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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 (Leeies *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. interleukin-1β (IL-1β), 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 (Breese *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 I 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 (+)-naltrexone, 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-preferring (P) rats decreased their binge drinking via a $GABA_A$ receptor mechanism (Liu *et al.*, 2011; 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 (Gilpin *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 (CRF_1) (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 naïve 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 Wistar 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 produced 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% isofluorane and placed the isolated brains quickly into ice-cold oxygenated high-sucrose cutting solution (composition in mM: sucrose, 206; KCl, 2.5; $CaCl_2$, 0.5; $MgCl_2$, 7; NaH_2PO_4, 1.2; $NaHCO_3$, 26; glucose, 5; HEPES, 5) gassed with 95% O_2 and 5% CO_2. We cut coronal slices (300 μm) 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; NaH_2PO_4, 1.25; $MgSO_4{\cdot}7H_2O$, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 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 $MgCl_2$, 0.5 EGTA, 2 ATP and 0.2 GTP (the latter two added fresh on the day of recording), pH 7.2–7.4, osmolarity 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 DNQX, 30 μM DL-AP5) and $GABA_B$ (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 $GABA_A$-mediated sIPSCs in the medial CeA of naïve 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–E 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 changed $GABA_A$ receptor function (Otis *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 then 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 naïve 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 naïve 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 neuroimmune 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 naïve 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 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.

