliferation, and altered gene expression, a process commonly referred to as reactive astrogliosis (23), which is associated with inflammation (24). Depending on the disease context, however, astrogliosis can be seen as either a positive event that promotes neuronal and glial survival via the production of neurotrophins and growth factors, or as a negative influence on regeneration via the inhibition of neuronal and glial growth and migration. The diffuse nature of reactive astrogliosis in the CNS suggests a role for either soluble mediators, such as cytokines, and/or the presence of an integrated astrocyte-to-astrocyte syncytium that enables the transfer of information across extended distances.

The cytokines for which the evidence is most compelling in the initiation and modulation of reactive astrogliosis include IL-1-beta, TNF-alpha, IFN-gamma and TGF-beta. Glial cells express receptors for all these cytokines, and each one appears to fulfill a different functional role in the astrocytes response (25). Activation of astrocytes in response to a neuropathologic process triggers the production of inflammatory cytokines, increases the expression of major histocompatibility complex II and augments the production of free radicals (16). This functional reprogramming may be essential for maintaining homeostasis and the local regulation of inflammatory and immune reaction (26). Among the cytokines, IL-1-beta has been considered to be an important mediator of inflammatory responses in the CNS. It is involved in Alzheimer's disease (27), and vastly produced under conditions of brain damage, disease, or stress (28). This cytokine is initially released by glial cells acting on astrocytes and microglia to induce the production of additional cytokines and growth factors, thereby promoting inflammatory activity in the brain (29, 30). IL-1-beta promotes glial scarring or astrogliosis when directly injected into CNS, thus suggesting a potential role of this cytokine in mediating astrocytic hypertrophy upon neuronal damage (31).

### 2.2. Innate immune response in the CNS: role of TLR/IL-1R signaling

The immune system encompasses a complex interrelated network of cellular, molecular, and chemical mediators that function to protect the body against environmental stress factors. These stressors can be as diverse as microorganisms (viral, bacterial, fungal agents), physical damage (burns), or environmental toxins (snake venoms, nonessential metals, chemicals). The first line of defense is innate or natural immunity. The inflammatory component of this response is important in recruiting cells from the immune system in the compromised area. The

mediators for this function are cytokines and chemokines. While cytokines mediate a specific response that is appropriately based on the type of foreign antigen that has penetrated the tissue, chemokines allow cells from the immune system to reach the area under attack.

Although the brain has been considered to be immunologically privileged, it is now well accepted however that immune surveillance indeed occurs in the normal CNS and that inflammatory responses can take place in the context of disease. The CNS presents a well organized series of innate immune reactions in response to systematic bacterial infections and cerebral injury (32, 33). Activation of the innate immune system is an important component of the inflammatory response. However, while the acute activation of inflammatory events is a necessary component of the CNS defense against foreign antigens, prolonged activation can lead to chronic inflammation, and this can eventually result in neuronal cell death. Inflammation occurs through the action of proinflammatory cytokines, and the production of these cytokines is initiated by signaling through TLRs that recognize host-derived molecules released from injured tissues and cells.

TLRs are a family of pattern-recognition receptors expressed in cells of the innate immune system, that allow for the recognition of conserved structural motifs on a wide array of pathogens (referred to as *pathogen-associated molecular patterns*) as well as some endogenous molecules. The recent emergence of studies examining TLRs in the CNS indicates that these receptors respond to molecules related to either microbial infection or host tissue injury (34), and that they do not only play a role in innate immunity in response to infectious diseases, but may also participate in CNS autoimmunity, neurodegeneration, and tissue injury (9). Therefore, given the importance of these receptors in the innate immune response, a great effort has been made to understand the regulation of the innate immune system, particularly the TLRs signaling mechanisms.

The TLRs, type I integral membrane glycoproteins, are a member of a large superfamily that includes the interleukin-1 receptors (IL-1Rs) (35, 36). TLRs and IL-1Rs have a conserved region of ~ 200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain (36-39). By contrast, the extracellular regions of TLRs and IL-1Rs differ markedly: the extracellular region of TLRs contains leucine-rich repeats motifs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains (see rev. Takeda and Akira, 2004) (40). TLRs interact with MyD88 (myeloid differentiation primary-response protein 88) (41), activating downstream signaling molecules which culminate in the activation of nuclear factor-kappa B (NF-kappaB), a transcription factor that regulates the expression of a wide array of genes involved in immune responses, as well as in the mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK) (42). Stimulation of MAPKs also results in the activation of the transcriptional factors NF-kappaB and AP-1 (activator protein-1) (43).

Notably, since NF-kappaB and AP-1 binding sites have been found in the promoters of many genes that are induced during inflammation, it has been claimed that the large number of specific receptors of the innate immune system exerts their manifold gene activations principally through the MAP kinases and the IKK-NF-kappaB pathways (36, 44, 45) (figure 1).

