

High Mobility Group Box 1/Toll-like Receptor 4 Signaling Increases *GABRB3* Expression in Alcohol Exposure

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Introduction: Prefrontal cortex (PFC) and striatal neurotransmitter homeostasis is affected by alcohol dependence. In this study, the microarray dataset from the Gene Expression Omnibus (GEO) database were downloaded. The prefrontal and striatum data were cross-analyzed to reveal the co-effects of alcohol dependence on the two brain regions of mice.

Methods: The GSE123114 microarray profile was downloaded from the GEO database, and differentially expressed genes (DEGs) between the two groups were acquired by GEO2R. KEGG analyses were performed to identify the pivotal pathways of these DEGs. Key differential gene expressions and their mechanism associated with alcohol exposure were investigated by an intraperitoneal alcohol model.

Results: A total of 13 overlapping DEGs from the PFC and striatal datasets of the GSE123114 microarray profile were identified, and they were significantly enriched in the morphine addiction pathway. The transcript levels and protein expression of *Gabrb3* were consistent with the microarray data both in the PFC and striatum. The transcript levels of *HMGB1*, *TLR4*, *TNFα* and *IL-1β* were upregulated in the PFC and striatum of mice in the alcohol group. The *HMGB1* inhibitor decreased *Gabrb3* transcript and protein levels as well as *TNFα* and *IL-1β* transcript levels both in the PFC and striatum in the intraperitoneal alcohol model mice.

Discussion: Through the reanalysis of GSE123114 microarray profile, we found that *Gabrb3* is a key gene associated with alcohol exposure. In further experiments, our findings suggest that alcohol exposure modulates *Gabrb3* expression through the *HMGB1/TLR4* pathway. Moreover, inflammation-associated factors, such as *IL-1β* and *TNFα*, may be related to the *HMGB1/TLR4*-mediated regulation of *GABRB3* expression in alcohol exposure.

Keywords: alcohol exposure, prefrontal cortex, striatum, *Gabrb3*, *HMGB1/TLR4* pathway

Introduction

Alcohol dependence is a chronic recurrent brain disease, with negative health, economic, and social consequences for millions of adults worldwide.¹⁻³ Emerging evidence suggests that alcohol dependence is related to dysregulation of limbic circuits, including pathways that connect with the prefrontal cortex (PFC) and striatum. It has been reported that repeated alcohol intake increases PFC dopamine release in mice, which plays a key role in facilitating learning associated with drug dependence.⁴ In particular, upregulation of *GLT-1* and *xCT* expression in the PFC is related to attenuation of chronic and relapse-like alcohol drinking.¹ In mammal, alcohol exposure is associated with reduced excitability in the prefrontal cortex.⁵

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Several studies suggest that dysregulation of the striatal neurotransmitter system is closely associated with alcohol dependence. For example, extracellular glutamate levels are increased⁶ and GLT-1 expression is decreased in the striatum after alcohol withdrawal.⁷ Ceftriaxone-induced upregulation of GLT-1 in the striatum also leads to reduced alcohol consumption in mice. Furthermore, upregulation of GLT-1 in the striatum induced by ceftriaxone also results in reduced alcohol drinking in mice. Together, these studies suggest that PFC and striatal neurotransmitter homeostasis are closely associated with alcohol dependence.

Although several researchers have investigated the impact of alcohol dependence on PFC or striatal gene expression individually, the co-effects of alcohol dependence on these two brain regions have not been reported. In this study, the GSE123114 microarray profile was downloaded from the GEO (Gene Expression Omnibus) database. The PFC and striatal data were cross-analyzed to identify the co-effects of alcohol dependence on the two brain regions of mice to provide insight into the molecular pathogenesis of the disorder.

Materials and Methods

Microarray Data

The GSE123114 microarray profile datasets were obtained from GEO database which is based on the GPL1261 (Mouse430_2) platform of the Affymetrix Mouse Genome 430 2.0 Array that includes 12 mouse samples, including three inflexible drinkers PFC, three light drinkers PFC, three inflexible drinkers striatum and three light drinkers striatum. In this part of the experiment, the three-bottle free-choice method (5 and 10% of ethanol (v/v) and water for 16 weeks) was carried out on all experimental male mice. On the last two weeks of the experiment, the bitter and aversive substance, quinine was adulterated in both ethanol solutions. According to their individual ethanol intake and preference mice were classified in three groups: “light drinkers” (preference for water at all experimental stages); “heavy drinkers” (ethanol preference decreased after adulteration of the ethanol solution with quinine), and “inflexible drinkers” (preference for ethanol at all experimental stages).^{8,9}

DEG Identification and Pathway Enrichment Analysis

Differentially expressed genes (DEGs) between the two groups were acquired by GEO2R. A *P*-value <0.05 and

FC=1.5 were set as the cutoff criteria. KEGG pathway enrichment analyses were performed to identify the potential pathways of the DEGs, with *P*<0.01 as the threshold.