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

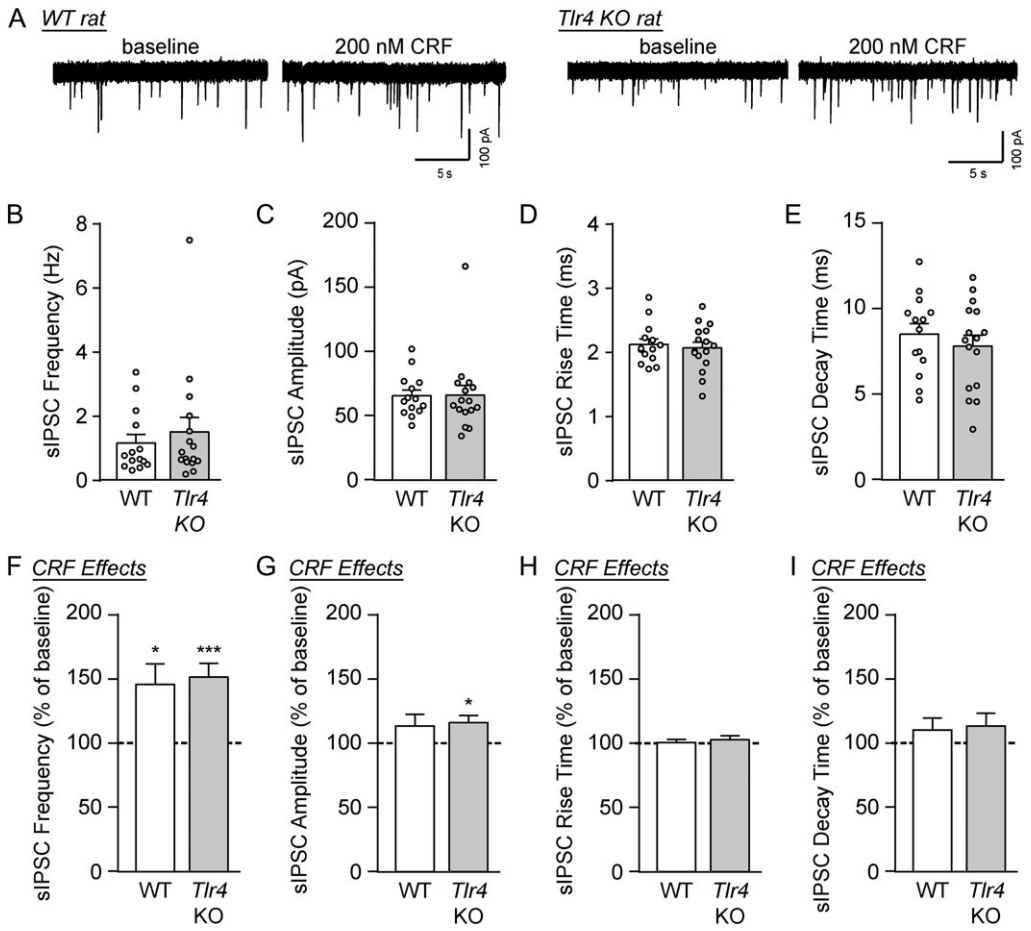

Fig. 1. CRF increased GABA transmission in the CeA of WT and *Tlr4* KO rats. (**A**) Representative sIPSC traces from CeA neurons of WT and *Tlr4* KO rats at baseline and during CRF (200 nM) superfusion. (**B–E**) Baseline sIPSC frequencies (B), amplitudes (C), rise times (D) and decay times (E) were similar across both genotypes (WT: 14 cells from three rats; *Tlr4* KO: 16 cells from five rats). (**F**) CRF significantly increased the sIPSC frequencies in WT rats (12 cells from three rats; one-sample *t*-test, $t_{(11)} = 2.86$, $^{*}P < 0.05$) and *Tlr4* KO rats (14 cells from five rats; $t_{(13)} = 4.84$, $^{***}P < 0.001$). There was no difference in the magnitude of drug effects between groups (unpaired *t*-test, $t_{(24)} = 0.31$, $P = 0.76$). (**G**) The mean sIPSC amplitude was increased by CRF in *Tlr4* KO rats (one-sample *t*-test, $t_{(13)} = 3.01$, $^{*}P < 0.05$), though the drug's effects were not significantly different across genotypes (unpaired *t*-test, $t_{(24)} = 0.27$, $P = 0.79$). (**H–I**) CRF had no effect on sIPSC kinetics across both genotypes. All data are presented as mean ± SEM.

Table 1. Basal $GABA_A$-mediated sIPSC parameters in the CeA of WT and *Tlr4* KO rats

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

There were no significant differences in baseline sIPSC frequencies, amplitudes, rise times and decay times of CeA neurons from naïve and restraint stress WT and KO rats.