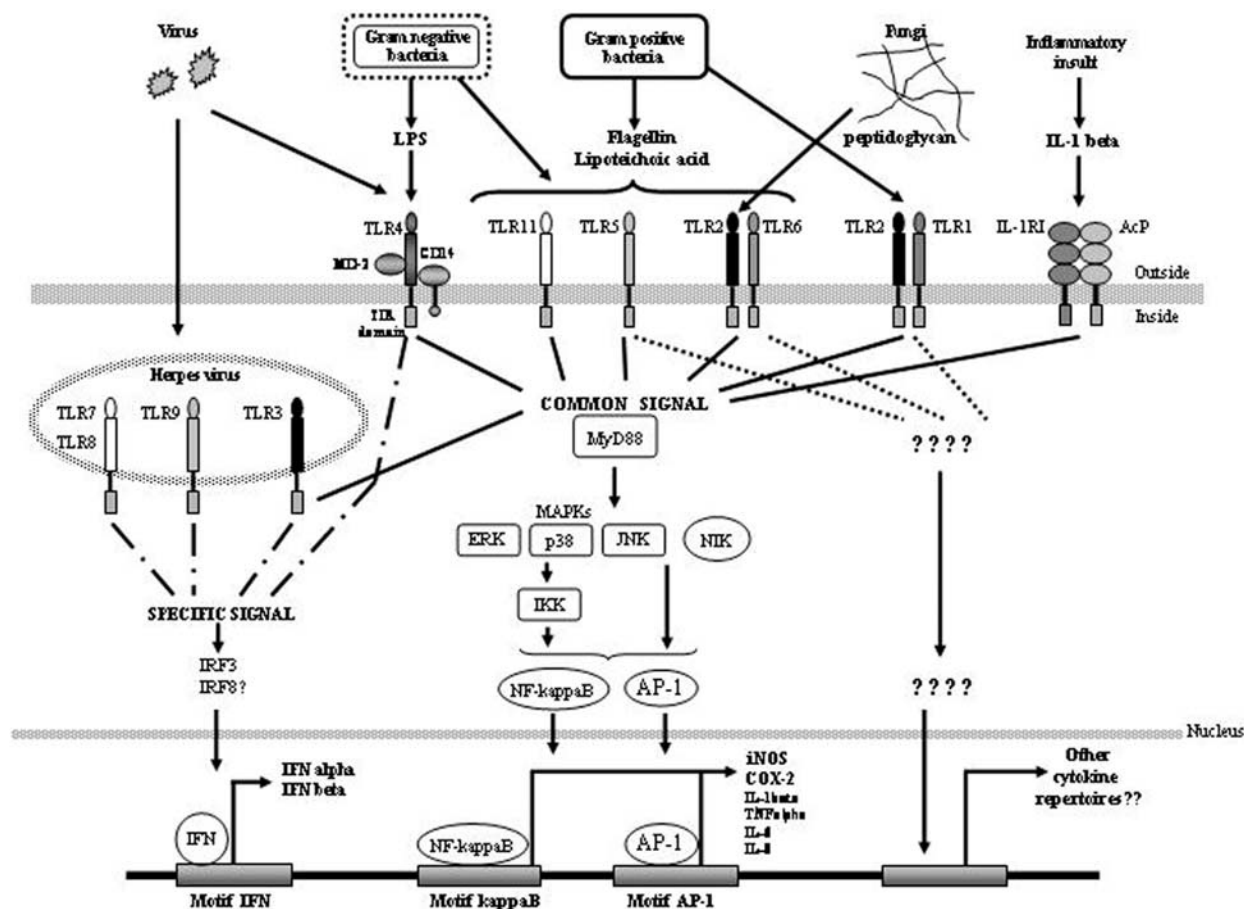
Among the 11 different human TLRs, TLRs 1, 2, 4, 5, 6 and 11 are expressed on the extracellular membrane as opposed to the intracellular localization of TLRs 3, 7, 8 and 9 (46-48). Each TLR recognizes specific components of bacterial and fungal pathogens, such as LPS (lipopolysaccharide) from Gram-negative bacteria, PGN (peptidoglycan) and LTA (lipoteichoic acid) from Gram-positive bacteria and fungi, which possess common structural features. Recent evidence indicates that TLRs are also involved in the recognition of viral invasion. Thus, TLR3 and TLR4 are presumably involved in the viral recognition, and these receptors utilize a unique signaling pathway that is independent of the common signaling adaptor MyD88, triggering the IRF-3 (interferon regulatory factor-3) downstream signaling activation and the subsequent induction of interferon-alpha and -beta (IFN) (49), in addition to a MyD88-dependent pathway that is common to all the TLR family. The same occurs with TLR7 (50) that is also involved in the viral recognition, as well as TLR9 (51). The different Toll signaling pathways are summarized in figure 1.

Notably, most of the TLRs, as well as the IL-1RI, have been identified in neural cells of the human and murine brain, although the expression of the different TLRs varies between neural cell types, species, cell maturation, and activation state (52-54). For example, while human adult and fetal microglia express a wide range of different TLR family members, the TLR expression profile is more restricted in human astrocytes (10, 11). However, a robust expression of TLR3 has been demonstrated in adult and fetal astrocytes (55, 56), along with low levels of expression for TLR 1, 4, 2, 5 and 9 on human fetal astrocytes (11). Conversely, emerging data suggest that broader repertoires of TLRs are expressed in murine astrocytes as compared with human astrocytes (52, 57). Another striking difference between microglia and astroglia is the fact that TLR are generally expressed in intracellular vesicles inside microglia, while they are found in the cell surface on astrocytes (10, 11). The different TLR profile in the two types of glial cells, as well as the distinct subcellular localization of some of TLRs, suggest the possibility of their differential role in the innate immune response (11). Indeed, recent findings point to a distinction in TLR expression between the two glial cell types and illustrate their divergent functional response to TLR ligands (11).