Animals

All experiments were approved by the Animal Ethics Committee of Jiamusi University and Chongqing Medical University and were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised in 1996). Six to eight week old C57BL/6J mice (males) were obtained from the Department of Laboratory Animal Science of Chongqing Medical University. All mice, with free access to food and water, were maintained in a temperature (21–22°C) and humidity-controlled room (55 ±5%) under a 12/12 h light/dark cycle.

Drug Treatment

All mice were randomly divided into control, alcohol and glycyrrhizin groups. Glycyrrhizic is a triterpenoid saponin which is present in large quantities in licorice root (*Glycyrrhiza radix*), and has been suggested to act as a direct HMGB1 antagonist. We used 0.9% saline to dissolve all drugs. Mice were treated with saline (control group), alcohol (20% v/v, 2 g/kg) or alcohol+glycyrrhizin (50 mg/kg)^{10,11} by intraperitoneal (ip) injection once per day for seven days.^{12–14} Glycyrrhizin was administered on days six and seven after alcohol injection. Glycyrrhizin and alcohol were administered as separate injections. On day eight, all mice were sacrificed and PFC and striatum were quickly isolated from mouse brain on ice and stored at –80°C.

Quantitative Real-time PCR (qRT-PCR) and Western Blot Analysis

TRIzol was used to isolate RNA from the PFC and striatal samples (Thermo Fisher Scientific, Rockford, IL, USA) and the PrimeScript RT master mix kit was used to reverse-transcribe the total RNA from the PFC and striatal samples (Takara, Otsu, Japan). The qRT-PCR primer sequences are listed in [Supplementary materials Table S1](#). qRT-PCR which using SYBR Premix Ex Taq II (Takara) was performed on a LightCycler 96 System (Roche, Basel, Switzerland). Fold change was calculated using the $\Delta\Delta C_t$ method. Transcript levels were calculated relative to β -actin.

As previously described,¹⁵ the PFC and striatum stored at –80°C were homogenized in ice-cold RIPA lysis buffer with the presence of phosphatase and protease inhibitors, then

centrifuged at 4°C to extract total protein. Primary antibodies, *GABRB3* (gamma-aminobutyric acid type A receptor subunit beta3; 1:1000) and *GAPDH* (1:10,000), were used for Western blotting analysis (Abcam, Cambridge, MA, USA). *GAPDH* was used as a loading control and enhanced chemiluminescence was used to detect signals.

Statistical Analysis

For qRT-PCR and Western blot analysis, one-way ANOVA with the LSD post hoc test was used to assess the differences among more than two groups; Student's *t*-test was used to assess the differences between two groups. $P < 0.05$ was considered statistically significant.

Results

Alcohol Exposure Increases Expression of Morphine Addiction-related Genes in the PFC and Striatum

Using the cutoff criteria described above, we extracted 229 DEGs between inflexible drinker mice and light

drinker mice from the PFC datasets of the GSE123114 microarray profile (Figure 1C). The most enriched KEGG pathways related to these 229 DEGs were “morphine addiction” (mmu05032, $P = 0.0000$), “amphetamine addiction” (mmu05031, $P = 0.0000$) and “dopaminergic synapse” (mmu04728, $P = 0.0000$) (Figure 1A). Using the same cutoff criteria, we extracted 32 DEGs between inflexible drinkers and light drinkers from the striatum datasets of the GSE123114 microarray profile (Figure 1C). The most enriched KEGG pathways related to the 32 DEGs were “arrhythmogenic right ventricular cardiomyopathy (ARVC)” (mmu05412, $P = 0.0032$), “GABAergic synapse” (mmu04727, $P = 0.0043$) and “morphine addiction” (mmu05032, $P = 0.0045$) (Figure 1B). The 13 overlapping DEGs from the PFC and striatum were subjected to KEGG enrichment analysis (Figure 1C). Again, the most enriched KEGG pathway was “morphine addiction” (mmu05032, $P = 0.0006$) (Figure 1D). More data about KEGG analysis results were shown in [supplementary materials Table S2-S4](#).

