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HMGB1/TLR Receptor Danger Signaling Increases Brain Neuroimmune Activation in Alcohol Dependence

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Abstract

Background—Innate immune gene expression is regulated in part through high mobility group box 1(HMGB1), an endogenous proinflammatory cytokine, that activates multiple members of the interleukin-1/Toll-like receptor (IL-1/TLR) family associated with danger signaling. We investigated expression of HMGB1, TLR2, TLR3 and TLR4 in chronic ethanol treated mouse brain, post-mortem human alcoholic brain, and rat brain slice culture to test the hypothesis that neuroimmune activation in alcoholic brain involves ethanol activation of HMGB1/TLR danger signaling.

Methods—Protein levels were assessed using Western blot, ELISA, immunohistochemical immunoreactivity (+IR), and mRNA levels were measured by real time PCR in ethanol-treated mice (5 g/kg/day, i.g., 10 days + 24 hr), rat brain slice culture, and post-mortem human alcoholic brain.

Results—Ethanol treatment of mice increased brain mRNA and +IR protein expression of HMGB1, TLR2, TLR3, and TLR4. Post-mortem human alcoholic brain also showed increased HMGB1, TLR2, TLR3, and TLR4+IR cells that correlated with lifetime alcohol consumption as well as each other. Ethanol treatment of brain slice culture released HMGB1 into the media and induced the proinflammatory cytokine, IL-1 β . Neutralizing antibodies to HMGB1 and small inhibitory mRNA to HMGB1 or TLR4 blunted ethanol induction of IL-1 β .

Conclusions—Ethanol-induced HMGB1/TLR signaling contributes to induction of the proinflammatory cytokine, IL-1 β . Increased expression of HMGB1, TLR2, TLR3, and TLR4 in alcoholic brain and in mice treated with ethanol suggests that chronic alcohol-induced brain neuroimmune activation occurs through HMGB1/TLR signaling.

Keywords

alcoholism; Toll-like receptors; ethanol; cytokines; interleukin-1; cerebral cortex

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Background

Neuroinflammation is implicated in the etiology of many brain diseases. Recent discoveries indicate that endogenous danger-associated molecular pattern agonists and Toll-like receptors (TLRs) contribute to neuroinflammation [1–3]. High-mobility group box 1 (HMGB1) is an endogenous danger signaling cytokine that acts on multiple interleukin-1/Toll-like receptors (IL-1/TLRs) [2, 4, 5]. The family of IL-1/TLRs signal through kinases that activate the proinflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) increasing expression of cytokines, oxidases, and other genes associated with innate immune responses [6]. Although there are multiple TLRs in the brains of mice and humans, TLR2, TLR3, and TLR4 are the most commonly studied. HMGB1/TLR danger signaling is associated with persistent chronic inflammatory diseases [7], and our studies find that once induced, neuroimmune activation in brain persists for long periods. For example, ethanol [8] and endotoxin [9] treatment of mice increases proinflammatory gene expression in brain that persist for weeks to months after treatment. Thus, we hypothesized that HMGB1/TLR danger signaling might contribute to ethanol-induced neuroinflammation and the neurobiology of alcoholism.

We have previously found that ethanol increases expression of brain proinflammatory genes through activation of the transcription factor, NF- κ B [6, 10]. Chronic ethanol-induced expression of brain proinflammatory cytokines and oxidases are linked to neurodegeneration [8, 11] and inhibition of neurogenesis [12]. In post-mortem human alcoholic brain we found increased expression of the proinflammatory chemokine, monocyte chemoattractant protein, MCP1 and markers of microglial activation [13], and increased expression of IL-1 β and inflammasome proteins [14] as well as increased expression of proinflammatory NADPH oxidase [15], indicating significant neuroimmune activation in alcoholic human brain. Further, transgenic mice lacking TLR4 are protected against ethanol-induced proinflammatory gene induction, glial activation, and neurotoxicity [16]. HMGB1/TLR danger signals may also impact responses to ethanol since knockdown of amygdala TLR4 with small inhibitory RNA (siRNA) blunts ethanol-dependent rat operant responding for ethanol [17] and acute behavioral responses to ethanol are altered in transgenic mice lacking TLR2 and/or TLR4 [18]. Studies in mice have found that neuroimmune activation increases ethanol drinking [19], whereas transgenic mice lacking innate immune genes drink less than their wild-type controls [20]. These studies suggest that the actions of ethanol in brain include neuroimmune activation consistent with danger signaling through TLR4 and perhaps other TLRs. Thus, alcoholic human brain shows neuroimmune activation and ethanol-induced neuroimmune activation is linked to drinking behavior, alcohol responses, TLR upregulation, and neurodegeneration.

We report here that ethanol treatment of mice or rat brain sections *in vitro* increase expression of HMGB1/TLRs. Post-mortem human alcoholic brain was found to have significantly more HMGB1, TLR2, TLR3, and TLR4 which correlated with lifetime alcohol consumption. Furthermore, ethanol was found to release HMGB1 from brain slice culture activating proinflammatory IL- β synthesis through TLR4. These findings support the hypothesis that ethanol activation of HMGB1/TLR danger signaling contributes to neuroimmune activation in alcoholic brain and to the neurobiology of alcoholism.

Methods and Materials

Animals

Eight-week old male (20–22 g) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). After at least 1 week of acclimation to the animal colony, mice were divided into control and ethanol groups. Mice were treated with water (control) or ethanol (5

g/kg, i.g., 25% ethanol w/v), with volumes matched, daily for 10 days and sacrificed 24 hr later. The average blood ethanol concentration (BEC) at 1 hr after the first ethanol treatment and the last ethanol treatment was 291 ± 16 mg/dl (w/v, n=10) and 301 ± 19 mg/dl (w/v, n=10), respectively. The BEC is high modeling alcoholic binge drinking [21]. Brain slice culture was prepared from P7 pups from timed pregnant Sprague-Dawley rats.