### 3. ETHANOL. BRAIN DAMAGE AND INFLAMMATION

#### 3.1. Ethanol and brain damage: role of inflammatory mediators

Alcohol is an addictive substance. Like other addictive drugs, its continued consumption can induce adaptive changes in the CNS that lead to tolerance, physical



**Figure 1.** Schematic representation of Toll-like receptor (TLRs) signaling pathways. Each TLRs recognizes specific components of bacterial and fungal pathogens, LPS (Gram-negative bacteria), PGN and LTA (Gram-positive bacteria). All TLRs except TLR3, 7, 8 and 9 are thought to share the MyD88-dependent pathway that activates NF-kappaB and AP-1, via MAPKs/NIK, leading to the induction of inflammatory mediators (iNOS, COX-2) and cytokines (IL-1-beta, TNF-alpha, IL-6, IL-8) genes. TLR3 and TLR4 are presumably involved in the viral recognition, and activate interferon regulatory factor-3 (IRF-3), and the subsequent induction of the IRF-3-dependent gene expression such as interferon-alpha and -beta (IFN). Although all TLRs share the cytoplasmic TIR domain, stimulation of TLRs leads to the homo-, heterodimerization or multimerization of TLRs for signaling. TLR4 and IL-1RI additionally recruit adapter molecules, such as MD-2 and CD14 (for TLR4) and AcP “Accessory protein” (for IL-1RI) to induce their signaling response.

dependence, sensitization, craving and relapse (58). In addition, alcohol is a toxic compound that can cause tissue damage and organ dysfunctions.

The brain is one of the major target organs for ethanol actions, and heavy alcohol consumption results in significant alterations of the brain structure, physiology and function. Alcoholics have reduced brain weight compared with nondrinking controls (59), and the degree of brain atrophy correlates with the rate and amount of alcohol consumed over a lifetime. The reduction in brain weight and volume has been attributed to a loss of white matter, which occurs primarily in the frontal lobe and is specifically susceptible to alcohol-related brain damage (3, 4), although a neuronal loss has also been documented in specific regions of the cerebral cortex, hippocampus and cerebellum from alcoholic brains (3, 60).

Current neuroimaging studies also indicate that chronic alcohol use induces important changes in brain morphology, such as cortical and subcortical atrophy, and confirm that frontal lobe structures are specifically vulnerable to the effects of ethanol with shrinkage in this area, largely owing to a loss of white matter (61). The mechanisms to reduce white matter loss remain unclear, although glial impairments in conjunction with astrocytic loss and death have been reported in the prefrontal cortex and hippocampus from human alcoholic brains (62-65). Changes in myelination might also occur during chronic alcoholism, since the gene expression of both the glial fibrillary acidic protein GFAP (a marker of astrocytes) and myelin-associated genes were down-regulated in the brain of alcoholics (66, 67). A recent report demonstrates that astrocytes promote myelination in response to electrical impulses (68), suggesting that alterations and cell death in

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astrocytes could cause deficits and loss of myelin in alcoholics.

Experimental evidence also demonstrates that alcohol is toxic for neural cells in culture (69-72) and that acute intoxication can cause brain damage, and even neurodegeneration in some cases (6, 73). Indeed, neural damage and neurodegeneration have been demonstrated in short-term binge drinking animal models, leading to neuronal loss (6) in specific brain regions including olfactory and forebrain corticolimbic association areas, entorhinal and pirirhinal cortex and hippocampus, brain areas that are involved in many aspects of learning and spatial memory. These results suggest that a drinking pattern, specifically binge drinking in which high blood and brain alcohol levels are achieved, is an important factor in the ethanol-induced neuropathology, and that alcoholic neurodegeneration could occur primarily as a result of binge drinking episodes (6, 73, 74).

The neuropathological processes underlying the effects of ethanol on neural damage are largely unknown, although several mechanisms and concurring factors have been proposed to contribute to neurodegeneration. Among the mechanisms proposed include: the participation of excitotoxic events and nitric oxide generation (75), the involvement of glial swelling and brain edema (74), and the production of free radicals causing oxidative stress (76). The third mechanism includes the ability of ethanol to enhance free radical species by inducing the cytochrome P450 2E1 (CYP2E1) (77), which leads to the generation of hydroxyethyl radicals (78), reactive oxygen species (ROS) (79) and the activation of NF-kappaB (76). Interestingly, the induction of both P450 2E1 (CYP2E1) and ROS was also noted in ethanol-exposed astrocytes (77), suggesting that glial activation might contribute to the ethanol induction of free radical species in the brain.

Recent findings also suggest the involvement of inflammation in ethanol-induced brain damage. These studies demonstrate that chronic ethanol treatment not only increases the levels of cytokines (IL-1-beta, TNF-alpha) and inflammatory mediators (iNOS and COX-2) in both the rat's brain and in cultured astrocytes, but also activates signaling pathways that are classically associated with inflammation (MAPKs, NF-kappaB, AP-1, see below). Notably these inflammatory events are associated with an increase in cell death (72), suggesting that activation of glial cells and the innate immune system by ethanol might trigger the production of toxic compounds such as ROS or nitric oxide (70), inflammatory cytokines and glutamate, which might contribute to ethanol-induced brain damage, similar to what occurs in several brain disorders and neurodegenerative diseases (7, 8, 80).