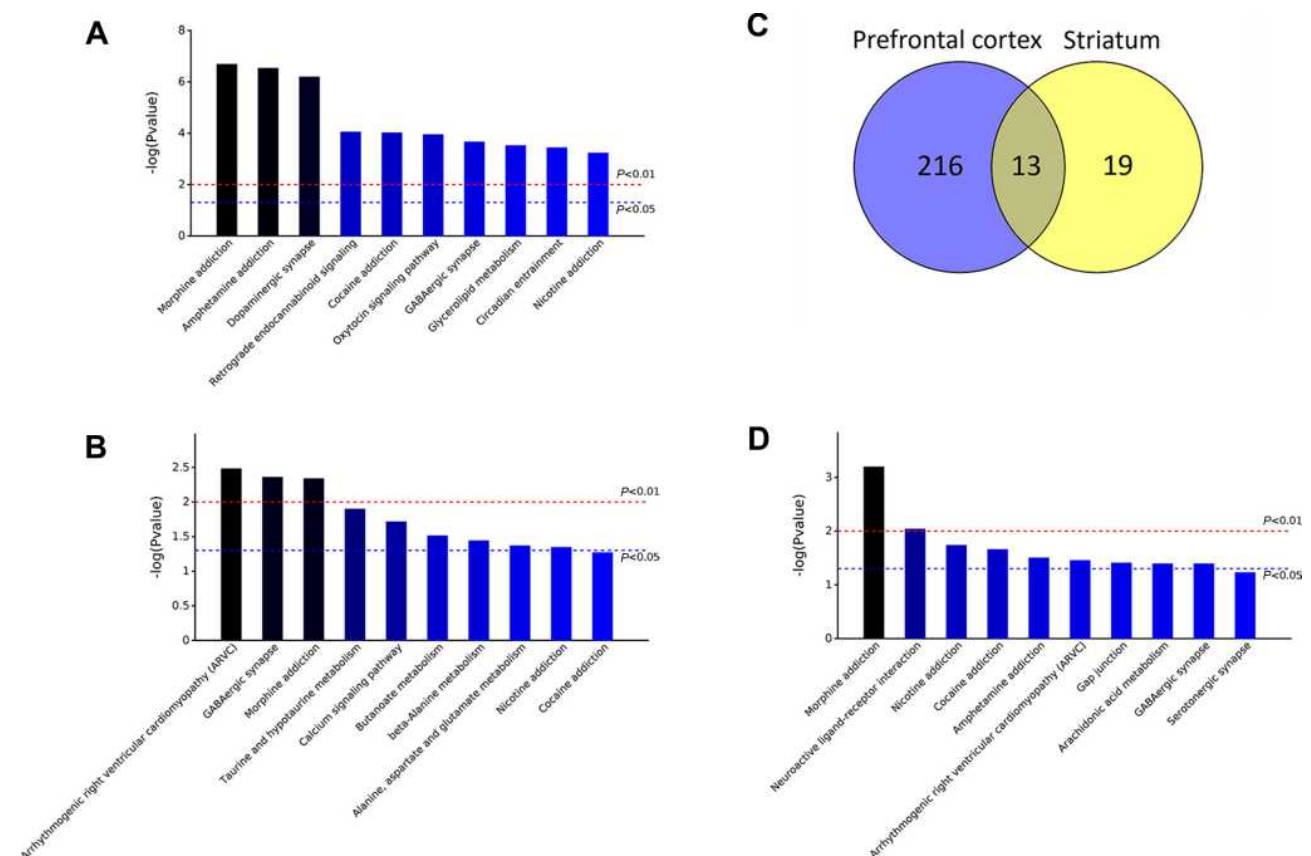


Figure 1 Alcohol exposure promote morphine addiction-related gene expressions in the PFC and striatum. **(A)** The most enriched KEGG pathways related to DEGs in PFC. **(B)** The most enriched KEGG pathways related to DEGs in striatum; **(C)** Venn diagram of 13 overlapping DEGs from PFC and striatum; **(D)** The most enriched KEGG pathways related to the overlapping differentially genes from PFC and striatum.

