Role of TLR4 in the Modulation of Central Amygdala GABA Transmission by CRF Following Restraint Stress

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Abstract

Aims: Stress induces neuroimmune responses via Toll-like receptor 4 (TLR4) activation. Here, we investigated the role of TLR4 in the effects of the stress peptide corticotropin-releasing factor (CRF) on GABAergic transmission in the central nucleus of the amygdala (CeA) following restraint stress.

Methods: Tlr4 knock out (KO) and wild-type rats were exposed to no stress (naïve), a single restraint stress (1 h) or repeated restraint stress (1 h per day for 3 consecutive days). After 1 h recovery from the final stress session, whole-cell patch-clamp electrophysiology was used to investigate the effects of CRF (200 nM) on CeA GABA_A-mediated spontaneous inhibitory postsynaptic currents (sIPSCs).

Results: TLR4 does not regulate baseline GABAergic transmission in the CeA of naive and stress-treated animals. However, CRF significantly increased the mean sIPSC frequencies (indicating enhanced GABA release) across all genotypes and stress treatments, except for the Tlr4 KO rats that experienced repeated restraint stress.

Conclusions: Overall, our results suggest a limited role for TLR4 in CRF’s modulation of CeA GABAergic synapses in naïve and single stress rats, though TLR4-deficient rats that experienced repeated psychological stress exhibit a blunted CRF cellular response.

Short Summary: TLR4 has a limited role in CRF’s activation of the CeA under basal conditions, but interacts with the CRF system to regulate GABAergic synapse function in animals that experience repeated psychological stress.

INTRODUCTION

The neuroimmune system plays an important role in the homeostatic regulation of brain physiology (Pribiag and Stellwagen, 2014; Becher et al., 2017), and its inflammatory responses have been implicated in numerous psychiatric disorders (major depressive disorder, post-traumatic stress disorder (PTSD), schizophrenia, substance use disorders) (Liu et al., 2014). Many of these disorders involve stress-related pathologies (Zorn et al., 2017), and a growing body of evidence indicates that acute stressors can induce a submaximal activation of the neuroimmune system (Calcia et al., 2016; Wohleb and Delpech, 2017), which can lead to long-term sensitization of the brain’s stress response to subsequent challenges (Frank et al., 2016). Stress also plays an important role in alcohol use disorders, particularly in alcohol drinking. Alcohol has anxiolytic
properties and is often used as ‘self-medication’ to cope with stress in non-dependent individuals (Leees et al., 2010), while stress is a primary trigger for alcohol craving and relapse in alcoholic patients (Blaine and Sinha, 2017).

There are several common mechanisms mediating the cellular and behavioral effects of alcohol and stress in the central nervous system (CNS), including neuropeptides (e.g. corticotropin-releasing factor (CRF), neuropeptide Y), neurotransmitters (e.g. γ-aminobutyric acid (GABA), norepinephrine) and the neuroimmune system (e.g. toll-like receptor 4 (TLR4), Toll-like receptor 4 (TLR4)) (Akira and Takeda, 2004; Palsson-Mcdermott and O’neill, 2004). Moreover, several studies have shown that pharmacological interventions or transgenic manipulations that target the immune system can ameliorate stress-induced (Breeze et al., 2008; Caso et al., 2008; Garate et al., 2013) and alcohol-related (Alfonso-loeches et al., 2010; Wu et al., 2011, 2012; Bajo et al., 2015; Blednov et al., 2015, 2017a, 2017b; Marshall et al., 2016) changes at the molecular, cellular and behavioral levels.

Importantly, the stress-induced immune response is initiated by TLR4 activation (Liu et al., 2014). Specifically, TLR4 activates the innate immune system, both peripherally and within the CNS, in response to endogenous danger-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1), S100, heat shock proteins (HSP) and exogenous microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS) (Akira and Takeda, 2004). TLR4 activation also triggers the production of several neuroimmune mediators, including type 1 interferons and cytokines (e.g. tumor necrosis factor α (TNFα), IL-1β, IL-6) (Akira and Takeda, 2004), and as such, plays a critical role in the regulation of the brain’s responses to both stress and inflammatory stimuli (Caso et al., 2008). Moreover, stress increases brain expression of several TLRs, including TLR4 (Garate et al., 2013; Tang et al., 2017), and both TLR4 and TLR2 mediate stress-induced priming of the neuroimmune system to subsequent challenge (Caso et al., 2008; Weber et al., 2013).