Evidence indicating that ethanol-induced inflammatory mediators in the brain originate from several studies performed mainly in neural cells in culture. These studies demonstrate that ethanol influences the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in both the brain and neural cells exposed to ethanol (72). Both enzymes are induced in

response to different stimuli including stress, inflammation or neural damage (81-83). Likewise, stimulation of TLRs signaling triggers NF-kappaB activation, which could lead to the induction of iNOS and COX-2 expression in the brain (84-86).

Ethanol is known to influence both the NO production and iNOS expression in a number of cellular systems, and in the presence or absence of additional stimuli such as cytokines or LPS. For example, depending on the cell type, ethanol can inhibit or potentiate cytokine-induced iNOS expression in immortalized astrocytes (87), and in BBB cells (88) respectively. However, in human astrogloma cells (A172), ethanol biphasically affects proinflammatory-induced iNOS expression (50 mM potentiates and 200 mM is inhibitory) (89, 90). Stimulation of both the NO production and iNOS expression also occurs as a direct effect of ethanol, as demonstrated in brain homogenates from the prenatal ethanol exposure model of guinea pigs (91), in brain lysates from chronic-alcohol fed rats (72) and in cerebral pial cultures (92). In fact, the induction of iNOS can occur upon 30 min of ethanol treatment in astrocytes (70).

The up-regulation of the COX-2 expression has also been observed in the rat's brain after acute (93) and chronic ethanol administration (72, 93, 94), as well as in ethanol-exposed astrocytes (70, 95). Interestingly, the study by Luo et al (2001) (95), demonstrated that ethanol selectively increased COX-2 levels in astrocytes, but not in neurons. However, a clearer demonstration that ethanol induces COX-2 in astrocytes is seen in a recent study showing a fast induction of COX-2 upon 30 min of ethanol treatment (70). This study also demonstrates that the activation of NF-kappaB is critical for the ethanol-induced up-regulation of iNOS and COX-2 in astrocytes, since the inhibition of NF-kappaB activity by either pyrrolidine dithiocarbamate (PDTC) or BAY 11-7082 suppresses the induction of iNOS and COX-2, suggesting a transcriptional regulation of these inflammatory mediators by NF-kappaB (70). Collectively, these results suggest that ethanol can induce rapid response genes (iNOS and COX-2), implicated in the generation of free radicals as well as other products that could increase neuronal oxidative stress and contribute to ethanol-induced brain damage. These findings also suggest that the activation of NF-kappaB is a critical component in the ethanol-mediated inflammatory mediators, at least in astroglial cells.

Although the mechanisms by which ethanol-induced inflammatory mediators in the brain are presently unknown, recent data suggest that ethanol could activate the innate immune response and TLRs signaling, resulting in both NF-kappaB activation and inflammatory mediators production (see below).

### 3.2. Ethanol activates signaling pathways and transcription factors involved in inflammatory brain damage

As previously commented, stimulation of the innate immune system triggers NF-kappaB activation (see figure 1) and the induction of numerous immune and

inflammatory response genes (96), encoding cytokines, chemokines, enzymes (iNOS and COX-2) (84-86) and adhesion molecules.

Several *in vitro* and *in vivo* studies clearly demonstrate that both acute and chronic ethanol treatments cause NF-kappaB activation. Short-term ethanol treatment (25-100 mM) has been shown to potentiate cytokine-induced NF-kappaB activity in human astroglial cells (97), as well as in long-term ethanol-treated rat astrocytes (72). Similarly, chronic ethanol treatment also induces NF-kappaB activation in the liver (98), brain (99), and in the cerebral cortex of ethanol-fed rats (72). In the last study mentioned, NF-kappaB stimulation was accompanied by a significant decrease in the cytoplasmic levels of IkappaB-alpha, and by elevated levels of cytokines and COX-2 and iNOS (72). These findings suggest that NF-kappaB is activated in response to challenge with both acute and chronic ethanol, although the mechanism involved in these effects remains unclear.

NF-kappaB activity is controlled at multiple levels, most notably by the regulation of its subcellular localization. In resting cells, NF-kappaB is retained in the cytoplasm and it is transported to the nucleus in response to a diverse range of stimuli (100, 101) where it binds specifically to kappaB enhancer elements of DNA and alters the expression of a great number of proinflammatory genes (102). Following stimulation, the duration of NF-kappaB activation may be transient or persistent, depending on the cellular stimulus and cell type. The temporal profile is of considerable clinical relevance because whereas rapid induction of NF-kappaB is beneficial for immune response, infection or injury, long-term activation has been demonstrated to be associated with chronic inflammatory diseases, such as multiple sclerosis. Notably, chronic ethanol treatment triggers a sustained NF-kappaB activation in both the rat's brain and astrocytes in culture, and this event is associated with elevated levels of inflammatory mediators, IL-1-beta and TNF-alpha (72). Likewise, a recent study demonstrated that NF-kappaB is sustained in astrocytes in response to stimulation with IL-1-beta (103), which suggests that elevated levels of IL-1-beta could mediate the persistent NF-kappaB activation observed in both astrocytes and the brain of alcohol-fed rats, and might contribute to a prolonged induction of inflammatory mediators and ethanol-induced damage in the brain.