All protocols and procedures in this study were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institute of Health regulations for the care and use of animals in research. Details on reagents and reagents sources and equipment are listed within the supplemental methods and materials section.

Brain Slice Culture

Organotypic hippocampal-entorhinal cortex (HEC) brain slice culture was prepared from P7 Sprague-Dawley rat pups as described previously [6, 22]. After 10 days in culture, slices were used for experimental treatments as described below.

Human tissue

Human post-mortem brain tissue was obtained from the New South Wales Tissue Resource Center in Australia [ethics committee approval number: X11-0107]. Paraffin sections of orbitofrontal cortex (OFC) were used in this study. The detailed patients' medical history is presented in Table 1. Human alcoholic patients, which averaged over 10 drinks per day with lifetime consumption of over 500 kg of alcohol, were compared to moderate drinkers who averaged less than 1 drink per day with lifetime consumption of less than 100 kg of alcohol. Total lifetime alcohol consumption provides an index of alcoholic neurodegeneration [23, 24], whereas there is no relationship among moderate drinkers [24, 25]. Only individuals with alcohol dependence not complicated by liver cirrhosis or nutritional deficiencies were included in this study. The most common cause of death was cardiovascular disease for both groups. Post-mortem interval (PMI) causes of death and alcohol consumption are documented. All psychiatric and alcohol use disorder diagnoses were confirmed using the Diagnostic Instrument for Brain Studies which complies with the Diagnostic Statistical Manual of Mental Disorders and has demonstrated reliability [26].

Real-time PCR analysis

Total RNA was extracted from mouse brain or rat brain slice culture. Real-time PCR was performed as described previously [27] and is in supplemental methods and materials section. The primer sequences used in this study were as follows:

Immunohistochemistry—Mouse brains were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and processed for immunostaining as described previously [27]. Table 3 describes the antibodies used for immunostaining of TLR2, TLR3, TLR4 and HMGB1. Immunolabeling was visualized by using nickel-enhanced 3,3'-diaminobenzidine (DAB).

Stereological cell counts—Immunoreactive (+IR) cells were quantified using unbiased stereological cell counts in six random regions of interest from each brain sample and were counted at 40X. Cell density (Nv) was determined following the optical disector method [28, 29], as described previously [8, 27]. More details on the cell counting methods are described in the supplement.

HMGB1 ELISA

At the conclusion of ethanol treatment, HEC slices were removed for real-time PCR or immunohistochemistry and culture media were used for ELISA detection of released HMGB1. HMGB1 ELISA was performed according to the manufacturer's protocol of ELISA kits (IBL, Germany).

TLR4 or HMGB1 knockdown with siRNA

Rat TLR4 or HMGB1 siRNA (Ambion, Grand Island, NY) was used to knockdown TLR4 or HMGB1 expression. Preparation of transfection reagents and transfection was performed as previously described [14]. Briefly, the transfection mixture was added to serum-free N2 medium at a final concentration of 20 nM siRNA + 6 μ l Lipofectamine 2000 to a total volume of 1.2 ml (500 μ l on top of slices and 700 μ l at bottom of the culture). Vehicle controls were treated with the same N2 medium containing negative control siRNA (Ambion, Grand Island, NY). After 24 hr transfection, siRNA-containing medium was replaced with regular serum-free N2-supplemented medium and the slices were cultured for 4 days in the absence or presence of ethanol (100 mM). At the end of experiments, the slices were removed for real-time PCR analysis.

Western blotting

For human Western blots, 30 mg of frozen OFC tissue was homogenized in 0.2 ml RIPA lysis buffer with protease inhibitor (1ml/10ml buffer) and centrifuged at 14000 rpm at 4°C for 1 hr. Protein in the supernatant was measured using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) and 25 μ g of protein from each denatured sample was subjected to SDS-PAGE on Bio-Rad Precast polyacrylamide mini-gel (4–15%) and blotted onto immunoblot PVDF membrane. Antibodies were used to identify TLR2, TLR3, TLR4, and HMGB1 bands (table 3). Bands were scanned with Odyssey Infrared imager from LI-COR Bioscience (Lincoln, NE). Band intensity was quantitated using Bioquant Imaging software and normalized to β -actin band intensity. To support antibody specificity, TLR2, TLR3, TLR4 and HMGB1 antibody preabsorption was performed using 0.5 μ g/ml of the corresponding blocking peptides.

Western blot analysis of HEC slices used lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9) plus protease inhibitor cocktail (Sigma) for 15 min and then disrupted with sonication (3 time, 2 min apart on ice). After centrifugation, the supernatant were collected, and protein determined using a Bio-Rad Bradford reagent kit. For Western blotting, an equal amount of protein (50 μ g) or culture medium (40 μ l) was mixed with 10/ μ l 5 $\times$ loading buffer, and separated using a 4–15% Tris mini-gel (Bio-Rad) and transferred onto PVDF membrane. After blocking with LI-COR blocking buffer overnight, the membrane was probed with anti-HMGB1 antibody (1:400, Abcam) at 4°C overnight. After washing, membrane was incubated with second antibodies coupled with fluorescence from LI-COR Bioscience (Lincoln, NE).

Statistical analysis

The data are expressed as mean $\pm$ SEM and statistical significance was assessed with a ANOVA followed by Bonferroni's t-test using the StatView program (Abacus Concepts, Berkeley, CA). A value of $p < 0.05$ was considered statistically significant. The association between HMGB1, TLR, and lifetime alcohol consumption was analyzed with the Pearson product-moment correlation coefficient (Pearson correlations (r)) to determine meaningful relationships.