TLR4 activation has been hypothesized to promote excessive alcohol drinking, as TLR4 levels are increased in the brains of both human alcoholics and ethanol-dependent rats (Crews et al., 2013). In humans, TLR4 brain expression is also correlated with the age of drinking onset and lifetime alcohol consumption (Crews and Vetreno, 2015), while LPS and cytokine serum concentrations are correlated with the cravings of alcohol-dependent patients (Leclercq et al., 2012, 2014). In rodents, TLR4 activation (via LPS injection) increased the ethanol intake of mice (Blednov et al., 2011). However, recent findings do not fully support a critical role for TLR4 in ethanol drinking (Alfonso-loeches et al., 2010; Harris et al., 2017; Blednov et al., 2017b). Mice lacking functional TLR4 or systemically administered (+)-naloxone, a TLR4 inhibitor, did not alter their ethanol intake (Alfonso-loeches et al., 2010; Harris et al., 2017; Blednov et al., 2017b). In addition, although systemic administration of another TLR4 antagonist, T5342126, decreased the ethanol consumption and preference for ethanol in mice, these changes were likely due to nonspecific effects, as evidenced by T5342126-related reductions in motor activity, saccharin intake (a more general reward-related behavior), and body core temperature (Bajo et al., 2016). Nonetheless, brain region-selective knockdown of TLR4 in the central nucleus of the amygdala (CeA) and paraventricular nucleus (PVN), but not the ventral tegmental area (VTA), of alcohol-prefering (P) rats decreased their binge drinking via a GABAγ receptor mechanism (Liu et al., 2013; June et al., 2015). Notably, alcohol consumption in these same P rats increased CeA expression of the stress peptide CRF, which in turn increased local TLR4 expression (June et al., 2015).

Given these recent findings and the prominence of the CeA CRF system in stress and anxiety (Glipin et al., 2015), we hypothesized that TLR4 signaling plays a role in CRF’s modulation of GABA transmission in the CeA after psychological stress. The CeA is primarily GABAergic (>95% of cell bodies) (Alheid, 2003), and we have previously reported that CRF enhances GABA release to a similar extent in the CeA of naive rats and rats that have undergone restraint stress, although restraint stress also reduces the baseline expression of type 1 CRF receptors (CRF1) (Ciccocioppo et al., 2014). In addition, we have shown that TLR4 activation enhances CeA GABA transmission (Bajo et al., 2014), while TLR4 deletion (i.e. TLR4-deficient rats) or antagonism slightly reduces it or has no effect, respectively (Harris et al., 2017). In the present study, TLR4-deficient rats were exposed to single or repeated restraint stress sessions, and CRF-induced CeA cellular responses were assessed. Our results suggest a limited role for TLR4 in CRF’s modulation of CeA GABAergic transmission in naive rats and rats exposed to a single stress session; however, TLR4-deficient rats that experienced repeated stress sessions exhibited a blunted CRF cellular response.

**MATERIALS AND METHODS**

**Animals**

We used 3–7 months old adult male TLR4-deficient rats (Tlr4 KO) and their littermate wild-type (WT) rats (weight: Tlr4 KO: 522.3 ± 20.6 g; WT: 543.2 ± 26.6 g). The Tlr4 KO rat line is on a C57Bl/6 background harboring a nonfunctional Tlr4 gene, as described previously (Ferguson et al., 2013; Harris et al., 2017). Heterozygous (Het) breeding pairs were produced at the University of Pittsburgh and shipped to The Scripps Research Institute for breeding. In this study, we used WT control (n = 12), and homozygous KO (n = 13) littermates used from HET pairs. Offspring were weaned at 21–28 days of age and genotyped by the Genotyping Center of America (Ellsworth, ME). The rats were housed in a temperature- and humidity-controlled room (6 am–6 pm lights on) with food and water available ad libitum. All care procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Institutional Animal Care and Use Committee policies of The Scripps Research Institute.

**Restraint stress**

We exposed the Tlr4 KO and WT rats to restraint stress for a single session (1 h) or for three repeated sessions (1 h per day for 3 consecutive days). For each restraint session, the rats were placed in a vented Plexiglas tube fitted with a tail slot to prevent unnatural body posture. The rats were transferred back to their home cages after each stress session for recovery. After the final stress session, the rats recovered for 1 h in their home cage and were then sacrificed for the electrophysiological studies.