Furthermore, despite the fact that the induction of NF-kappaB is directly regulated by IKK, there is evidence demonstrating that IKKs themselves are also activated through phosphorylation by an upstream kinase(s). Candidates for this kinase include NF-kappaB-inducing kinase (NIK) and mitogen-activated protein kinase (MAPK) pathways (p44/p42 ERK, JNK and p38) (104-106) (see Figure 1). Cumulative data demonstrate the participation of the MAPKs pathway in inflammation processes (107). Moreover, the stimulation of this pathway, apart from activating NF-kappaB, can also trigger the activator protein-1 (AP-1) transcriptional activity. Phosphorylation of the AP-1 family by MAPKs (108),

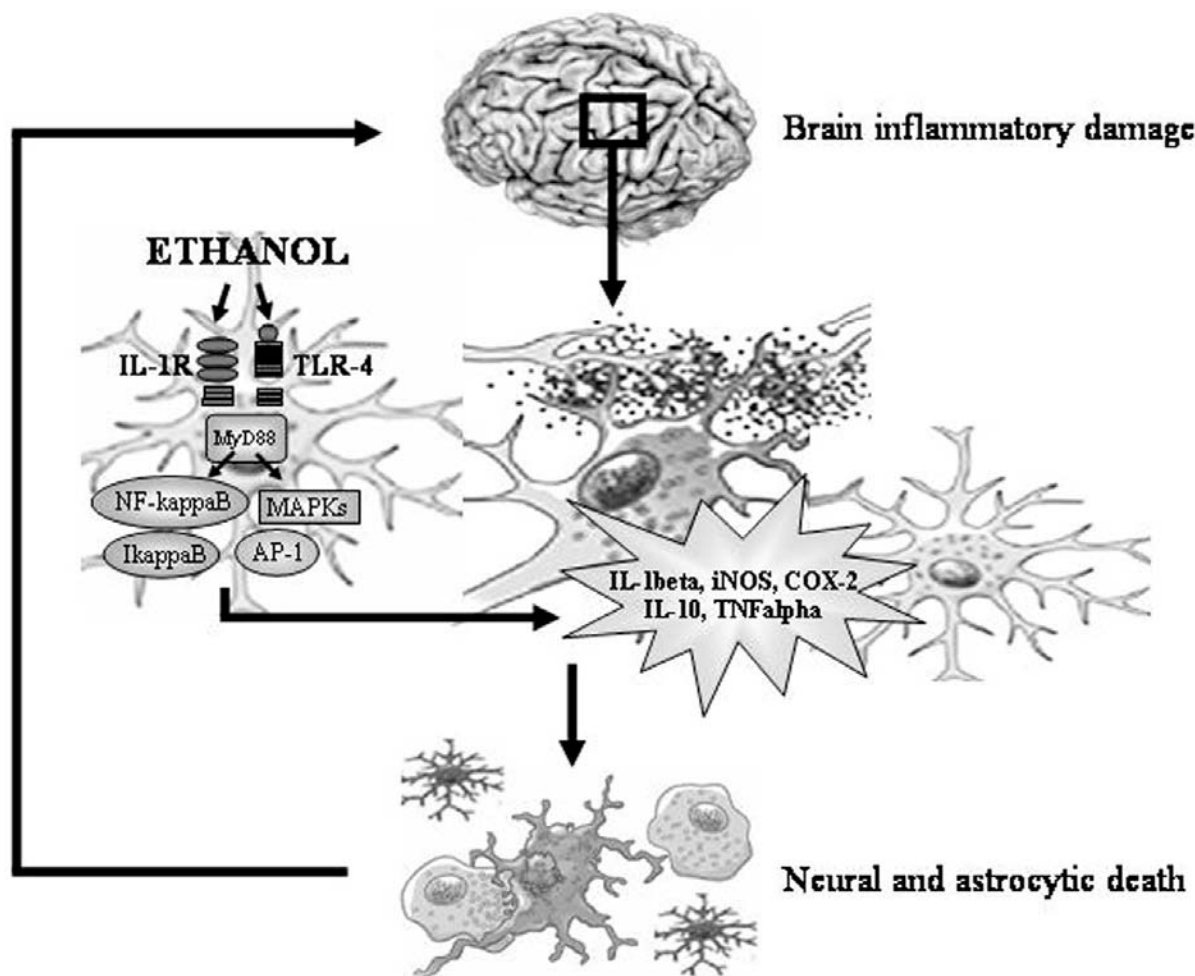
results in the transcriptional activation of genes of the jun and fos-family. Ethanol withdrawal hyperactivity is associated with the expression of the early genes c-fos and c-jun (109) with transient selective increases in the DNA-binding activity of immediate early genes (IEGs)-encoded AP-1 in the brain (110, 111). Chronic ethanol treatment increases the MAPKs and AP-1 expression in the liver, brain and astrocytes (72, 112), and these effects are associated with inflammation and cell damage. Finally, although the activation of MAPKs can mediate the inflammation and release of neurotoxic molecules, it is obvious that the activation of MAPK under certain circumstances may be beneficial for the plasticity of the CNS. Nevertheless, based on the few *in vivo* and *in vitro* studies available to date, the MAPKs which mediate the activation of glial cells by ethanol with neurotoxic consequences represent promising targets for pharmacotherapy for acute brain insults and neuroinflammatory injury induced by ethanol.