Alcohol Exposure Increases Gabrb3 Expression in the PFC and Striatum

According to KEGG enrichment analysis (Figure 1D), two genes, *Gabrb3* and *Drd1*, among the 13 overlapping DEGs were enriched in the morphine addiction pathway. The effect of alcohol exposure on the expression of *Gabrb3* and *Drd1* was investigated by intraperitoneal alcohol model. The mRNA expression of *Gabrb3* was consistent with the microarray data, both in the PFC and striatum (*Gabrb3*: F1, 10=0.011, $P=0.024$; Figure 2C) (*Gabrb3*: F1, 10=1.429, $P=0.015$; Figure 2D). The transcript levels of *Drd1* in the striatum were increased in the alcohol group mice, but no difference was detected in the PFC (*Drd1*: F1, 10=0.230, $P=0.013$; Figure 2D) (*Drd1*: F1, 10=0.168, $P=0.745$; Figure 2C). The protein expression of *Gabrb3* were both increased in the PFC and striatum of the Alcohol group mice (F1, 4=1.198, $P=0.015$, Figure 2A) (F1, 4=3.467, $P=0.037$,

Figure 2B). These results indicate that alcohol exposure regulates *Gabrb3* signaling in the PFC and striatum.

Alcohol Exposure Upregulates HMGB1 and TLR4 in the PFC and Striatum

It has been reported that the HMGB1/TLR4 pathway is related to the alcohol-mediated regulation of *GABRB3* expression in the mouse brain,¹⁶ and HMGB1 induces TNF α and IL-1 β expression through TLR4 activation in alcohol dependence.¹⁷ To analyze the mechanisms by which alcohol exposure regulates *Gabrb3* expression in the PFC and striatum, we examined HMGB1/TLR4 signaling, which is closely associated with alcohol exposure. The transcript levels of HMGB1 and TLR4 were both upregulated in the PFC and striatum of the alcohol group mice (HMGB1: F1, 10=11.673, $P=0.017$; TLR4: F1, 10=0.779, $P=0.030$; Figure 3A) (HMGB1: F1, 10=6.003, $P=0.009$; TLR4: F1,

