Neuropharmacology 65 (2013) 134–142

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Thalamic glutamatergic afferents into the rat basolateral amygdala exhibit increased presynaptic glutamate function following withdrawal from chronic intermittent ethanol

Daniel T. Christian [a], Nancy J. Alexander [a], Marvin R. Diaz [a,b,1], Brian A. McCool [a,b,*]

[a] Department of Physiology and Pharmacology, Wake Forest School of Medicine, Winston-Salem, NC, USA
[b] Neuroscience and Alcohol Research Training Programs, Wake Forest School of Medicine, Winston-Salem, NC, USA

ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised form
4 September 2012
Accepted 5 September 2012

Keywords:
Strontium substitution
Coefficient of variation
Whole-cell patch-clamp electrophysiology
Paired-pulse ratio
Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)
γ-D-glutamylglycine (γDGG)

ABSTRACT

Amygdala glutamatergic neurotransmission regulates withdrawal induced anxiety-like behaviors following chronic ethanol exposure. The lateral/basolateral amygdala receives multiple glutamatergic projections that contribute to overall amygdala function. Our lab has previously shown that rat cortical (external capsule) afferents express postsynaptic alterations during chronic intermittent ethanol exposure and withdrawal. However, thalamic (internal capsule) afferents also provide crucial glutamatergic input during behavioral conditioning, and they have not been studied in the context of chronic drug exposure. We report here that these thalamic inputs express altered presynaptic function during withdrawal from chronic ethanol exposure. This is characterized by enhanced release probability, as exemplified by altered paired-pulse ratios and decreased failure rates of unitary events, and increased concentrations of synaptic glutamate. Quantal analysis further implicates a withdrawal-dependent enhancement of the readily releasable pool of vesicles as a probable mechanism. These functional alterations are accompanied by increased expression of vesicle associated protein markers. These data demonstrate that chronic ethanol modulation of glutamate neurotransmission in the rat lateral/basolateral amygdala is afferent-specific. Further, presynaptic regulation of lateral/basolateral amygdala thalamic inputs by chronic ethanol may be a novel neurobiological mechanism contributing to the increased anxiety-like behaviors that characterize withdrawal.

1. Introduction

Emotion-related signal processing occurs in the amygdala's lateral/basolateral subdivisions (BLA; Sah et al., 2003), which form the primary input nuclei of this brain region. Information flow into the BLA occurs via anatomically distinct glutamatergic afferents relaying cortical and subcortical information (Rainnie et al., 1991) onto glutamatergic principal neurons. Following local processing, these pyramidal cells drive physiological and psychological manifestations of anxiety and fear (Davis et al., 1994). Thus, the regulation of glutamate signaling arising from different afferents can have a profound impact on fear learning and anxiety (Bauer et al., 2002). For example, qualitatively distinct types of information carried by cortical (external capsule, EC) and subcortical/thalamic (internal capsule, IC) afferents suggests they make differential contributions to BLA neuron activity. In support of this, *in vivo* fear conditioning induces afferent-specific alterations of synaptic transmission at EC and IC inputs (Boatman and Kim, 2006). Afferent-specific pre- and postsynaptic mechanisms also govern the initiation and expression of BLA glutamatergic synaptic plasticity *in vitro* and *in vivo* (Sah et al., 2008; Sigurdsson et al., 2007).

Pathological conditions can also target input-specific mechanisms at BLA glutamatergic synapses. Chronic intermittent ethanol (CIE) exposure significantly increases the postsynaptic function of *N*-methyl-D-aspartate (NMDA) (Läck et al., 2007), kainate (Läck et al., 2009), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Christian et al., 2012) glutamate receptors. And withdrawal (WD) from CIE exposure produces treatment-specific increases of anxiety-like behavior regulated by BLA glutamatergic signaling (Läck et al., 2007). Recent data suggests that glutamatergic signaling alterations may be input-specific. For example, the

* Corresponding author. Department of Physiology and Pharmacology, Wake Forest Baptist Health School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA. Tel.: +1 336 716 8534; fax: +1 336 716 8501.
E-mail address: bmmcool@wakehealth.edu (B.A. McCool).
[1] Current address: Department of Neurosciences, University of New Mexico Health Sciences Center, Albuquerque, NM, USA.

0028-3908/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2012.09.004

external capsule (cortical) glutamatergic afferents exhibit only CIE/WD-dependent increases in postsynaptic AMPAR function (Christian et al., 2012) without any presynaptic alterations (Christian et al., 2012; Läck et al., 2007). However, nothing is known about the impact of CIE/WD on thalamic IC-inputs. Since IC (thalamic) inputs express presynaptic forms of plasticity (Zinebi et al., 2002), we hypothesized that CIE and WD could enhance presynaptic function at IC–BLA glutamatergic afferents.

2. Methods

2.1. Animals

Male Sprague Dawley rats (Harlan, Indianapolis, IN, USA) ~5–6 weeks of age (100–150 g) were housed in groups for all experiments. Animals were given 5 days to recover from the shipping and were then placed into our experimental groups (below). At the time of tissue preparation, animals were ~7–8 weeks of age (170–200 g). All experimental procedures conformed to NIH Guidelines for the Care and Use of Laboratory Animals and were reviewed and approved by the WFUSM Animal Care and Use Committee.

2.2. CIE and WD exposure

CIE and WD exposures were similar to previous reports (Christian et al., 2012). Ethanol vapor (~37 mg/l) or room air exposures (CON) were conducted during the light-phase of the light/dark cycle for 10 consecutive days, 12 h/day (Läck et al., 2007). Ethanol vapor exposed animals were euthanized either while still intoxicated (CIE) or 24 h after the last ethanol exposure (WD). CIE animal trunk blood was collected and analyzed using a commercially available alcohol dehydrogenase assay kit (Genzyme, Middleton WI, USA). Mean blood-ethanol concentrations were 185.34 ± 5.75 mg/dl.

