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Chronic intermittent ethanol and withdrawal differentially modulate basolateral amygdala AMPA-type glutamate receptor function and trafficking

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ABSTRACT

The amygdala plays a critical role in the generation and expression of anxiety-like behaviors including those expressed following withdrawal (WD) from chronic intermittent ethanol (CIE) exposure. In particular, the BLA glutamatergic system controls the expression of both innate and pathological anxiety. Recent data suggests that CIE and WD may functionally alter this system in a manner that closely parallels memory-related phenomena like long-term potentiation (LTP). We therefore specifically dissected CIE/WD-induced changes in glutamatergic signaling using electrophysiological and biochemical approaches with a particular focus on the plasticity-related components of this neurotransmitter system. Our results indicate that cortical glutamatergic inputs arriving at BLA principal via the external capsule undergo predominantly post-synaptic alterations in AMPA receptor function following CIE and WD. Biochemical analysis revealed treatment-dependent changes in AMPA receptor surface expression and subunit phosphorylation that are complemented by changes in total protein levels and/or phosphorylation status of several key, plasticity-associated protein kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). Together, these data show that CIE- and WD-induced changes in BLA glutamatergic function both functionally and biochemically mimic plasticity-related states. These mechanisms likely contribute to long-term increases in anxiety-like behavior following chronic ethanol exposure.

1. Introduction

For human alcoholics, withdrawal-associated anxiety is a major risk factor for relapse. The amygdala plays a critical role in the generation and expression of anxiety-like behaviors including those expressed following withdrawal from chronic ethanol exposure (Läck et al., 2005; Santucci et al., 2008). The lateral and basolateral subdivisions (BLA) form the primary input nuclei in the amygdala's emotion-related neural circuitry (Sah et al., 2003) and receive highly processed sensory information via cortical afferents coursing into the region from the external capsule (EC) (Rainnie et al., 1991). The BLA subsequently sends significant excitatory glutamate projections into areas like the central amygdala and the bed nucleus of the stria terminalis to drive the physiological and psychological manifestations of anxiety and fear (Davis et al., 1994). EC glutamatergic inputs are therefore a principal regulator of BLA principal neuron activity and help modulate the expression of anxiety-like behaviors included learned emotional responses. For example, experimental manipulation of BLA glutamatergic neurotransmission blocks innate anxiety responses as well as the acquisition/expression of learned fear in rodents (Rodrigues et al., 2001). We have recently demonstrated that inhibition of BLA glutamatergic neurotransmission can likewise down regulate the expression of anxiety-like behaviors following withdrawal (WD) from chronic intermittent ethanol (CIE) exposure (Läck et al., 2007). Thus, the BLA glutamatergic system functions to regulate the expression of anxiety in both natural and pathological states.

Recent work has focused on glutamate neurotransmission in the BLA as a general target of modification by CIE exposure and WD. CIE/WD robustly increase both *N*-Methyl-D-aspartate receptor (NMDAR) and kainate receptor locally-evoked synaptic function in BLA pyramidal neurons (Floyd et al., 2003; Läck et al., 2008, 2009). However, both kainate receptor-dependent and NMDAR-dependent plasticity are occluded following both CIE and WD (Läck et al., 2009; Stephens et al., 2005). Specific functional changes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR) in response to CIE/WD have only been recently examined. In these

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studies, it was determined that the amplitude of AMPAR mediated miniature excitatory post-synaptic currents (mEPSC) increased following WD (Läck et al., 2007). These functional studies suggest that CIE/WD up-regulate AMPAR function to induce plasticity-like state. This would suggest that chronic ethanol exposure engages the BLA glutamate system in a manner similar to experience-dependent long-term potentiation (LTP). Given that the BLA receives numerous glutamatergic inputs and the anatomical origins of mEPSCs are not clear, it is uncertain whether the increased AMPAR function can be attributed to specific afferents or represents a more generalized feature of BLA glutamatergic synapses during/following chronic ethanol exposure.