Results

Increased expression of danger signaling HMGB1 and TLR2, TLR3, and TLR4 in ethanol-treated mouse brain

To investigate the effects of chronic binge-like ethanol treatment on danger signal expression, C57BL/6 mice were treated for 10 days with a binge drinking dose of ethanol. Ethanol treatment of mice increased brain TLR2 mRNA $241 \pm 26\%$ ($p < 0.05$), TLR3 mRNA $167 \pm 17\%$ ($p < 0.05$), and TLR4 mRNA $233 \pm 29\%$ ($p < 0.01$) (Figure 1). To determine protein expression in orbital frontal cortex (OFC) and entorhinal cortex (ENT), we determined immunoreactivity (+IR) for each of these proteins. The controls in both OFC (Figure 1C) and ENT (images not shown) showed low levels of diffuse staining of small punctate spots with a few cellular silhouettes for all three TLRs. Ethanol treatment increased staining, particularly cellular staining (Figure 1C). In OFC, ethanol treatment increased TLR2+IR cells $176 \pm 10\%$ ($p < 0.01$), TLR3+IR cells $151 \pm 10\%$ ($p < 0.01$) and TLR4+IR cells $174 \pm 12\%$ ($p < 0.01$). In ENT, ethanol treatment increased TLR2+IR cells $165 \pm 9\%$ ($p < 0.01$), increased TLR3+IR cells $183 \pm 9\%$ ($p < 0.01$), and increased TLR4+IR cells $221 \pm 13\%$ ($p < 0.01$). Increases in TLR2, TLR3, and TLR4 mRNA, and cell staining +IR are consistent with ethanol treatment of mice inducing expression of all three TLRs in cortex.

HMGB1 is a commonly expressed cellular protein [4]. In mice treated with chronic ethanol, brain HMGB1 mRNA increased to $219 \pm 20\%$ ($p < 0.01$) of control brain mRNA (Figure 3A). Immunohistochemical staining of protein +IR found OFC increased $181 \pm 9\%$ ($p < 0.01$) and ENT $195 \pm 5\%$ ($p < 0.01$) (Figure 3B). In controls, there were at least two types of cellular staining: (1) large cells with strong membrane staining silhouettes, and (2) smaller cells with more homogenous staining. Both forms of cellular staining appeared to increase with ethanol treatment. Thus, chronic ethanol treatment of mice increases brain HMGB1 mRNA and the number of HMGB1+IR cells in OFC and ENT. These findings suggest that chronic ethanol treatment of mice increases danger signaling agonist HMGB1 as well as danger signaling receptors TLR2, TLR3, and TLR4 in cortex.

Increased expression of danger signaling HMGB1 and TLR2, TLR3, and TLR4 in post-mortem human alcoholic brain

In previous studies, we found that post-mortem human alcoholic brain has increased expression of the proinflammatory chemokine MCP1 and microglial markers [13], increased expression of IL-1 β and inflammasome proteins [14] and increased expression of proinflammatory NADPH oxidase (NOX) [15]. We studied post-mortem human OFC to determine if HMGB1/TLR danger signaling molecules were increased in alcoholic brain (Table 1, Figure 4 and 5). Moderate drinking controls showed little TLR2+IR staining. TLR3+IR controls showed a few cells and stained processes, but overall low levels of staining. TLR4+IR in controls showed large and small cellular silhouettes (images not shown). Alcoholic brains showed increased TLR2+IR cells $208 \pm 15\%$ ($p < 0.01$), TLR3+IR cells $259 \pm 18\%$ ($p < 0.01$), and TLR4+IR cells $356 \pm 73\%$ ($p < 0.01$), compared to moderate drinking controls. Western blot analysis of protein expression levels also indicated increased levels of TLR2 ($147 \pm 14\%$, $p < 0.05$), TLR3 (180 ± 27 , $p < 0.05$) and TLR4 ($184 \pm 24\%$, $p < 0.01$) (Figure 4). Thus, both methods of quantification found significant increases in post-mortem human alcoholic OFC. Similar to that found in ethanol-treated mouse brain, post-mortem alcoholic brain OFC showed increased expression of HMGB1+IR cells ($213 \pm 21\%$, $p < 0.05$, Figure 5) and Western blot HMGB1 protein ($151 \pm 18\%$, $p < 0.01$, Figure 5). Surprisingly, most labeled cells appeared to have neuronal morphology promoting further analysis in alcoholic brain where staining was prominent. Double confocal immunohistochemistry using NeuN to identify neuronal cells indicated that NeuN+IR colocalization with $90 \pm 2.4\%$ of TLR2+IR cells, $89.4 \pm 1.8\%$ of TLR3+IR cells, $80.6 \pm 1.5\%$ of TLR4+IR cells, and $82.7 \pm$

4.3% of HMGB1+IR cells (Supplemental Figure S1). Increased expression of the cytokine HMGB1, as well as TLR2, TLR3, and TLR4, suggest increased HMGB1/TLR signaling in alcoholic brain.

Our findings of increased HMGB1, TLR2, TLR3, and TLR4 in chronic ethanol-treated mouse brain as well as human alcoholic brain prompted a comparison of human lifetime alcohol consumption with expression of HMGB1/TLR danger signaling molecules. HMGB1, TLR2, TLR3, and TLR4 all showed significant correlations with lifetime alcohol consumption (Table 4, Figure 6). Moderate drinking control subjects consumed much less lifetime alcohol with the heaviest drinking control consuming approximately 100 kg of alcohol. Alcoholics in our study consumed between 500 and 5,000 kg of alcohol providing a broad range of consumption that shows a progressive increase in HMGB1 expression with increased lifetime drinking (Figure 6). HMGB1 and TLR3 showed the strongest correlations with lifetime alcohol consumption, although TLR2 and TLR4 were significantly correlated. The correlation of HMGB1 as well as TLR2, TLR3, and TLR4 with lifetime ethanol consumption is consistent with ethanol induction of these proteins in brain.