Neural injury and cell death have been associated with many neurological and neuroinflammatory disorders (113), and several reports demonstrate that one of the mechanisms implicated in ethanol-induced neurotoxicity is that through apoptosis promotion. Our recent studies suggest the role of inflammation in ethanol-induced cell death in both the brain and astrocytes, as we found that ethanol-induced activation in MAPKs, NF-kappaB, AP-1 was associated with increased apoptosis in both the brain and astrocytes (71, 72). Furthermore, we were able to prevent ethanol-induced apoptosis in astrocytes by blocking the receptors TLR4 and IL-1RI (71), suggesting the involvement of the innate immune response in ethanol-induced astrocytic death. Nevertheless, although apoptosis occurs in neurodegenerative disorders (114, 115), we cannot rule out the possibility that ethanol also causes necrotic cell death since it has been suggested that necrosis often triggers a prominent inflammatory reaction, while apoptosis results in the uneventful removal of dying cells, with little or no inflammation (116).

Figure 2 illustrates the cascade of events by which ethanol, through the stimulation of TLRs signaling pathways mediated by glial activation, triggers the production of inflammatory mediators and toxic compounds that could exacerbate an inflammatory response leading to astrocytic and neural death.

### 3.3. Ethanol and innate immune system

Ethanol has an immunomodulatory effect that increases host susceptibility to infections by producing specific defects in both innate and cellular immunity (117, 118). Moreover, ethanol adversely impacts the immune response to other traumatic catabolic insults such as burn, haemorrhage and ischemia/reperfusion (119-121). Ethanol is known to alter cytokine levels in a variety of tissues including plasma, liver, lung and brain (122). Furthermore, recent studies demonstrate that the effects of ethanol on the innate immune system are mediated by the ethanol-induced disruption of TLRs signaling response (123).



**Figure 2.** Potential mechanism of ethanol-induced brain damage. Ethanol triggers signaling inflammatory responses (MyD88, MAPKs, NF-kappaB and AP-1), and the production of inflammatory mediators and toxic compounds (IL-1-beta, iNOS, COX-2, IL-10, TNF-alpha) by activating TLR4 and IL-1RI in glial cells, and this could exacerbate the inflammatory response leading to brain damage by astrocytic and neuronal death.

Many studies have consistently described that ethanol acutely suppresses the cytokine/chemokine response. For example, acute ethanol treatment suppresses the LPS-induced increase of TNF-alpha, IL-1-beta and IL-6 in blood, as well as *in vitro* responsiveness of various types of isolated cells, including macrophages and monocytes (124, 125). Recent findings demonstrate that ethanol suppresses the production of cytokines and chemokines in macrophages and monocytes by impairing the signaling response associated with the activation of TLR3 (126), TLR4 (127), TLR2 and TLR9 (128). Specifically, acute ethanol treatment has been shown to inhibit p38 and ERK pathway associated with the activation of TLR4, TLR2, and TLR9 (128), and suppress TLR3 signaling, resulting in an inhibition of interferon-related amplification and of several effector molecules of innate immunity and inflammation (126). Recent findings suggest that ethanol may affect inflammatory pathways differently depending on the complexity of TLR-mediated signals (129). These studies show that while acute ethanol

treatment attenuates TLR4 but not TLR2-induced TNF-alpha and NF-kappaB, the production of TNF-alpha increases when both TLR4 and TLR2 activation are present. These studies also demonstrate that while ethanol inhibits IRAK and ERK phosphorylation in TLR4-stimulated cells, JNK and AP-1 nuclear binding are augmented in the presence of combined TLR4 and TLR2 stimulation. These results show that the complex interactions between TLRs may determine attenuation or augmentation of inflammatory responses by acute ethanol treatment. Similarly, Frost et al (2005) (130) reported that acute ethanol regulates the TLR2 and TLR4 mRNA expression differently in a tissue-specific manner and that these changes might influence the subsequent host response to an invading bacterial pathogen.

In contrast to the general depressing effects of acute ethanol treatment on the innate immune system and TLRs response, chronic ethanol abuse often leads to an exaggerated inflammatory response, especially by the liver

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(131) characterized by elevated levels of proinflammatory cytokines in this tissue (132), and in plasma (133, 134). The gastrointestinal tract seems to be the initial target of ethanol, leading to an activation of the innate immune response. Alcohol consumption impairs the intestinal barrier function, leading to elevated plasma levels of endotoxins such as lipopolysaccharide (LPS) (135). Increased exposure to LPS and TLR4 activation seems to significantly contribute to the activation of the innate immune response in liver. In addition, chronic ethanol also exacerbates the response of Kupffer cells to LPS, resulting in an increased production of inflammatory cytokines, especially TNF- $\alpha$  (see rev., Nagy et al., 2003) (112). Recent findings have identified a number of intermediates in the TLR4 signaling cascade that are affected by chronic ethanol, including an increased expression of CD14, as well as an enhanced activation of NF- $\kappa$ B and the MAPK family members, p44/p42 ERK and p38 (see rev., Nagy et al., 2003) (112). Furthermore, although the importance of TLR4 in the development of alcohol liver disease has been demonstrated in knockout mouse models, in which the deficiency of TLR4 protects against alcohol-induced liver injury (136), recent studies demonstrate that chronic ethanol treatment upregulates multiple TLRs such as TLR1, TLR2, TLR4, TLR6, TLR7, TLR8 and TLR9 in mice liver, suggesting that the ethanol-exposed liver is sensitive to inflammation induced by multiple bacterial products recognized by TLRs (137).