There is a large body of literature describing the cellular and molecular mechanisms governing the generation and expression of LTP of glutamatergic synapses in the BLA (Blair et al., 2001; Maren, 2003; Sah et al., 2008). Post-synaptic forms of LTP can be initiated by several distinct mechanisms including NMDAR-dependent forms (Yu et al., 2008) as well as NMDAR-independent (Chapman and Bellavance, 1992) including those initiated by synaptic kainate receptors (Läck et al., 2008). Regardless of the initiation mechanism, the different forms of post-synaptic LTP are all characterized by increased AMPAR function and/or trafficking which are essential for the expression of post-synaptic LTP in the BLA (Makino and Malinow, 2009; Malenka, 2003; Malinow, 2003; Yu et al., 2008). In regions like the hippocampus, plasticity-associated increases in AMPAR function are strictly governed by the phosphorylation status of amino acid residues within specific AMPAR subunits (GluA1–4) (Boehm and Malinow, 2005; Wang et al., 2005). In this context, a multitude of protein kinases (Appleby et al., 2011; Boehm et al., 2006) appear to contribute to the regulation of AMPAR function and trafficking (Ahn and Choe, 2009; Boehm and Malinow, 2005). Given that CIE/WD alter glutamate receptor systems involved in LTP, we examined parallels between plasticity-related glutamatergic signaling components altered by CIE/WD. We specifically hypothesize that CIE/WD engage the BLA AMPAR system, including plasticity-associated kinases, similar to LTP.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 100–150 g (approximately 5–6 weeks of age) were group housed for the duration of the experiments. All experimental procedures were reviewed and approved by the WFUSM Animal Care and Use Committee and are consistent with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. Chronic intermittent ethanol exposure & withdrawal

Chronic ethanol exposure was conducted in a manner similar to previous reports (Läck et al., 2009, 2007). Briefly, animals were placed into air-tight, Plexiglas enclosures in their home cages and exposed to either ethanol vapor (~37 mg/L) or room air during the light cycle (12 h/day) for 10 consecutive days. Animals receiving this chronic intermittent treatment were divided into two subgroups: 1) those euthanized while still intoxicated at the end of the last exposure (CIE) and 2) those euthanized 24 h after the last exposure (WD). Control (CON) animals were housed in identical Plexiglas enclosures, exposed only to room air, and euthanized during the same weeks as the CIE and WD animals. Trunk blood was collected from the CIE group at euthanasia and analyzed for blood ethanol levels. At the time of brain extraction, blood ethanol levels in the CIE animals were 185.33 ± 5.75 mg/dL as determined by a commercially-available alcohol dehydrogenase assay (Genzyme, Middleton WI, U.S.A.).

2.3. Electrophysiology methods

2.3.1. Slice preparation

Electrophysiology experiments used coronal brain slices containing the amygdala from animals anesthetized using isoflurane (3%) and decapitated according to a Wake Forest Baptist Health institutional IACUC-approved protocol. Brains were removed and incubated in ice-cold sucrose-modified artificial cerebral spinal fluid (aCSF) equilibrated with 95% O_2 and 5% CO_2 containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 6H_2O$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 100 ketamine, and 10 glucose. Brains were sliced (400 μm) in the same solution on a Leica VT1200S Vibratome (Leica, Germany) or Vibratome Series 3000 (Vibratome, St. Louis, MO, U.S.A.) then submerged in room-temperature (~25 °C), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 10 glucose, and 2 $CaCl_2 \cdot 2H_2O$. Slices were maintained in aCSF for ~1 h before recording. Experiments were performed 1–5 h after preparation of the BLA slices. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Tocris (Ellisville, Missouri), or United States Pharmacopeia (Rockville, Maryland).

2.3.2. Whole-cell patch clamp recording

Methods for whole-cell voltage clamp recordings from BLA neurons within slices were similar to those reported previously (Läck et al., 2007). Slices were placed in a recording chamber continuously perfused with room-temperature aCSF at a rate of 2.0 ml/min. Pipette resistances were 6–12 MΩ when electrodes were filled with an internal solution containing (in mM): 122 Cs-gluconate, 10 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 $CaCl_2$, 4 Mg-ATP, 0.3 Na-GTP, and 2 QX314-(Cl), pH 7.25, osmolarity 280–290 mOsm or 145 K-Gluconate, 5 NaCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES, 2 Mg-ATP, 0.1 Na-GTP, pH 7.25, osmolarity 280–290. Data were acquired via Axopatch 700B or Axopatch 200B amplifiers (Axon Instruments, Foster City, CA) and stored for later analysis using pClamp software (Axon Instruments, Foster City, CA). Glutamatergic synaptic currents were pharmacologically isolated using GABAergic antagonists. Only neurons with a high membrane capacitance (>100 pF) and low access resistance (<30 MΩ) were considered presumptive principal neurons (Washburn and Moises, 1992) and included in the current analysis. Synaptic responses were evoked using constant-voltage activation of concentric bipolar stimulating electrodes (FHC Inc., Bowdoin, ME) as previously described (Läck et al., 2009). Mean stimulation intensities across all experiments were 31 ± 3 mV and did not statistically vary across the different treatment groups ($P > 0.05$, $F(2,31) = 1.92$ One Way ANOVA).

