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Innate immune factors modulate ethanol interaction with GABAergic transmission in mouse central amygdala

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ABSTRACT

Excessive ethanol drinking in rodent models may involve activation of the innate immune system, especially toll-like receptor 4 (TLR4) signaling pathways. We used intracellular recording of evoked GABAergic inhibitory postsynaptic potentials (eIPSPs) in central amygdala (CeA) neurons to examine the role of TLR4 activation by lipopolysaccharide (LPS) and deletion of its adapter protein CD14 in acute ethanol effects on the GABAergic system. Ethanol (44, 66 or 100 mM) and LPS (25 and 50 μg/ml) both augmented eIPSPs in CeA of wild type (WT) mice. Ethanol (44 mM) decreased paired-pulse facilitation (PPF), suggesting a presynaptic mechanism of action. Acute LPS (25 μg/ml) had no effect on PPF and significantly increased the mean miniature IPSC amplitude, indicating a postsynaptic mechanism of action. Acute LPS pre-treatment potentiated ethanol (44 mM) effects on eIPSPs in WT mice and restored ethanol's augmenting effects on the eIPSP amplitude in CD14 knockout (CD14 KO) mice. Both the LPS and ethanol (44–66 mM) augmentation of eIPSPs was diminished significantly in most CeA neurons of CD14 KO mice; however, ethanol at the highest concentration tested (100 mM) still increased eIPSP amplitudes. By contrast, ethanol pretreatment occluded LPS augmentation of eIPSPs in WT mice and had no significant effect in CD14 KO mice. Furthermore, (+)-naloxone, a TLR4-MD-2 complex inhibitor, blocked LPS effects on eIPSPs in WT mice and delayed the ethanol-induced potentiation of GABAergic transmission. In CeA neurons of CD14 KO mice, (+)-naloxone alone diminished eIPSPs, and subsequent co-application of 100 mM ethanol restored the eIPSPs to baseline levels. In summary, our results indicate that TLR4 and CD14 signaling play an important role in the acute ethanol effects on GABAergic transmission in the CeA and support the idea that CD14 and TLR4 may be therapeutic targets for treatment of alcohol abuse.

1. Introduction

Recent evidence points to a role for neuroimmune mechanisms, and particularly the innate immunity mediated by the toll-like receptors (TLRs), in ethanol effects and drinking (for review see (Crews et al., 2011; Harris and Blednov, 2012). TLR4 plays an especially critical role in innate immunity and this pathway is activated by both exogenous [e.g., LPS, exhibiting a microbe-/pathogen-associated molecular pattern (MAMP/PAMP)] and endogenous signals or ligands [e.g., high mobility group box 1, heat shock proteins, nucleic acids and fibronectin, exhibiting damage-associated molecular patterns (DAMPs)] (Piccinini and Midwood, 2010). Activation of TLR4 triggers transcriptional activation of pro-interleukin-1β and TNFα and initiation of the innate immune response (Hanamsagar et al., 2012; Kielian, 2009).

Evidence supporting a key role of TLR4 in alcohol effects and drinking includes the following: 1) the alcohol-induced activation of glia, induction of inflammatory mediators, apoptosis, and behavioral and anxiety impairments seen in WT mice are not found in TLR4 deficient mice (Alfonso-Loeches and Guerri, 2011;

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Alfonso-Loeches et al., 2010, 2012; Blanco and Guerri, 2007; Blanco et al., 2005; Fernandez-Lizarbe et al., 2013, 2009; Pascual et al., 2011; Valles et al., 2004; Wu et al., 2012); 2) TLR4 is involved in excessive alcohol drinking in preclinical models (Blednov et al., 2011a, 2012; Liu et al., 2011; Mulligan et al., 2006); 3) alcohol releases endogenous ligands for TLR4 in brain (Crews et al., 2013; He and Crews, 2008; Vetreno and Crews, 2012); and 4) LPS leaks from the gut in human alcoholics to activate pro-inflammatory signaling that may contribute to neuroinflammation and neurodegeneration (Qin et al., 2007). Examination of the TLR4 inflammatory pathways suggests possible approaches to study ethanol effects and drinking. Notably, knockout of the accessory protein CD14, that plays a critical role in LPS activation of TLR4, reduces ethanol preference and blocks the LPS-induced increase in ethanol drinking seen in wild type (WT) mice (Blednov et al., 2011a, 2012). The reduction of TLR4 expression in the central nucleus of the amygdala (CeA) modulates ethanol binge drinking in a mouse model via a $GABA_A$ receptor effect (Liu et al., 2011). Moreover, it has been shown that TLR4–MyD88 signaling is involved in the acute behavioral actions of alcohol, as both pharmacological inhibition of TLR4 signaling with (+)-naloxone, a TLR4-MD-2 complex inhibitor and genetic deficiency of TLR4 or MyD88 significantly reduced the sedation and motor impairment induced by a single dose of alcohol in mice (Wu et al., 2012). Ethyl glucuronide, an ethanol metabolite, causes TLR4-dependent pain allodynia that can be blocked by (+)-naloxone (Lewis et al., 2013). Naloxone has two stereotactic isoforms, (+)-naloxone and (−)-naloxone, that are both potent TLR4 signaling inhibitors (Hutchinson et al., 2008). Whereas (+)-naloxone is selective for TLR4, (−)-naloxone also acts on opioid receptors (Hutchinson et al., 2008). Because another opioid antagonist, naltrexone, is now used in the treatment of alcohol addiction (Jarosz et al., 2013; Thorsell, 2013), there has been an effort to evaluate the therapeutic potential of TLR4-MD-2 complex specific (+)-naloxone and (+)-naltrexone.