At present, the mechanisms by which ethanol can either inhibit or augment TLR signaling have not been elucidated, but some findings support the idea that ethanol might affect the early steps in the TLRs signaling, such as the TLRs clustering and recruitment into the lipid rafts (see below). Although the exact nature of interaction between TLRs (e.g. TLR4 and TLR2) and their agonists has not yet been fully elucidated to date, it is clear that any changes in the physical or chemical state of the cell membranes would affect cell processes that involve membrane-associated proteins. Ethanol interacts with membrane lipids and influences the function of membrane proteins (138). Acute ethanol treatment is known to increase membrane fluidity (139), which may interfere with receptor clustering and signal transduction processes. Conversely, the fluidizing effects of ethanol during chronic ethanol exposure are counterbalanced by changes in membrane lipids toward more saturated fatty acids (140), allowing TLRs specific ligands binding and exacerbating the inflammatory response.

In summary, the results presented demonstrate that the effects of ethanol on both the immune system and TLRs are complex, and they depend on the ethanol dose, treatment duration (acute vs. chronic), type of cell and tissue analyzed, as well as the concomitant presence of pathogens and their characteristics. Clearly, further studies are required to ascertain the molecular and cellular mechanisms underlying the different effects of ethanol on the TLRs immune response.

### 3.4. Role of TLR4/IL-1RI signaling in ethanol-induced inflammatory response in brain and in astrocytes.

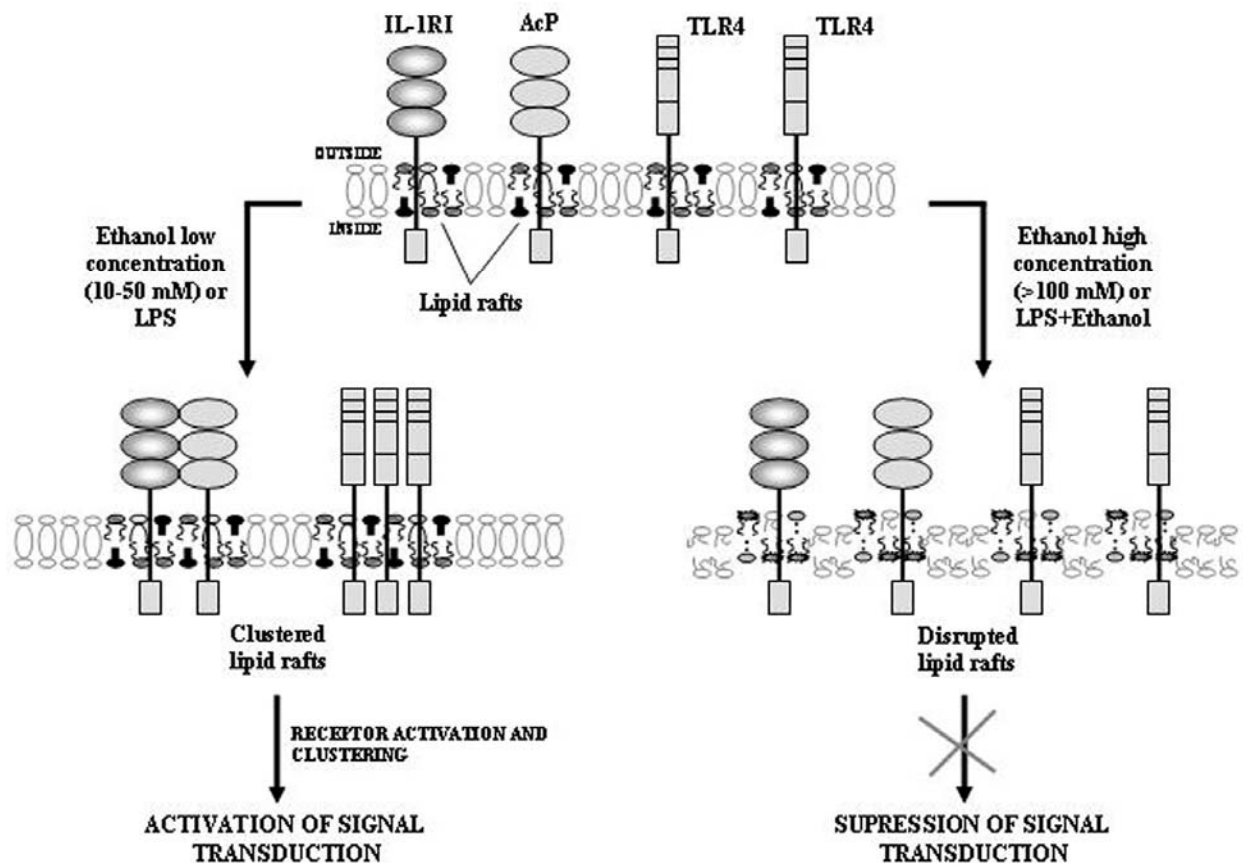
As already commented, the CNS exhibits well organized innate immune reactions, and glial cells, in

response to injury (32, 33) and infections (141, 142), are capable of mounting a quick and effective response to control an infection until the cells of the peripheral adaptive immune system can be recruited (143, 144). Microglia and astrocytes respond functionally with cytokine and chemokine production (57, 145) and they are capable of contributing to an inflammatory environment in the CNS (16, 146) following a variety of infectious or inflammatory insults (52).