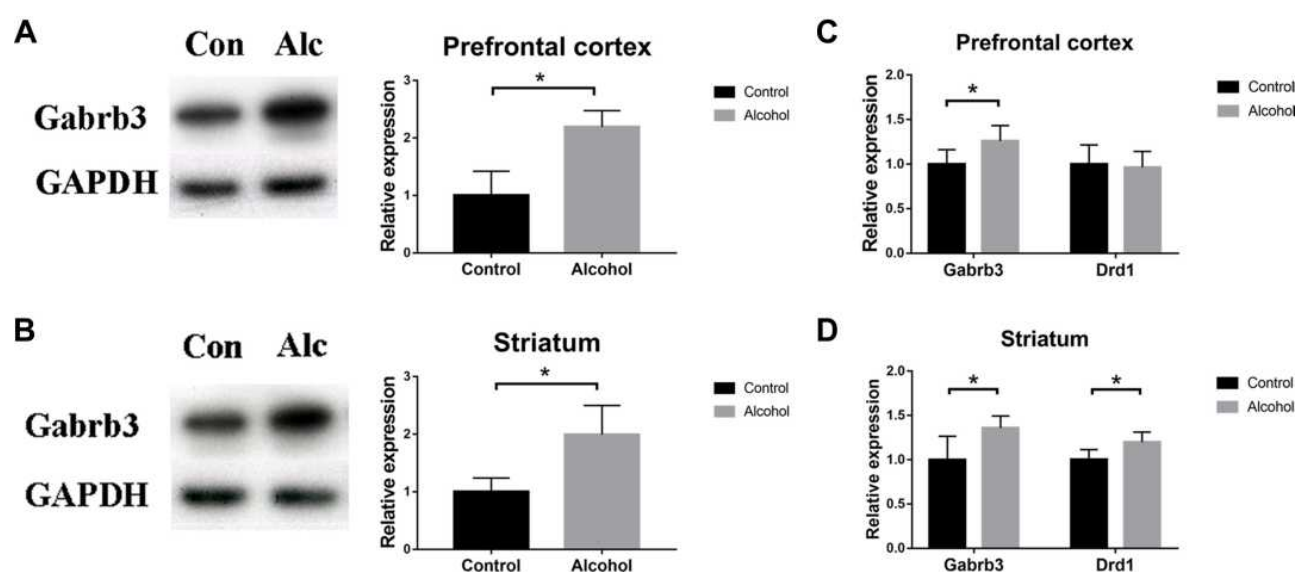


Figure 2 Alcohol exposure promoted *Gabrb3* expression in prefrontal cortex and striatum. (A) Effect of alcohol exposure on *Gabrb3* protein levels in prefrontal cortex (n=3); (B) Effect of alcohol exposure on *Gabrb3* protein expression in striatum (n=3); (C) *Gabrb3* and *Drd1* transcript levels in prefrontal cortex (n=6). (D) *Gabrb3* and *Drd1* transcript levels in striatum (n=6). * $P<0.05$.

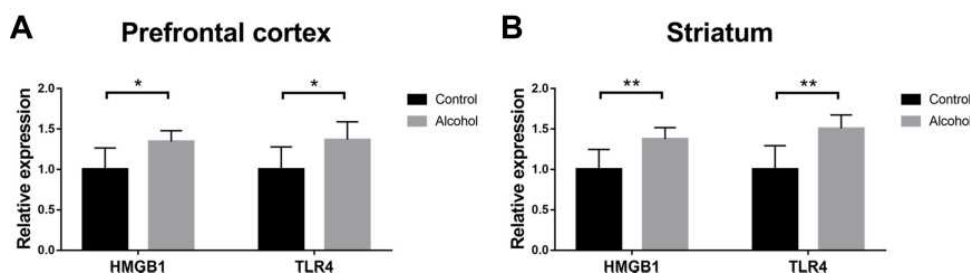


Figure 3 Alcohol exposure promoted HMGB1 and TLR4 expression in prefrontal cortex and striatum. (A) Effect of alcohol exposure on HMGB1 and TLR4 transcript levels in prefrontal cortex (n=6); (B) Effect of alcohol exposure on HMGB1 and TLR4 transcript levels in striatum (n=6). * $P<0.05$; ** $P<0.01$.

10=1.857, $P=0.004$; Figure 3B). Also, the expression levels of inflammatory response-related factors, IL-1 β and TNF α , were significantly increased in the PFC and striatum of mice in the alcohol group [TNF α : (F2, 15=7.465, $P=0.006$); control vs alcohol, $P=0.005$; IL-1 β : (F2, 15=7.399, $P=0.006$); control vs alcohol, $P=0.011$; Figure 4C] [TNF α : (F2, 15=7.519, $P=0.005$); control vs alcohol, $P=0.005$; IL-1 β : (F2, 15=4.492, $P=0.030$); control vs alcohol, $P=0.017$; Figure 4D]. These results indicate that alcohol exposure regulates HMGB1/TLR4 signaling in the PFC and striatum.

HMGB1 Inhibitor Decreases the Inflammatory Response and Gabrb3 Expression in the PFC and Striatum of Alcohol Exposure Mice

In order to further analyze the regulation mechanism of *GABRB3* expression mediated by alcohol exposure, the effect of HMGB1 inhibitor on *GABRB3* expression was detected by qRT-PCR and Western blotting. The HMGB1 inhibitor decreased Gabrb3 protein expression and transcript levels both in the PFC and striatum of alcohol exposure mice [Gabrb3: (F2, 6=7.747, $P=0.022$); alcohol vs glycyrrhizin, $P=0.011$; Figure 4A] [Gabrb3: (F2, 15=5.982, $P=0.012$); alcohol vs glycyrrhizin, $P=0.005$; Figure 4C] [Gabrb3: (F2, 6=5.226, $P=0.048$); alcohol vs glycyrrhizin, $P=0.026$; Figure 4B] [Gabrb3: (F2, 15=3.731, $P=0.048$); alcohol vs glycyrrhizin, $P=0.045$; Figure 4D]. Also, the expression of inflammatory response-related factors, IL-1 β and TNF α , was

significantly inhibited by HMGB1 inhibitor in the PFC and striatum of the alcohol group mice [TNF α : (F2, 15=7.465, $P=0.006$); alcohol vs glycyrrhizin, $P=0.004$; IL-1 β : (F2, 15=7.399, $P=0.006$); alcohol vs glycyrrhizin, $P=0.002$; Figure 4C] [TNF α : (F2, 15=7.518, $P=0.005$); alcohol vs glycyrrhizin, $P=0.004$; IL-1 β : (F2, 15=7.337, $P=0.006$); alcohol vs glycyrrhizin $P=0.024$; Figure 4D]. These results indicate that HMGB1/TLR4 signaling may be related to the regulation of Gabrb3 expression by alcohol exposure in the PFC and striatum.