**Whole-cell recordings**

We anesthetized the rats with 3–5% isoflurane and placed the isolated brains quickly into ice-cold oxygenated high-sucrose cutting solution (composition in mM: sucrose, 206; KCl, 2.5; CaCl2, 0.5; MgCl2, 7; NaH2PO4, 1.2; NaHCO3, 26; glucose, 5; HEPEs, 5) gassed with 95% O2 and 5% CO2. We cut coronal slices (300μm) containing the CeA using a Leica 1200S vibratome cutter (Leica...
Microsystems, Buffalo Grove, IL) and incubated them in artificial cerebrospinal fluid (ACSF; composition in mM: NaCl, 130; KCl, 3.5; NaH2PO4, 1.25; MgSO4·7H2O, 1.5; CaCl2, 2.0; NaHCO3, 24; glucose, 10) at 37°C for 30 min. The slices were then incubated at room temperature for a minimum of 30 min prior to their use.

We recorded spontaneous inhibitory postsynaptic currents (sIPSCs) mediated by GABA A receptors in the medial subdivision of the CeA using whole-cell voltage-clamp electrophysiology, as described previously (Ciccocioppo et al., 2014). Briefly, we visualized CeA neurons using infrared/DIC optics followed by digitization and image enhancement via an upright, fixed-stage Olympus microscope (Olympus Scientific Solutions Americas Corp, Waltham, MA) and a CCD camera (Exi Aqua, QImaging, Surrey, BC, Canada).

For the recordings, we used borosilicate glass micropipettes (Warner Instruments, Hamden, CT and King Precision, Claremont, CA) filled with an internal solution containing (in mM): 145 KCl, 10 HEPES, 2 MgCl2, 0.5 EGTA, 2 ATP and 0.2 GTP (the latter two added fresh on the day of recording), pH 7.2–7.4, osmolality 290–305 mOsm and with input resistances of 2.5–5 MΩ (access resistance <20 MΩ, compensated 60–80%). GABA A-sIPSCs were pharmacologically isolated by adding glutamatergic (20 µM DNBQX, 30 µM DL-AP5) and GABA B1 (1 µM CGP 55845A) receptor blockers to the bath. We applied a maximal effective concentration of CRF (200 nM; Tocris, Ellisville, MO) (Roberto et al., 2010; Ciccocioppo et al., 2014) by adding a known concentration of a stock solution directly to the bath and we took all the measures before (baseline) and during 15 min of CRF superfusion. For data acquisition, we used the Multiclamp 700B and pClamp 10.2 software (Molecular Devices, Sunnyvale, CA).

Data analysis and statistics
To analyze the data we used MiniAnalysis 5.1 software (Synaptosoft, Leonia, NJ). For statistical analyses, we used GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and applied the one-sample t-test, two-tailed unpaired t-test, or two-way ANOVA, with statistical significance accepted at \( P < 0.05 \).

RESULTS
We recorded GABA A-sIPSCs in the medial CeA of naive WT and Tlr4 KO rats. There were no significant differences in baseline sIPSC frequencies, amplitudes, rise times and decay times of CeA neurons from naive WT and KO rats (unpaired t-test; Fig. 1A and Table 1), indicating that TLR4 does not regulate basal GABAergic transmission in this region.

To investigate the potential role of the TLR4 system in CRF signaling in the CeA, we exogenously applied a maximally effective concentration (200 nM) of CRF (Roberto et al., 2010; Ciccocioppo et al., 2014) onto the recording cells for 15 min. CRF significantly increased the sIPSC frequency in naïve WT rats (to 146.1 ± 16.1% of baseline; one-sample t-test, \( t_{11} = 2.86, P < 0.05 \)) and KO rats (to 151.9 ± 10.7% of baseline; one-sample t-test, \( t_{13} = 4.84, P < 0.001 \)) to the same extent in both groups (unpaired t-test, \( t_{24} = 0.31, P = 0.76 \); Fig. 1A and F). The mean sIPSC amplitude was significantly increased by CRF in Tlr4 KO rats compared to the pre-drug baseline (to 116.5 ± 5.5% of baseline; one-sample t-test, \( t_{13} = 3.01, P < 0.05 \)), but not in WT rats (113.8 ± 9.1%, \( P = 0.16 \)); however, CRF’s effects were not significantly different between the two genotypes (unpaired t-test, \( t_{24} = 0.27, P = 0.79 \); Fig. 1G). Finally, CRF had no effect on sIPSC kinetics (Fig. 1H–I). For these experiments, increases in sIPSC frequencies reflect increases in GABA release probabilities, while altered amplitudes and kinetics denote changes in GABA A receptor function (Oits et al., 1994). Therefore, TLR4 does not mediate CRF’s facilitation of basal GABA release at CeA synapses.