HMGB1 and TLRs are required for ethanol induction of proinflammatory interleukin-1 β (IL-1 β)

To determine if the increased in expression of HMGB1/TLR danger signaling proteins actually increased neuroimmune gene expression, we assessed ethanol effects on danger signaling in slice culture. We have previously found that ethanol treatment of brain slice culture increases NF- κ B-DNA binding and transcription of multiple neuroimmune cytokines, oxidases, and proteases [11, 30]. We first determined whether ethanol treatment induced HMGB1 in rat brain slice cultures. Ethanol treatment increased HMGB1 mRNA about 4.5-fold (Figure 7). We had limited hippocampal sections from human post-mortem brain, but staining of individual samples from moderate drinking controls and alcoholics suggest similar increases in HMGB1+IR in hippocampus (Figure 7) to those found in cortex (Figure 5). Ethanol-treated rat brain slice culture showed that expression of HMGB1 colocalizes with neurons (NeuN+IR) using confocal microscopy (i.e. red-HMGB1 and green-NeuN coincide forming yellow, Figure 7). Thus, ethanol treatment of slice culture increases HMGB1 mRNA that histochemically appears to be in neurons.

Our previous studies found that ethanol-induced neuroimmune activation is amplified by signals that converge upon NF- κ B transcription of proinflammatory genes that form positive loops of activation [6] increasing transcription of multiple cytokines, cytokine receptors and TLRs, oxidases and proteases [8, 11, 15, 30]. To investigate the role of HMGB1/TLR signaling, we followed IL-1 β which we have previously found to be induced by ethanol in brain slice culture and in mice treated with ethanol [11, 14]. Ethanol increased HMGB1 protein within the brain slices as well as the media as determined using Western blots and ELISA (Figure 8). Ethanol treatment of brain slice culture also increased expression of IL-1 β mRNA, similar to previous studies [11]. Interestingly, ethanol increased IL-1 β expression was blocked by the addition of a neutralizing HMGB1 antibody (Figure 8). Neutralizing antibody binds HMGB1 preventing its interaction with receptors suggesting that ethanol released HMGB1 contributes to ethanol induction of IL-1 β . We used small inhibitory mRNA to knockdown expression of HMGB1 or TLR4, a key TLR in HMGB1 proinflammatory responses. We found that knockdown of HMGB1 or TLR4 blocked ethanol induction of IL-1 β . These findings suggest that ethanol releases HMGB1 that stimulates TLR4 leading to induction of IL-1 β .

Coincident induction of danger signaling molecules by ethanol

Our previous studies have found that ethanol induces brain neuroinflammatory responses through increased NF- κ B transcription of proinflammatory genes including TLRs, cytokines such as TNF α , IL-6, IL-1 β , and the chemokine MCP1, as well as oxidases COX and NOX, and other innate immune genes that contribute to persistence and amplification of brain neuroimmune responses [6, 10]. The finding that all of the HMGB1/TLR danger signaling molecules investigated in this study were increased by ethanol and correlated with lifetime ethanol consumption prompted assessment of correlations among the four HMGB1/TLR danger signaling molecules. Interestingly, HMGB1+IR cells in post-mortem human brain were correlated with TLR2, TLR3, and TLR4+IR cell expression ($r=0.85$, $p<0.0001$; Figure 9) and to each other (Table 4). The correlated increases in HMGB1 expression across all 3 TLRs is consistent with other findings of proinflammatory responses involving overlapping amplification of signals that contribute to persistent neuroimmune signaling [6]. Our finding that TLR2, TLR3, and TLR4 all correlate with increased HMGB1 and each other suggest that all are upregulated together as neuroimmune activation is amplified across cells and innate immune genes. This is consistent with ethanol exposure increasing neuroimmune activation through positive loops of innate immune gene induction that amplify multiple HMGB1/TLR danger signals and other neuroimmune genes in parallel (Figure 10).

Discussion

We report here that chronic ethanol treatment of mice increases HMGB1 mRNA and cellular expression of protein in OFC (i.e., HMGB1+IR). We also found HMGB1+IR increased in the human post-mortem OFC. HMGB1 is a common nuclear protein that is released by active and passive processes and has cytokine-like properties expressed through activation of IL-1/TLRs that stimulate NF- κ B transcription of innate immune genes [2, 4]. Using cultured brain slices, we found that ethanol increased HMGB1 in medium, consistent with ethanol both inducing and releasing HMGB1. We have previously found that chronic ethanol treatment increases expression of brain NF- κ B proinflammatory target genes including COX [31], NOX [15], multiple cytokines, proteases, and oxidases [8, 11, 15] as well as increasing NF- κ B-DNA binding *in vivo* [10] and in brain slice culture [30]. Although HMGB1 can bind to and activate multiple TLRs, signaling is complex due to neuroimmune amplification within and across cells that release additional HMGB1, other cytokines, and molecules that contribute to the response [2, 4]. In previous studies, we found ethanol pretreatment of mice potentiated TLR3 agonist Poly I:C [32] and TLR4 agonist LPS [8] induced proinflammatory gene induction. Although HMGB1 can activate multiple receptors and HMGB1 injected into brain causes cognitive deficits through multiple receptors [33], proinflammatory activation of innate immune cells by HMGB1 requires TLR4 [34, 35]. Many studies have suggested that brain neuroimmune activation requires microglia activation through TLR4 [36, 37]. Microglial TLR4 is also critical for activation of astrocyte proinflammatory responses [38] perhaps due to basal expression of TLR4 on microglia, but not other brain cells [39]. Ethanol activation of NF- κ B proinflammatory transcription in isolated microglia [40] and mouse brain [16] is dependent upon TLR4. Since microglia are known to express high basal levels of TLR4 and to rapidly release IL-1 β in response to danger signals [4, 41], it is possible that the pivotal role of TLR4 for ethanol responses is due to the critical role of TLR4 in HMGB1 microglial activation and the resulting amplification of responses by astrocytes and neurons (Figure 10). Although HMGB1 signaling is complex, our findings are consistent with ethanol releasing HMGB1 that activates TLR4 receptors that increase NF- κ B transcription of IL-1 β and other proinflammatory genes that lead to neuroimmune activation (Figure 10).