Discussion

By analyzing GSE123114 microarray profile, we found that the morphine addiction pathway was significantly different in both the PFC and striatum. The chronic use of morphine is characterized by adaptive changes in neurons and neuronal communication. Morphine can cause indirect excitation of dopamine neurons by reducing inhibitory synaptic transmission mediated by GABAergic neurons. Differences in the morphine addiction pathway suggested that there are similar pathological processes and corresponding gene expression changes in alcohol-dependent mice. Furthermore, we identified Gabrb3 of morphine addiction pathway as a key gene. *GABRB3* is a gamma-aminobutyric acid receptor, a major inhibitory neurotransmitter in human central nervous system.¹⁸ As a GABA-gated chloride ion channel,¹⁹ it plays an important role in the formation of functional inhibitory GABAergic synapses and participates in synaptic

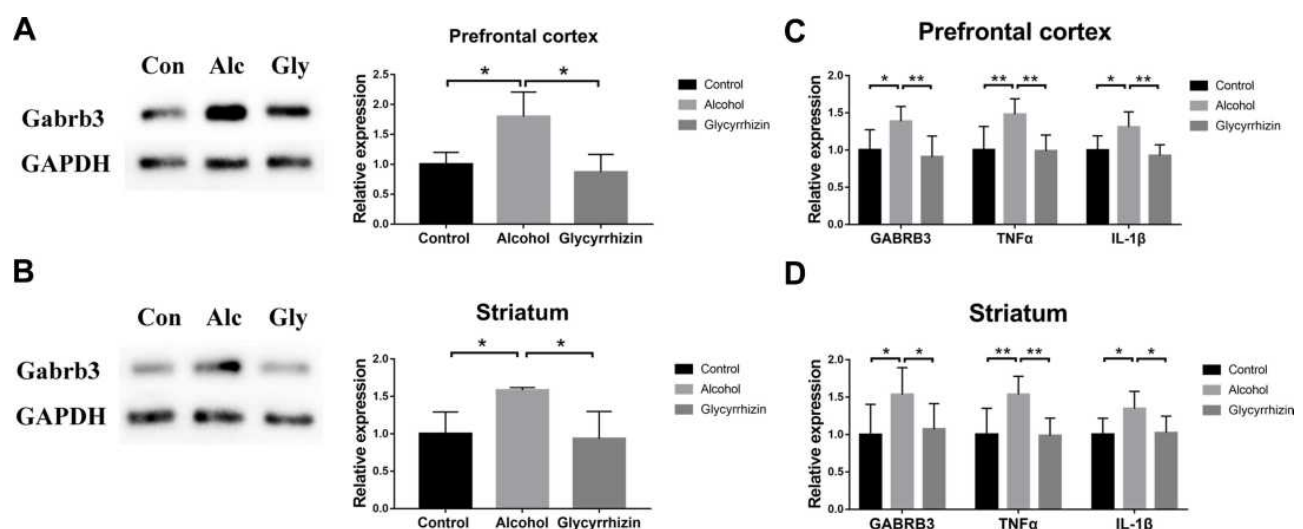


Figure 4 HMGB1 inhibitor decreased inflammatory response and Gabrb3 expression in PFC and striatum of alcohol exposure mice. (A) Effect of HMGB1 inhibitor on Gabrb3 protein levels in PFC (n=3); (B) Effect of HMGB1 inhibitor on Gabrb3 protein expression in striatum (n=3); (C) Effect of HMGB1 inhibitor on Gabrb3, TNF α and IL-1 β transcript levels in PFC (n=6). (D) Effect of HMGB1 inhibitor on Gabrb3, TNF α and IL-1 β transcript levels in striatum (n=6). * $P<0.05$; ** $P<0.01$.

inhibition.¹⁹ Also, it participates in mediating the cellular response to histamine,²⁰ somatosensation, and the production of antinociception.²¹