As TLR4 regulates stress-induced immune responses (Liu et al., 2014; Cheng et al., 2016) and can interact with CRF signaling in the CeA after alcohol exposure (June et al., 2015), we examined the effects of CRF on CeA activity in Tlr4 KO and WT rats exposed to either single (1 h) or repeated (1 h per day for 3 consecutive days) restraint stress and a final 1 h recovery period (Fig. 2A). Restraint stress did not alter the baseline sIPSC properties of WT and KO rats compared to their naive counterparts, as measured by two-way ANOVA (Fig. 2B–E and Table 1), indicating that these paradigms do not impact baseline CeA GABAergic transmission.

Bath application of CRF significantly increased the mean sIPSC frequencies compared to baseline across all genotype and treatment groups by one-sample t-test (WT/single restraint: \( t_{11} = 4.40, P < 0.01 \); KO/single restraint: \( t_{10} = 3.09, P < 0.05 \); WT/repeated restraint: \( t_{14} = 3.70, P < 0.01 \), except for the Tlr4 KO rats that experienced repeated restraint stress (\( t_{11} = 1.27, P = 0.23 \); Fig. 3A and B). Despite this within-group difference, a two-way ANOVA revealed no significant main effects of genotype or restraint stress on CRF’s facilitation of the mean sIPSC frequency, and no significant interaction. CRF also increased the sIPSC amplitude compared to baseline only in WT rats that experienced a single restraint session (one-sample t-test, \( t_{11} = 2.49, P < 0.05 \)), but two-way ANOVA revealed no significant effects of CRF on sIPSC amplitudes across all animal and treatment groups (Fig. 3C). Similarly, CRF increased the sIPSC rise and decay time in the CeA of single stress Tlr4 KO rats (one-sample t-test, \( t_{10} = 2.38, P < 0.05 \); Fig. 3D) and single stress WT rats (one-sample t-test, \( t_{11} = 2.25, P < 0.05 \); Fig. 3E), respectively. Comparison by two-way ANOVA revealed a significant main effect of genotype on CRF-induced sIPSC rise times (\( F_{1,70} = 4.59, P < 0.05 \)), but no other significant main effects or interactions with regard to CRF’s effects on sIPSC kinetics. Overall these data suggest a limited role for TLR4 in CRF’s modulation of CeA GABA synapses, mainly on the postsynaptic side of transmission, in naive rats and rats exposed to a single stress session; however, the TLR4-deficient rats that experienced repeated stress sessions exhibited a blunted CRF facilitation of GABA release.

DISCUSSION
A growing body of evidence indicates that submaximal activation of the neurotransmitter system, such as that triggered by psychological stress, can sensitize the brain’s response to subsequent challenges (Weber et al., 2013; Frank et al., 2016). Therefore, here we investigated the role of TLR4, a potent regulator of the innate immune system, on CRF-induced changes in GABAergic signaling in the CeA, with a particular focus on whether previous stress exposure primes cellular responses. We found that CRF facilitates CeA GABA release similarly in naive and stress-treated WT and Tlr4 KO rats, with the exception of the repeated stress-treated Tlr4 KO rats where CRF’s effects were blunted. Thus, TLR4 signaling has a limited postsynaptic role in CRF’s facilitation of the CeA under basal conditions, but interacts with the CRF system to regulate GABAergic synapse function in animals that experience repeated psychological stress.