2.3.3. Paired-pulse ratio

Paired-pulse ratios (PPR) were calculated with two equal-intensity electrical stimulations with a range of inter-pulse intervals (25–100 ms). A normalized ratio of the amplitudes of the evoked EPSCs was taken (second amplitude − first amplitude, divided by the first) to provide a conservative estimate of the second response amplitude (Schulz et al., 1995). All values are expressed as mean ± SEM.

2.3.4. Strontium substitution

Experiments were conducted in aCSF where strontium (3.0 mM) was substituted for calcium (2.0 mM). This substitution allowed for the characterization of electrically evoked asynchronous EPSC (aEPSC) responses (Choi and Lovinger, 1997). A bipolar stimulator was placed in the external capsule and electrical square wave stimulation (0.4 ms) was applied every 30 s to elicit aEPSCs. Events were collected and stored for offline data analysis (Clampfit 10.2, Mini Analysis) (Synaptosoft Fort Lee NJ). Semi-automated aEPSC analysis was conducted on responses starting 50 ms after the stimulation artifact to include responses during a 400 ms window (e.g. 50 ms–450 ms post-stimulus) as previously described (Choi and Lovinger, 1997). Only this time interval was extracted for analysis using commercially-available software (Mini-Analysis, SynaptoSoft, Decatur, GA) to allow for the resolution of individual aEPSC events. Some recordings utilized bath application of insulin (1.0 μM) as described in the text.

2.4. Western blots

Western blot procedures were similar to those in previously published reports (Diaz et al., 2011). Briefly, lysis buffer (10 mM Tris pH 7.4, 2.0% sodium dodecyl sulfate, 1 mM EDTA pH 8, protease inhibitors for mammalian tissue (Sigma, St. Louis, MO), and phosphatase inhibitor cocktail 1 & 2 [Sigma]) was added to BLA samples dissected from CON, CIE, and WD coronal brain slices at 7 μl/mg tissue, disrupted by brief sonication on ice, and incubated at 4 °C on a rotisserie mixer for 1 h. Protein yield was quantified using a BCA assay (Pierce Chemical, Rockford, IL).

Five to twenty micrograms of total protein was loaded on to 4–20% sodium dodecyl sulfate precast polyacrylamide gels (Pierce Chemical, Rockford, IL) or 8–16% GELS (Biorad), separated, and transferred to a nitrocellulose membrane (Hybond N; Amersham, Piscataway, NJ). Membranes were blocked with Tris buffered saline (TBS)-T (150 mM NaCl, 5.2 mM Na_2HPO4, 1.7 mM KH_2PO_4, 0.05% Tween-20) containing 5% bovine serum albumin (BSA) or 5% milk. Subsequently, blots were incubated overnight at 4 °C or for 3 h at 25 °C in TBS-T/1.0% BSA containing target specific primary antibodies. The following antibodies exhibited specificity for the protein targets as indicated by immunoreactive bands at expected molecular weights: PKCγ (1:2000) and Neurogranin (1:2000) from Abcam (Cambridge, MA); GluA1 (1:3000), GluA1 phosphoSer845 (1:1000), GluA2/3 (1:4000), and GluA2 phosphoSer831 (1:1000) from Chemicon (now Millipore, Billerica, MA); PKCα (0.5 μg/ml) from Millipore; CaMKIIα phosphosThr305/306 (1:1000) from PhosphoSolutions (Aurora, CO); CaMKIIα (1:20,000) and CaMKIIα phosphoThr286 (1:1000) from Thermo Scientific (Waltham, MA); GluA2 phosphoSer880 (1:1000) and Neurogranin phosphoSer36 (1:1000) from Upstate (now Millipore). Following extensive washing with TBS-T, the blots were exposed to HRP-labeled secondary antibodies for one hour at