We found increased expression of HMGB1, TLR2, TLR3, and TLR4 in OFC of human post-mortem alcoholic brain and chronic ethanol-treated mouse brain. Similar to other

reports, we found low levels of TLR expression in non-diseased human brain [42]. Other human postmortem studies report low levels of expression in healthy brain and broad brain regional increases in the expression of multiple TLRs across various brain diseases [42]. Proinflammatory gene induction is linked to neurodegeneration and the brains of transgenic mice lacking TLRs are protected against brain insults including ethanol [16, 43–45]. For instance, in models of stroke, mice lacking TLR2 or TLR4 have reduced infarct size and neuroimmune activation [43, 44], whereas in normal mice stroke models increase brain TLR2 and TLR4 expression [34, 44] and brain injection of HMGB1 increases infarct size [34]. Increased HMGB1 expression has been reported in hippocampal tissue from patients with temporal lobe epilepsy [46]. These studies found that glutamate hyperexcitability increases release of neuronal HMGB1 that activates TLR4 receptors releasing the proconvulsant IL-1 β , increasing seizure severity, and a long-lasting decrease in seizure threshold contributing to persistent risk of additional seizures [4, 41, 46]. Chronic alcohol treatment is also known to reduce seizure threshold, perhaps due to HMGB1/TLR induction. Similarly, neuroimmune microglial activation is associated with increased spinal cord hyperexcitability in models of chronic pain [47] that also finds persistent endogenous release of HMGB1 associated with the hyperexcitability [48]. Chronic ethanol also impacts chronic pain [49]. We studied human and mouse frontal cortex, the brain region most associated with alcoholic neurodegeneration [24, 50] and found increased expression of HMGB1/TLRs. We have previously established ethanol induced neuroimmune activation is a key component of neurodegeneration associated with ethanol exposure [6, 12]. Our studies of post-mortem human alcoholic brain find increased microglial markers and proinflammatory MCP-1 in multiple brain regions [13], increased IL-1 β and inflammasome proteins [14], and increased NADPH oxidase gp91, an innate immune oxidase linked to neurodegeneration [15]. In addition to neurodegeneration, HMGB1/TLR signaling links neuroimmune activation to components of addiction [6, 51]. Genetic studies have identified NF- κ B and proinflammatory genes as contributing to a genetic preference for drinking alcohol [52] and human genetic studies have identified alleles of NF- κ B1, a gene encoding a subunit of NF- κ B, are associated with increased risk of alcoholism [53] and other studies have linked innate immune genes to alcoholism [54]. Transgenic animals lacking innate immune genes show reduced alcohol drinking [6, 19] and altered acute ethanol motor and sedative responses [18]. Further, endotoxin produces persistent neuroimmune activation [9] and increased ethanol drinking in mice [1, 19]. Lever pressing for ethanol by ethanol-dependent rats is reduced by viral vector siRNA knockdown of TLR4 in brain [3, 17] and naltrexone, a drug that reduces alcohol drinking in rodents and is used to treat alcoholism, that also blunts ethanol induced neuroimmune activation [15], likely through a recently discovered antagonist action on TLR4 receptors [55]. These findings suggest increased HMGB1/TLR signaling contributes to alcoholic pathology.

We found that HMGB1+IR cells in OFC correlated with lifetime alcohol consumption and with TLR2, TLR3, and TLR4+IR cells. Alcoholic subject's lifetime alcohol consumption varied by over 10 fold and was far greater than the highest control. The correlation of HMGB1/TLR expression with lifetime alcohol consumption is consistent with alcohol induction of persistent neuroimmune activation. Neuroimmune activation by ethanol [8] or endotoxin [9] persists for long periods. HMGB1 and the TLRs all correlated with each other consistent with proinflammatory amplification of neuroimmune signals through positive loops of activation (Figure 10). Although TLR4 is critical for initiation of microglial activation, amplification through positive loops likely involves multiple proinflammatory cytokines and HMGB1/TLR danger signals that are upregulated in multiple brain cell types as neuroimmune activation spreads across the brain. Neuroimmune activation is associated with drug reward and addiction [6, 51, 56], depression-negative affect [57] and neurodegeneration [45, 58] consistent with HMGB1/TLR signaling contributing to the alcoholic pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Chronic ethanol treatment of C57BL/6 mice increases mRNA and protein expression (+IR) of TLR2, TLR3, and TLR4

(A) Levels of brain TLR2, TLR3, and TLR4 mRNA were measured by real-time PCR as described in the methods. Ethanol treatment significantly increased brain mRNA for TLR2, TLR3, and TLR4. (B) Immunoreactive (+IR) cells were quantified using unbiased stereological cell counts as described in the methods and supplement. TLR2, TLR3, and TLR4+IR cells were counted in mouse orbital frontal cortex (OFC). Ethanol significantly increased the number of TLR2, TLR3, and TLR4+IR positive cells. (C) Representative images of immunohistochemical staining for TLR2, TLR3 and TLR4 in the OFC region of control and ethanol-treated mice. Note staining for TLR in ethanol treated animal labels membranes of fairly large cells as well as smaller cells. * $p < 0.05$, ** $p < 0.01$, compared with water control group. Scale bar=50µm.