There are several lines of evidence implicating TLR4 in stress-induced CRF signaling in the brain. Most notably, the hypothalamic–pituitary–adrenal (HPA) axis comprises the body’s neuroendocrine system, connecting the pituitary gland to the adrenal gland and the brain, and is involved in the regulation of stress responses. In addition, TLR4 is expressed in various brain regions, including the CeA, where it may play a role in stress-induced immune responses. Studies have shown that TLR4 activation can modulate immune cell function, which in turn affects stress responses. Therefore, TLR4 may serve as a potential target for the development of new therapeutic strategies for stress-related disorders.
stress response (Stephens and Wand, 2012), and its stimulation by TLR4 increased adrenal glucocorticoid secretion (Vakharia and Hinson, 2005; Kanczkowski et al., 2013), hypothalamic CRF gene expression (Singh and Jiang, 2004; Loum-Ribot et al., 2006) and serum CRF levels (Goebel et al., 2011). Moreover, early life stress (Tang et al., 2017) or LPS exposure (Mouihate et al., 2010) increased hypothalamic CRF expression in adult rodents and sensitized their pain and stress responses, respectively, indicating that TLR4 has long-term influence over the brain’s CRF signaling. Acute and chronic stressors also upregulate extra-hypothalamic CRF expression in several brain regions, including the CeA (Sterrenburg et al., 2011), where alcohol-induced activation of the CRF system increased local TLR4 expression (June et al., 2015). Surprisingly, here we found that repeated restraint stress did not alter CRF’s facilitation of GABA release in the

![Image](https://academic.oup.com/alcalc/article-abstract/53/6/642/4788587)

**Table 1.** Basal GABA<sub>A</sub>-mediated sIPSC parameters in the CeA of WT and Tlr4 KO rats

<table>
<thead>
<tr>
<th>Genotype (# cells)</th>
<th>Frequency (Hz)</th>
<th>Amplitude (pA)</th>
<th>Rise time (ms)</th>
<th>Decay time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive WT (n = 14)</td>
<td>1.15 ± 0.26</td>
<td>65.84 ± 4.44</td>
<td>2.12 ± 0.09</td>
<td>8.53 ± 0.62</td>
</tr>
<tr>
<td>Tlr4 KO (n = 16)</td>
<td>1.50 ± 0.45</td>
<td>66.18 ± 7.50</td>
<td>2.07 ± 0.09</td>
<td>7.83 ± 0.65</td>
</tr>
<tr>
<td>Single stress WT (n = 12)</td>
<td>1.37 ± 0.33</td>
<td>60.11 ± 4.74</td>
<td>2.33 ± 0.12</td>
<td>6.37 ± 0.61</td>
</tr>
<tr>
<td>Tlr4 KO (n = 13)</td>
<td>1.15 ± 0.20</td>
<td>78.03 ± 10.41</td>
<td>2.30 ± 0.13</td>
<td>7.70 ± 0.80</td>
</tr>
<tr>
<td>Repeated stress WT (n = 15)</td>
<td>1.14 ± 0.18</td>
<td>74.72 ± 12.98</td>
<td>2.35 ± 0.11</td>
<td>7.81 ± 0.73</td>
</tr>
<tr>
<td>Tlr4 KO (n = 12)</td>
<td>0.98 ± 0.10</td>
<td>72.63 ± 6.60</td>
<td>2.08 ± 0.12</td>
<td>9.36 ± 1.02</td>
</tr>
</tbody>
</table>

There were no significant differences in baseline sIPSC frequencies, amplitudes, rise times and decay times of CeA neurons from naive and restraint stress WT and KO rats.
CeA of WT rats (compared to their naïve counterparts), but blunted it in TLR4-deficient rats (compared to the naïve KO rats and the repeated stress WT rats). While these results in the WT rats (after both single and repeated stress restraint) match our previous findings (Ciccocioppo et al., 2014), the results of our KO studies indicate that repeated psychological stress reveals a novel role for TLR4 signaling in the CeA CRF system.

Notably, a parallel study in our laboratory examined the role of TLR4 in acute ethanol's facilitation of CeA GABAergic transmission after LPS injection and found the opposite results; LPS treatment reduced ethanol's actions in WT rats, but had no effect in the KO rats (Harris et al., 2017). It is, therefore, possible that our current findings in the TLR4-deficient rats reflect long-term compensatory changes in other TLR pathways (e.g., TLR2) or downstream TLR4 signaling (e.g., myeloid differentiation primary response 88 (MyD88), protein kinase B (Akt)). More likely, TLR4 is both temporally and spatially regulated, and our restraint stress paradigms may not have robustly activated it in WT rats (vs. its activation by LPS in our previous work (Harris et al., 2017)). In support of this possibility, Knapp et al. recently observed brain region-specific dynamic changes in TLR4 expression in rats recovering from a single 1 h restraint stress. They reported that cortical TLR4 mRNA levels were elevated 4 h (but not 2 h or 8 h) after the stress, and this increase was similar to that observed in the cortex of ethanol-withdrawn rats; but TLR4 gene expression was unchanged in the amygdala, hippocampus or hypothalamus at the 4 h post-stress.