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

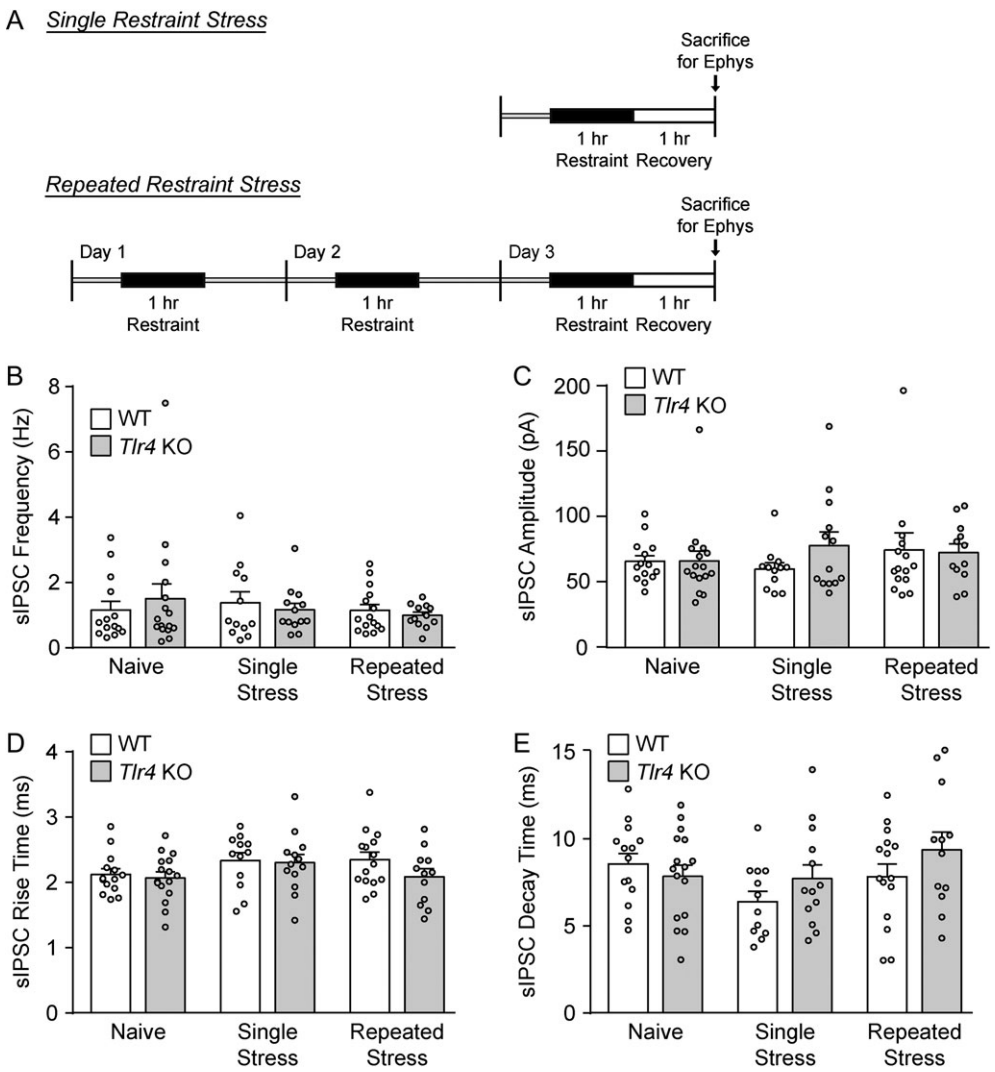

Fig. 2. Restraint stress does not alter basal GABAergic transmission in the CeA of WT and *Tlr4* KO rats. (**A**) Diagrams illustrating the single and repeated restraint stress paradigms. (**B**) The basal sIPSC frequency was not affected by restraint stress. Two-way ANOVA showed no significant main 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 ($F_{(1,76)} = 0.57$, $P = 0.45$) 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.

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 GABA 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