Figure 2. Chronic ethanol treatment of C57BL/6 mice (5 g/kg, i.g., daily for 10 days) increases TLR2, TLR3, and TLR4+IR cells in entorhinal cortex

Immunoreactive (+IR) cells were quantified in the entorhinal cortex (ENT) using unbiased stereological cell counts as described in the methods and supplement. Ethanol increased the number of TLR2, TLR3 and TLR4 +IR cells. ** $p < 0.01$, compared with water control group.

Figure 3. Ethanol increases HMGB1 expression in mouse brain

Chronic ethanol treatment of C57BL/6 mice (5 g/kg, i.g., daily for 10 days) increased mRNA and protein expression (+IR) of brain HMGB1. (A) HMGB1 gene expression was measured by real-time PCR. Chronic ethanol increased brain HMGB1 mRNA by approximately 2-fold. (B) Quantitative evaluation of HMGB1+IR in the OFC and ENT of control and ethanol-treated mice. Immunoreactive cells were quantified using unbiased stereological cell counts as described in the methods and supplement. The number of HMGB1+IR cells was increased 1.8 fold in OFC and 2-fold in ENT. (C) Representative images of immunostaining for HMGB1 in the OFC and ENT regions of water and ethanol-treated mice. ** $p < 0.01$, compared with water control group. Scale bar=50 μ m.

Figure 4. Increased expression of TLR2, TLR3, and TLR4 in post-mortem human alcoholic brain

See Table 1 for human subject details. (A) Quantification of TLR2, TLR3, and TLR4+IR cells. Immunoreactive (+IR) cells were quantified using unbiased stereological cell counts as described in the methods and supplement. The OFC of human post-mortem alcoholic brain had significantly more TLR2, TLR3, and TLR4 + IR cells, compared to the OFC of human moderate drinking controls (n=10 per group) (B) Quantification of Western blot analysis relative to β-actin. (n=8 per group). Although IHC and Western blots are from different subregions of OFC and different methodologies, Pearson's correlation of +IR vs. Western blot intensity was significant across subjects for TLR4 ($r=0.61$; $p<0.05$) and TLR3 ($r=0.56$, $p<0.05$), but TLR2 did not reach significance. (C) Images of Western blot. C is control and A is alcoholic with the # being individual patient. (C4+P = control patient 4 with peptide preabsorption; A4+P = alcoholic patient 4 with peptide preabsorption) Note that peptide preabsorption blocks antibody supporting specificity of binding. Alcoholic patients have more TLR2, TLR3, and TLR4 protein expression.

Figure 5. HMGB1 expression is increased in the post-mortem human alcoholic brain
 (A) Quantification of HMGB1 immunoreactivity. Immunoreactive (+IR) cells were quantified using unbiased stereological cell counts as described in the methods and supplement. The number of HMGB1+IR cells was significantly increased in alcoholic brain (n=10), compared to human moderate drinking controls. (B) Quantification of Western blot analysis relative to β -actin. (n=8 per group). * $p < 0.05$, ** $p < 0.01$, relative to moderate drinking controls. (C) Images of Western blot. C is control and A is alcoholic with the # being individual patient. (C4+P = control patient 4 with peptide preabsorption; A4+P = alcoholic patient 4 with peptide preabsorption) Note that peptide preabsorption blocks antibody supporting specificity of binding. Alcoholic patients have more HMGB1 protein expression.

Figure 6. HMGB1+IR cells in OFC correlate with lifetime alcohol consumption

Shown are individual HMGB1+IR cell counts vs. lifetime alcohol consumption. Note that control subjects are clustered along the y axis due to low lifetime alcohol consumption values and similar levels of HMGB1+IR. Alcoholic subjects show several fold variation in HMGB1+IR and over 10 fold variation in lifetime alcohol consumption. TLR2, TLR3, and TLR4+IR also correlate with lifetime alcohol consumption (Table 4). The derived straight line is shown ($r = 0.82; p < 0.001$). In previous studies, we have shown that neuroimmune activation persists for long periods once activated [8, 9]. The persistence of neuroimmune activation likely contributes to progressive amplification of HMGB1/TLR induction with repeated drinking that leads to cumulative increases [10] that result in correlations with lifetime alcohol consumption.

Figure 7. Ethanol induction of HMGB1 in rat brain slice culture

Brain slice culture was prepared and used after 2 weeks maturation in culture. Control or ethanol (100mM) treated slices were collected following treatment and assessed for protein or mRNA. (A) HMGB1 mRNA expression determined by real-time PCR as described in the methods. Note ethanol increased HMGB1 mRNA about 4.5 fold ($p < 0.05$). (B-top images) Immunohistochemical double labeling of brain slice culture of control (left) and ethanol (ETOH-100mM, right) treated slices for HMGB1 (red) and neuronal marker NeuN (green). Note ethanol treatment of brain slices culture increases HMGB1+IR (red). Green is the neuronal markers NeuN that combined with HMGB1 should appear yellow (red-green merged) that represent HMGB1 expressing neurons (upper right image). (C – bottom images) Immunohistochemistry of human hippocampus. A human moderate drinking control (bottom-left) and alcoholic (bottom-right) brain section after immunohistochemistry for HMGB1 (red) and neuronal marker NeuN (green). Note alcoholics show increased HMGB1 (red) that is almost entirely expressed within NeuN (green) expressing neurons such that all appear yellow. Arrows note HMGB1+IR in the cytoplasm consistent with cellular release of nuclear HMGB1.