**Fig. 2.** Restraint stress does not alter basal GABAergic transmission in the CeA of WT and Tlr4 KO rats. (A) Diagrams illustrating the single and repeated restraint stress paradigms. (B) The basal sIPSC frequency was not affected by restraint stress. Two-way ANOVA showed no significant main effects of genotype ($F_{1,76} < 0.001$, $P = 0.98$) and restraint stress treatment ($F_{2,76} = 0.45$, $P = 0.64$) on the basal sIPSC frequencies in the CeA of WT and Tlr4 KO rats, as well as no significant interaction between these factors ($F_{2,76} = 0.56$, $P = 0.57$). (C) Neither genotype ($F_{1,76} = 0.57$, $P = 0.46$) nor stress treatment ($F_{2,76} = 0.41$, $P = 0.67$) had significant main effects on sIPSC amplitudes, and there was no significant interaction ($F_{2,76} = 0.75$, $P = 0.48$). (D–E) There were no significant main effects of genotype (rise time: $F_{1,76} = 1.72$, $P = 0.19$ and decay time: $F_{1,76} = 1.46$, $P = 0.23$) or stress treatment (rise time: $F_{2,76} = 2.10$, $P = 0.13$ and decay time: $F_{2,76} = 2.23$, $P = 0.11$) on the sIPSC kinetics, and no significant interactions (rise time: $F_{2,76} = 0.66$, $P = 0.52$ and decay time: $F_{2,76} = 1.48$, $P = 0.24$). For all data presented in this figure, the WT/single stress group comprised 12 cells from four rats, Tlr4 KO/single stress group of 13 cells from four rats, WT/repeated stress group of 15 cells from five rats, Tlr4 KO/repeated stress group of 12 cells from four rats, and the naïve data were taken from Fig. 1B–E. All data are presented as mean ± SEM.
time point, as well as after withdrawal (Knapp et al., 2016). Therefore, our overall findings suggest that single (1 h) and repeated restraint stress paradigms (1 h per day for 3 days) may not induce TLR4 signaling and do not produce adaptive changes in CeA GABAergic signaling in WT rats, similar to the effects of a more potent restraint stress paradigm (6 h per day for 10 days) (Reznikov et al., 2009). Thus, our data, in combination with the work of others, suggest that while moderate stress activates the HPA axis (Leggett et al., 2007) and induces brain region-specific gene expression (Wang et al., 2010; Knapp et al., 2016), a more severe stress exposure may be needed to robustly engage the TLR4 system and induce adaptive changes in CeA neurotransmitter (GABA) and neuropeptide (CRF) systems of WT rats.

In conclusion, here we report a role for TLR4 in CRF’s modulation of CeA spontaneous GABAergic transmission in naive and single stress-treated rats, though TLR4-deficient rats that experienced repeated stress sessions exhibit a blunted CRF cellular response. Given these current findings and our previous observation that TLR4 activation reduces acute ethanol’s actions on CeA GABA signaling (Harris et al., 2017), we speculate that the TLR4 system may mediate a synergistic interaction between chronic alcohol exposure and stress in the CeA. Notably, Breese and colleagues report that in
a protocol comprising three acute withdrawals from chronic ethanol exposure, either restraint stress or LPS injection can be substituted for the initial two withdrawal periods to produce an anxiogenic phenotype that is not observed after a single ethanol withdrawal, LPS injection or restraint stress session (Breeze et al., 2006; Knapp et al., 2016). Therefore, a systematic determination of how common neuroimmune components, such as TLR4, independently regulate the cellular and behavioral effects of alcohol and stress, is critical to our overall understanding of the role of these neuroimmune factors and their therapeutic potentials in protecting against stress-induced relapse and reducing alcohol-stress disorder comorbidity (e.g. PTSD) in humans.

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CONFLICT OF INTEREST STATEMENT

None declared.

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