Alleles of *GABRB3* in Caucasian (non-Hispanic) subjects have been associated with alcohol dependence.²² Alcohol dependence patients with the *GABRB3* G1+ allele reported significantly lower drinking refusal self-efficacy in situations of stronger mood-related alcohol expectancy (AE) and social pressure relating to negative affective change.²³ In Korea, the *GABRB3* G1- alleles are more commonly in children of alcoholics (COAs) than in non-COAs.²⁴ In African Americans, CpGs in *GABRB3* are hypermethylated in alcohol dependence patients compared with controls.²⁵ Together, these studies indicate that *GABRB3* is closely related to alcohol dependence. In in vitro experiments, alcohol increases the expression of the *GABRB3* gene in human embryonic stem cells.²⁶ In in vivo experiments, adult male offspring born to Sprague Dawley dams that received ethanol had elevated expression of the *Gabrb3* gene in the hippocampus.²⁷ These studies provide further evidence that *GABRB3* is related to the regulation of brain function by alcohol. In this research, functional analysis of DEGs from GSE123114 microarray profile revealed that *Gabrb3* is the key gene of alcohol dependence in both the PFC and striatum. In the PFC and striatum of alcohol exposure mice, *GABRB3* expression was significantly increased at both the transcript and protein levels, consistent with the microarray profile. Together, all these findings suggest that *Gabrb3* is a critical novel gene in alcohol exposure.

It has been reported that the expression of *Gabrb3* is elevated in the male offspring frontal cortex of ethanol-fed wild-type dams, but is unaffected in the offspring of ethanol-fed TLR4(-/-) dams.¹⁶ These results indicate that the TLR4 pathway is related to the alcohol-mediated regulation of *GABRB3* expression in the mouse brain.

Toll-like receptors are the most well characterized pattern recognition receptors that recognize PAMPs and DAMPs, including HMGB1 and heat shock proteins.²⁸ TLR4 has been identified as a critical receptor related to ethanol-induced neuropathology.^{28,29} HMGB1, a nuclear chromatin binding protein, is a endogenous TLR4 agonist which has been identified as a key immune mediator in alcohol dependence.²⁸ Extracellular HMGB1 released as an innate immune mediator activates innate immune responses through direct binding to TLR4 or RAGE.³⁰ It has been reported that the expression of TLR4 and HMGB1 is increased both in chronic ethanol-administered mice and the human postmortem alcoholic brain.³¹ In this experiment, the expression

levels of HMGB1 and TLR4 were significantly increased in the PFC and striatum of mice in the alcohol group. These results are consistent with previous research indicating increased HMGB1/TLR4 signaling in the alcoholic brain. To examine whether changes in HMGB1/TLR4 signaling are associated with increased *Gabrb3* gene expression in alcoholic brain, we assessed the effects of an HMGB1 inhibitor on *Gabrb3* expression in alcohol exposure mice. The HMGB1 inhibitor decreased *Gabrb3* expression in both the PFC and striatum of these mice. Together, these results reveal that alcohol exposure modulates *Gabrb3* expression through the HMGB1/TLR4 pathway.

Previous studies have reported that HMGB1 induces TNF α and IL-1 β expression through TLR4 activation in alcohol dependence,¹⁷ and that inflammation inhibits GABA transmission in CNS diseases.³² In this study, the HMGB1 inhibitor decreased TNF α and IL-1 β expression both in the PFC and striatum of alcohol exposure mice, consistent with previous studies. These results indicate that HMGB1 may regulate *Gabrb3* expression through the inflammatory response pathway. It has been reported that activation of Akt is involved in GABA(A) receptor anchoring in neurons,³³ and that Akt1 deficiency modulates GABAergic functions and reduces GABAAR subunit expression in the hippocampus.³⁴ In glioma cells, TNF α -induced oxidative stress-dependent Akt signaling affects actin cytoskeletal organization,³⁵ and in the CNS, IL-1 β enhances the expression of GABAA receptor in cell surface by the Akt pathway.³⁶ Collectively, these findings suggest that inflammation-related factors in the PFC and striatum of alcohol-dependent mice, such as TNF α and IL-1 β , may regulate *Gabrb3* expression through the Akt pathway. However, further study is needed to test this hypothesis.

Conclusion

Our findings suggest that *Gabrb3* is the key gene associated with alcohol exposure in mice, and reveal that alcohol exposure modulates *Gabrb3* expression through the HMGB1/TLR4 pathway. Moreover, inflammation-related factors, such as IL-1 β and TNF α , may be related to the regulation of *GABRB3* expression by the HMGB1/TLR4 pathway in alcohol exposure.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current

journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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