Figure 8. Ethanol releases HMGB1 into media and activates IL-1 β synthesis through TLR4 (A) Increased HMGB1 protein level in ethanol-treated HEC slice culture cell lysate and medium. Left: HMGB1 released into culture medium, as assessed by ELISA, was increased by approximately 3-fold in ethanol group (100 mM, 4 days). * $p < 0.001$ compared with control; $n = 4$. This experiment was replicated 5 times and produced similar results. Right: Representative Western blots of HMGB1 protein from HEC brain slice culture cell lysates and medium. As depicted in right immunoblot, ethanol exposure increased HMGB1 protein in both cell lysates and release into the culture medium. (B) Ethanol induction of IL-1 β is blocked by siRNA and neutralizing antibodies. Left: mRNA levels of the proinflammatory cytokine IL-1 β , relative to negative siRNA-treated controls. Ethanol induction of IL-1 β was blocked by siRNAs that knockdown either TLR4 or HMGB1 (* $p < 0.001$ compared with control, $n = 4$). Shown on the right are mean $\pm$ SEM of proinflammatory cytokine IL-1 β mRNA level, relative to control that was treated with control antibody chicken IgY (10 μ g/ml) for HMGB1 neutralization (IBL, Germany). Ethanol induction of IL-1 β mRNA is blocked by HMGB1 neutralizing antibody (* $p < 0.0001$ compared to Control; # $p < 0.0001$ compared with EtOH).

Figure 9. HMGB1+IR cells in OFC correlate with TLR2, TLR3 and TLR4+IR cells
 Shown are individual subjects HMGB1+IR cells vs. TLR2, TLR3 or TLR4+IR cells. Across subjects, HMGB1 correlated with TLR expression ($r = 0.85$; $p < 0.0001$). Note that HMGB1+IR varies several fold across subjects. Each subject's TLR levels (e.g., TLR2, TLR3, and TLR4) arrange in vertical points over each subject's HMGB1 level. Although all correlate with HMGB1, the TLRs showing the greatest induction varies across subjects. For example, on the right are 3 subjects with the highest HMGB1 expression (alcoholics), two showing $TLR3 > TLR4 > TLR2$ and one with $TLR4 > TLR3 > TLR2$. Thus, among the TLRs examined, all are increased without any apparent priority once activation has occurred. The correlation of HMGB1 with TLR is consistent with neuroimmune loops of amplification coordinating induction of all TLR. Activation of innate immune proinflammatory signaling through multiple pathways is known to converge on signals leading to transcription of multiple TLRs, proinflammatory cytokines, and their receptors that spread across cells through paracrine and autocrine activation of proinflammatory signaling. The parallel induction of TLR is a well-known mechanism of amplification of innate immune signals. Our discovery of primarily neuronal localization of these signaling molecules suggest that neuroimmune signaling amplification of multiple TLRs also occurs among neurons in brain and is not unique to monocytes or microglia.

Figure 10. Schematic of ethanol-induced release of HMGB1 and activation of TLR4 on neurons and microglia IL-1 β creating positive loops of amplification of innate immune cascades

Ethanol (alcohol) is shown releasing HMGB1 from the neuronal nucleus that stimulates TLR4 on glia and neurons. Microglia contribute to the initiation of neuroimmune cascades [59], and under basal conditions, are the only brain cells that express TLR4 in mice [37, 39]. TLR4 are critical for HMGB1 activation of innate immune cells [38], although the effect on neurons is poorly understood. Ethanol activation of innate immune gene induction is blocked by minocycline, an inhibitor of microglial activation [15], inhibitors of NF- κ B [10], inhibitors of IL-1 β [14], naltrexone [15], and in microglial culture for transgenic mice lacking TLR4 [40], as well as in ethanol-treated mouse brain of transgenic mice lacking TLR4 [16]. We found HMGB1 is expressed primarily in neurons, consistent with studies finding that neuronal hyperexcitability releases HMGB1 [46]. HMGB1/TLR4 activation releases IL-1 β , which activates its own receptors linked to proinflammatory transcription cascades as well as releasing additional HMGB1 contributing to positive loops of amplification of innate immune gene induction. In previous studies, we found ethanol-induced NF- κ B transcription of IL-1 β , TNF α , MCP1, and IL-6 as well as oxidases, proteases, and other innate immune genes [11]. Illustrated are our findings reported here that ethanol releases HMGB1, and neutralizing antibody to HMGB1 and siRNA knockdown of HMGB1 block ethanol induction of IL-1 β . Also, TLR4 siRNA knockdown blocked ethanol induction of IL-1 β . This schematic illustrates ethanol initiation of positive loops of activation between neurons and microglia. Astrocytes are activated as well and contribute to altered neurotransmission although not illustrated for simplicity. Neuroimmune activation increases TLR expression, which is primarily neuronal. HMGB1 is an agonist at multiple TLRs and other brain neuroimmune receptors such as the receptor for glycation signals (RAGE) [60] and (MAC-1) [61]. We found that chronic ethanol and post-mortem human alcoholic brain had increased levels of TLR2, TLR3, and TLR4 as well as HMGB1 consistent with chronic neuroimmune activation that includes astrocytes, microglia, and neurons. Activation of microglia increases neuronal excitatory synaptic potentials [62] as well as initiating glial glutamate release [63] and neuronal extrasynaptic NMDA 2B receptors are sensitized by HMGB1/TLR4 increased phosphorylation [46]. Further, cytokines are known to inhibit glial glutamate transporters [22]. These findings support the schematic illustration of increased HMGB1/TLR signaling leading to hyper-excitability due to increased glutamate from glia and sensitized neuronal glutamate receptors. These findings support hypotheses linking alcohol dependence to a hyperglutamate state [64, 65].