The CeA, a major component of the extended amygdala (Heimer and Alheid, 1991), is a brain region known to be critically involved in anxiety and fear-conditioning, as well as in alcohol and drug dependence (Davis and Shi, 1999; Koob and Volkow, 2010; Rosen, 2004). In alcohol dependence, the CeA participates in the learning of stimulus-reward responses and mediation of alcohol's motivational effects, self-administration, and stress-induced reinstatement of drinking (Koob, 1998; Koob and Volkow, 2010). The great majority of CeA neurons are GABAergic, and the GABAergic system is a key player in ethanol effects in the CeA (Nie et al., 2004, 2009; Roberto et al., 2008, 2003, 2004a; Siggins et al., 2005).

Knocking out TLR4 or CD14 reduced ethanol drinking and ethanol-related behavior in rodents (Alfonso-Loeches et al., 2010; Blednov et al., 2011a, 2012; Pascual et al., 2011). Importantly, decreased TLR4 expression in CeA but not in basolateral amygdala (BLA) reduced ethanol binge drinking, indicating a critical role of the TLR4 system in the CeA for ethanol drinking (Liu et al., 2011). Although TLR4 receptors are expressed primarily by microglia in the CNS (Lehnardt et al., 2002; Chakravarty and Herkenham, 2005; Pascual et al., 2012), several studies also have shown neuronal expression of TLR4 (Acosta and Davies, 2008; Okun et al., 2011; Rolls et al., 2007; Tu et al., 2011). In the CeA, TLR4 receptors appear to be expressed in neurons, and their expression is regulated by the α2 $GABA_A$ subunit (Liu et al., 2011). Overall, these findings indicate that both TLR4 and the GABAergic system, and their cellular interactions in the CeA, may play an important role in ethanol drinking. However, little is known about the cellular aspects of TLR4 activation on neurophysiology and GABAergic transmission or on ethanol-induced potentiation of GABAergic transmission in the CeA (Bajo et al., 2008; Cruz et al., 2011; Roberto et al., 2012, 2003, 2004b). Therefore, in the present study we explored these issues using electrophysiological methods in CeA slices from CD14 KO mice, with exogenous administration of LPS and the TLR4 antagonist (+)-naloxone to activate and inhibit TLR4, respectively. We report that acute ethanol effects on GABAergic transmission in the CeA involve, or are mimicked by, components of the innate immune system such as TLR4 and CD14.

2. Methods

2.1. Slice Preparation

We prepared *in vitro* brain slices (300 and 400 μm thick for whole-cell and sharp electrode recordings, respectively) containing CeA as previously described (Bajo et al., 2008, 2011) from male (20–30 weeks old; 25–31 g) C57Bl/6J mice (Jackson Laboratory and the rodent breeding colony of The Scripps Research Institute) and from male CD14 KO mice (provided by Drs. Blednov and Harris of The University of Texas at Austin; see (Blednov et al., 2011a)). For more detailed information on the mice and slice preparation, see the Supplemental Information (SI). We conducted all mouse breeding and care procedures in accordance with the Institutional Animal Care and Use Committee (IACUC) policies of The University of Texas at Austin and The Scripps Research Institute.

2.2. Electrophysiology

2.2.1. Intracellular recording of evoked responses

We recorded from CeA neurons with sharp micropipettes containing 3 M KCl (65–80 mΩ resistance) using current-clamp mode. The CeA is divided into medial and lateral subdivisions, but they cannot be easily identified in acute slices maintained *in vitro* (Sah et al., 2003). Therefore, we recorded from both subdivisions and did not distinguish between neurons from the two subdivisions. We held most neurons near their resting membrane potential (RMP), acquired data with an Axoclamp-2A preamplifier (Axon Instruments, now Molecular Devices, Sunnyvale, CA) and analyzed the recordings using pClamp software (Molecular Devices). We evoked pharmacologically-isolated $GABA_A$ receptor-mediated inhibitory postsynaptic potentials (eIPSPs) by stimulating locally within the CeA through a bipolar stimulating electrode, positioned medially close to the lateral globus pallidus or internal capsule, while superfusing the slices with the glutamate receptor blockers, 6-cyano-7-nitroquinoxaline-2,3-dione (DNQX, 20 μM) and DL-2-amino-5-phosphonovalerate (DL-AP5, 30 μM), and a $GABA_B$ receptor antagonist (CGP 55845A; 1 μM).

To determine half-maximal eIPSP amplitudes, we generated input/output (I/O) curves by measuring eIPSP amplitudes at 5 incrementally-increasing stimulus strengths, threshold to maximum stimulation. We measured the eIPSP amplitude I/O curves before (control), during and after (washout) drug application. We also used the paired-pulse facilitation (PPF) protocol to determine if the ethanol and LPS effects on eIPSPs were mediated by pre- or postsynaptic mechanisms. We examined PPF using 100 ms interstimulus intervals with the stimulus strength adjusted to give a 50% maximal amplitude of the first eIPSP, as determined from the I/O relationship. We calculated PPF as the ratio of the second eIPSP amplitude over that of the first eIPSP*100 (see SI). It has been shown that changes in the PPF ratio vary inversely with the presynaptic release of transmitter (Bonci and Williams, 1997; Mennerick and Zorumski, 1995; Salin et al., 1996).

2.2.2. Whole-cell patch-clamp recording of miniature IPSCs

We also recorded spontaneous action potential-independent $GABA_A$ergic mIPSCs (miniature inhibitory postsynaptic currents) to verify pre- versus postsynaptic mechanisms of action of LPS. Generally, a change in the frequency of mIPSCs suggests an altered