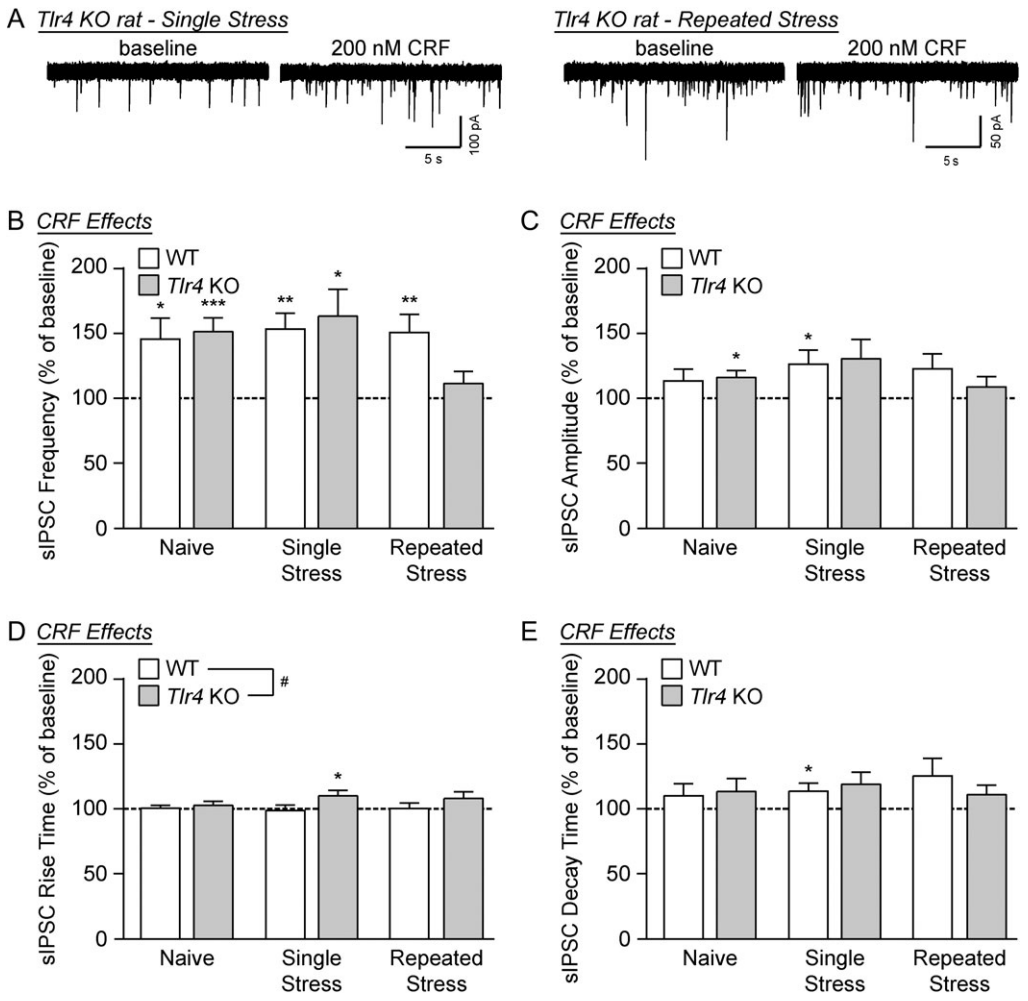

Figure 3. CRF facilitation of GABA release is reduced in the CeA of *Tlr4* KO rats following repeated restraint stress. (**A**) Representative sIPSC traces recorded in the CeA of *Tlr4* KO rats that experienced either single or repeated restraint stress, at baseline and during CRF (200 nM) superfusion. (**B**) CRF significantly increased sIPSC frequencies in all restraint stress-treated animals by one-sample *t*-test (WT/single stress: $t_{(11)} = 4.40$, $^{**}P < 0.01$; KO/single stress: $t_{(10)} = 3.09$; $^{*}P < 0.05$; WT/repeated stress: $t_{(14)} = 3.70$; $^{**}P < 0.01$), except for the repeated stress *Tlr4* KO rats ($t_{(11)} = 1.27$, $P = 0.23$). Despite this difference, two-way ANOVA across both genotypes (WT and *Tlr4* KO) and all three treatment groups (naïve, single stress and repeated stress) showed no significant main effects (genotype: $F_{(1,70)} = 0.47$, $P = 0.50$; stress treatment: $F_{(2,70)} = 1.94$, $P = 0.15$), and no significant interaction ($F_{(2,70)} = 1.94$, $P = 0.15$). (**C**) The sIPSC amplitude was also increased by CRF in single stress WT rats (one-sample *t*-test, $t_{(11)} = 2.49$, $^{*}P < 0.05$), but two-way ANOVA revealed no significant main effects of genotype ($F_{(1,70)} = 0.08$, $P = 0.78$) or stress treatment ($F_{(2,70)} = 1.01$, $P = 0.35$), and no interaction ($F_{(2,70)} = 0.50$, $P = 0.61$). (**D**) CRF increased the sIPSC rise time in single stress *Tlr4* KO rats (one-sample *t*-test, $t_{(10)} = 2.38$, $^{*}P < 0.05$). In addition, by two-way ANOVA there was a significant main effect of genotype ($F_{(1,70)} = 4.59$, $^{\#}P < 0.05$) on CRF-induced rise times, which was associated with no main stress treatment effect ($F_{(2,70)} = 0.29$, $P = 0.75$) and no stress x genotype interaction ($F_{(2,70)} = 0.62$, $P = 0.54$). (**E**) The sIPSC decay time was also increased by CRF in single stress WT rats (one-sample *t*-test, $t_{(11)} = 2.25$, $^{*}P < 0.05$), but two-way ANOVA revealed no significant main effects of genotype ($F_{(1,70)} = 0.05$, $P = 0.82$) or stress treatment ($F_{(2,70)} = 0.22$, $P = 0.80$), and no interaction ($F_{(2,70)} = 0.58$, $P = 0.56$). For all data presented in this figure, the WT/single stress group comprised 12 cells from four rats, *Tlr4* KO/single stress group of 11 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 was taken from Fig. 1F–I. 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 naïve 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 (Breese *et al.*, 2008; 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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