Table 1

Patient characteristics of human post-mortem brains

Group	Age at Death	Sex	PMI	Clinical Cause of Death	Lifetime Ethanol (gm)
Control	60	Male	28	Ischemic heart disease	0
Control	62	Male	46	Ischemic heart disease	5000
Control	50	Male	30	Coronary artery disease	5500
Control	50	Male	40	Haemopericardium	9000
Control	43	Male	66	Aspiration pneumonia	13000
Control	46	Male	29	Acute myocardial infarction	17300
Control	24	Male	43	Undetermined (but consistent with idiopathic cardiac arrhythmia)	22000
Control	48	Male	24	Ischemic heart disease	59000
Control	44	Male	50	Ischemic heart disease	69000
Control	53	Male	16	Dilated cardiomyopathy	102000
Carbon monoxide intoxication/Alcohol					
Alcoholic	43.5	Male	43.5	Intoxication	506000
Alcoholic	44	Male	15	Ischemic heart disease	639000
Alcoholic	42	Male	41	Combined bromoxynil and alcohol toxicity	1052000
Alcoholic	49	Male	44	Ischemic heart disease	1181000
Alcoholic	45	Male	7.5	Drowning	1271000
Alcoholic	49	Male	16	Coronary artery thrombosis	1278000
Alcoholic	51	Male	27	Gastrointestinal hemorrhage	1863000
Alcoholic	50	Male	17	Ischaemic heart disease	1958000
Alcoholic	61	Male	23.5	Atherosclerotic cardiovascular disease	3158000
Alcoholic	61	Male	59	Myocarditis	5811000

Brains were collected by the New South Wales Tissue Resource Center brain donor program at the University of Sydney. Alcohol consumption rates varied with time, such that the reporting of alcohol consumption also varied. The alcohol consumption rate was recorded in grams of ethanol/day, and calculated from the reported number of standard drinks consumed per day. A range of maximum and minimum consumption rates was obtained from hospital records and questionnaires to family members. A mean of the maximum and minimum rates was used for classification (alcoholics consumed > 80 g/day, controls consumed little or no alcohol). The age of commencement of drinking was assumed to be 25 years of age in all cases, except where other ages were recorded. The 25 year age minimum allowed for increasing consumption at earlier ages until a regular drinking pattern was established. Any period(s) of known abstinence was subtracted from the potential drinking years. Only recorded periods of abstinence greater than six months were considered in our calculations. The duration of alcohol consumption was calculated for each case. Lifetime alcohol consumption (expressed in kilograms of 100% alcohol) was calculated by multiplying the duration and the mean consumption rate.

Table 2

Real-time PCR primers

	Primer pairs
Mouse TLR2	5'-TGC TTT CCT GCT GAA GAT TT-3' 5'-TGT ACC GCA ACA GCT TCA GG-3'
Mouse TLR3	5'-TTG TCT TCT GCA CGA ACC TG-3' 5'-GGC AAC GCA AGG ATT TTA TT-3'
Mouse TLR4	5'-ACC TGG CTG GTT TAC ACG TC-3' 5'-GTG CCA GAG ACA TTG CAG AA-3'
Mouse HMGB-1	5'-CCA TTG GTG ATG TTG CAA AG-3' 5'-CTT TTT CGC TGC ATC AGG TT-3'
Mouse β -actin	5'-GTA TGA CTC CAC TCA CGG CAA A-3' 5'-GGT CTC GCT CCT GGA AGA TG-3'
Rat HMGB1	5'-ATGGGCAAAGGAGATCCTA-3' 5'-ATTCTCATCATCTCTTCT-3'
Rat IL-1 β	5'-GAAACAGCAATGGTCGGGAC-3' 5'-AAGACACGGGTTCATGGTG-3'
Rat β -actin	5'-CTACAATGAGCTGCGTGTGGC-3' 5'-CAGGTCCAGACGCAGGATGGC-3'

Table 3

Summary of antibodies used in the present study

Antibody	Manufacturer	Host	Species detected	Dilution	Application
TLR2 (M-15)	Santa Cruz	Goat	Mouse	1:200	IHC
TLR2 (N17)	Santa Cruz	Goat	Human	1:200	IHC/WB
TLR3 (N-14)	Santa Cruz	Goat	Mouse, Human	1:200	IHC
TLR3	Abcam	Rabbit	Human	1:500	WB
TLR4	Abcam	Rabbit	Mouse, Human	1:500	IHC
TLR4 (C-18)	Santa Cruz	Goat	Human	1:200	WB
HMGB-1 (K-12)	Abcam	Rabbit	Rat, Human	1:400	IHC/DFL/WB
HMGB-1	Santa Cruz	Goat	Mouse, Human	1:200	IHC
HMGB-1	Abcam	Rabbit	Human	1:500	WB
β -actin	Cell Signaling Inc.	Rabbit	Human	1:500	WB
NeuN	Chemicon	Mouse	Rat, Human	1:100	DFL

IHC, immunohistochemistry; DFL, double fluorescent staining; WB, Western blot

Table 4

Correlations of lifetime alcohol consumption and danger signals HMGB1, and TLR2, TLR3, and TLR4 in post-mortem human OFC.

DANGER SIGNAL	Lifetime Alcohol Consumption (kg)	TLR2	TLR3	TLR4
TLR2	0.66 $p=0.002$	----	----	----
TLR3	0.83 $p<0.001$	0.89 $p<0.0001$	----	----
TLR4	0.62 $p<0.005$	0.90 $p<0.0001$	0.85 $p<0.0001$	----
HMGB1	0.83 $p<0.001$	0.83 $p<0.0001$	0.94 $p<0.0001$	0.80 $p<0.0001$

Lifetime alcohol consumption of moderate drinking controls and alcoholics is shown in Table 1. Correlations between lifetime alcohol consumption with HMGB1, TLR2, TLR3, and TLR4 +IR cells within the orbital frontal cortex. Drinking history from family interviews and a follow back technique was used to assess drinks per year and years drinking. Lifetime alcohol consumption (expressed in kilograms of 100% alcohol) was calculated by multiplying the duration and the mean consumption rate (See methods for more details). Correlations were determined using Pearson Correlation coefficients and 2 tailed significance. The correlation coefficient is above the p value in each applicable table cell.