TLR4 and IL-1RI trigger common signaling pathways (figure 1), and both are absolutely necessary in the expression of a large number of genes that are characteristic of inflammation. TLR4 is critical for the LPS-induced innate immune response and injury in the CNS (147), and members of the interleukin-1 (IL-1) family of cytokines are key mediators in both the regulation of host defense responses and development of inflammation in response to acute and chronic injury to the brain (148). Results in our laboratory have shown that both chronic and acute ethanol are capable of inducing intracellular signal-transduction events associated with the TLR4 and IL-1RI signaling response in both the brain and astrocytes (70-72). Specifically, these studies demonstrate that ethanol at physiological relevant concentrations such as those found in alcoholics (10-50 mM, approximately 0.05-0.2 g/dl), rapidly stimulate the phosphorylation of IRAK, p44/p42 ERK, SAPK/JNK and p38 MAPK, followed by the activation of NF- $\kappa$ B and AP-1, and also the transcriptional activation of iNOS and COX-2. Interestingly, these events were associated with cell death (71). Conversely, high ethanol concentrations (>100mM, 0.4 g/dl) inhibit the TLR4/IL-1RI response or have no effect (71). Blocking TLR4 and IL-1RI abolishes most of the inflammatory signals events and prevents cell death, supporting the notion that ethanol-induced inflammatory mediators in the brain are induced via the activation of glial TLR4/IL-1RI signaling pathways (71).

How does ethanol interact with TLR4/IL-1RI to either activate or inhibit their signaling response remains uncertain. Nevertheless, some recent findings suggest that ethanol might influence the early steps in the TLRs signaling, affecting clustering and recruitment of TLRs into the lipid rafts (127, 149). Lipid rafts are membrane microdomains enriched in cholesterol and sphingomyelin which are known to be involved in signal transduction by promoting the recruitment and clustering of specific receptors (150, 151). Recent reports demonstrate the presence of TLR2 and TLR4 within lipid rafts and their subsequent clustering in response to LPS (152, 153), supporting their role in the innate immune response (152-155). Indeed, disruption of lipid rafts leads to an inhibition of TLRs internalization and signaling (152). Interestingly, recent findings demonstrate that high concentrations of ethanol can perturb membrane lipid microdomains, interfering with lipid raft clustering and leading to the suppression of TLR4 signaling (127, 149). However, low concentrations of ethanol might facilitate the aggregation and interaction of proteins within the membrane, promoting receptor recruitment into lipid rafts, and leading to dimerization and signaling.





**Figure 3.** Suggested model by which ethanol mediates activation of TLR4/IL-1RI through its interactions with lipid rafts. We hypothesized that low concentrations of ethanol (10-50 mM) or LPS might facilitate TLR4 and IL-1RI aggregation and the recruitment into lipid rafts, leading to the clustering of these receptors and the activation of its signal transduction. On the contrary, high ethanol concentrations (>100 mM) or the LPS plus ethanol combination, can perturb membrane lipids, including lipid rafts, resulting in a disruption of the receptor clustering which suppress TLR4 and IL-1RI activation by its ligands binding and signaling.

Furthermore, several studies demonstrate that ethanol suppresses both cytokine-induced iNOS expression (87, 156) and LPS-induced NO production (157) and NF-kappaB activation, but not IFN-alpha-induced signaling in glial cells (158). These results confirm previous findings demonstrating that ethanol affects inflammatory pathways differently depending on the complexity of TLR-mediated signals (129). In addition, these findings also suggest that ethanol could interfere with the TLR4 recruitment into the lipid rafts when other stimuli (e.g. LPS, cytokines) are present, leading to an inhibitory rather than an additive effect on the TLR signaling transduction.

Since lipid rafts are considered to be important for TLRs signaling, alterations in the membrane lipid composition, such as those occurring after chronic ethanol intake, would be expected to lead to different ethanol effects on TLRs response. Indeed, while acute ethanol treatment down-regulates the production of pro-inflammatory cytokines induced by LPS in human monocytes (159, 160), chronic ethanol treatment (7 days *in vitro*) results in the augmentation of LPS-induced TNF-alpha (122). A schematic model of the suggested effects of

low and high concentrations of ethanol in the absence or presence of LPS on both lipid rafts and the signal transduction of TLR4 and IL-1RI is provided in figure 3.

To summarize, although the ethanol effects on TLRs response are complex, some data suggest that these receptors, particularly TLR4/IL-1RI, are important targets of ethanol-induced inflammatory damage in many organs, including the brain.

#### 4. CONCLUSIONS AND PERSPECTIVES

Results of studies reviewed in this article indicate that ethanol consumption activates a wide range of inflammatory mediators and signaling pathways in the brain, which are associated with inflammation and the immune response. In particular, ethanol-induced inflammatory responses seem to involve activation of LPS and IL-1-beta specific receptors, such as TLR4 and IL-1RI. It is important to emphasize that the actions of ethanol on inflammation and host defense mechanisms are strictly time-, dose- and cell type-dependent. However, there is still much to be understood about the nature of CNS

inflammation and molecular effects of ethanol in the brain before any clinical treatment development may be successfully explored. Further studies are required to understand the mechanisms by which ethanol activates TLR4/IL-1RI, as well as to ascertain whether other TLRs are also involved in the ethanol-induced inflammatory damage in both the brain and astrocytes. In the search for answers, it is necessary to use as many kinds of tools as possible, bearing in mind that specific deficits may be observed only with certain methods, specific tissue regions, and conditions of ethanol administration. Although these results contribute to our understanding of ethanol-related brain injury, they may also lead to the potential of new treatment and/or intervening strategies to restore ethanol-induced brain damage.

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**Role of TLR4/IL-1RI in ethanol-induced brain inflammation**

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