2.3. Electrophysiology methods

2.3.1. Slice preparation

Coronal brain slices containing the amygdala were taken for electrophysiology experiments from anesthetized animals (3% isoflurane) following decapitation in accordance with an approved Wake Forest Baptist Health Institutional ACUC protocol. Brains were incubated and sliced (400 μm) on a Leica VT1200S (Leica, Germany) or Vibratome Series 3000 (Vibratome, St. Louis, MO) in ice-cold sucrose modified artificial cerebral spinal fluid (aCSF) containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 6H_2O$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 0.10 ketamine, and 10 glucose, equilibrated with 95% O_2 and 5% CO_2. Slices were incubated for ~1 h in room temperature (~25 °C), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH2PO4, 2 $MgSO_4$, 26 $NaHCO_3$, 10 glucose, and 2 $CaCl_2 \cdot 2H_2O$ before initiation of recordings (1–5 h postpreparation). Sigma-Aldrich (St. Louis, MO) and Tocris (Ellisville, MO) purveyed all chemical reagents.

2.3.2. Whole-cell patch-clamp recording

BLA slices were transferred to a submersion-type recording chamber and perfused with room temperature aCSF (2.0 ml/min) for whole-cell voltage clamp recordings similar to previously published reports (Christian et al., 2012). Recording electrodes were filled with an internal solution containing (in mM): 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; pipette open tip resistances were 6–12 MΩ. Data were acquired via Axopatch 700 b or 200 b amplifiers (Molecular Devices, Foster City, CA) and analyzed offline via pClamp software (Molecular Devices). Inclusion criteria for presumptive principal neurons included high membrane capacitances (>100 pF) and low access resistances in the whole-cell configuration (<20 MΩ; Washburn and Moises, 1992) leading to the inclusion of ~80% of all cells analyzed. Synaptic responses were electrically evoked using either concentric bipolar stimulating electrodes (FHC Inc, Bowdoin, ME) or electrodes fabricated from theta tube borosilicate glass (World Precision Instruments, Inc., Sarasota, FL) with constant-voltage stimulation as previously described (Christian et al., 2012; Läck et al., 2009). Glutamatergic synaptic currents were pharmacologically isolated by continuous perfusion of slices with 100 μM picrotoxin.

2.3.3. Paired-pulse ratio

Two stimuli of equal intensity were evoked with a range of interpulse intervals (25–500 ms), with evoked excitatory postsynaptic current (EPSC) amplitudes being used to calculate paired-pulse ratio's (PPR). A conservative estimate of amplitudes to minimize contamination of second response amplitude by the first-response decay at short time intervals ((peak 2 – peak 1)/peak 1) was utilized for all PPR calculations (Schulz et al., 1995). Several studies utilized bath application of pharmacological agents during the paired-pulse experiments to further characterize presynaptic mechanisms associated with alterations of glutamatergic synaptic function following CIE and WD.

2.3.4. Collision protocol

Paired electrical stimuli of equal intensity were delivered at an interpulse interval of 500 ms paired at a single afferent (e.g. EC_1–EC_2; IC_1–IC_2) or mixed at multiple afferents (e.g. EC_1–IC_2; IC_1–EC_2). Response decay did not influence amplitudes at these longer interpulse intervals, peak EPSC amplitudes were used to calculate PPR's (second amplitude – first amplitude, divided by the first) at both IC and EC afferents. Ratios were calculated across all afferent stimulation patterns and examined for facilitation or depression during the second stimulation at each input (EC_1–EC_2; IC_1–IC_2) independent of stimulation pattern.

2.3.5. SR^{2+} evoked responses

Some electrophysiology experiments were conducted in aCSF where strontium (2.0 mM) was substituted for calcium (2.0 mM) to allow the characterization of electrically evoked asynchronous EPSC (aEPSC) responses (Choi and Lovinger, 1997a; Miledi, 1966) at specific afferents.

2.3.6. Synaptic fatigue

A 2.0-Hz stimulation (15 min) was applied to the IC–BLA input following a stable baseline period to generate synaptic rundown. EPSC amplitudes were analyzed for standard deviations and utilized in a Coefficient of Variance (CV) analysis (Deng et al., 2010; Faber and Korn, 1991) to characterize alterations of synaptic function. Standard deviation and mean amplitude values were calculated from 10 consecutive EPSC events grouped into bins of 5 consecutive event (50 sweeps total/bin) groups over the duration of the protocol.

2.4. Western blot methods

Western blot methods are similar to those previously reported (Christian et al., 2012). Importantly, single blots contained BLA protein from single animals (1 per lane) containing CON, CIE, and WD groups (4 per group). Within each blot, group specific expression (4 lanes) was averaged and normalized to CON mean expression (percent CON). Several experiments were performed in duplicate to increase power. Antibodies to protein targets (primary concentration utilized) exhibited specificity as indicated by immunoreactive bands at expected molecular weights: VGLUT1 (1:40,000), VGLUT2 (1:7000) from Synaptic Systems (Goettingen, Germany), synaptobrevin I (0.5 μg/ml), synaptobrevin II (1:1000), syntaxin 1 (1:3000), synaptotagmin I (1.0 μg/ml) and synaptotagmin II (1:750) from Abcam (Cambridge, MA), and SNAP25 (1:1000) from Chemicon (Now Millipore, Billerica, MA). VGLUT1/2 protein samples were run as above with the exception that protein samples were not heated prior to loading per manufacturer's instructions.

2.5. Statistics

All values are expressed as mean ± SEM. Primary statistical analyses were conducted using 2-way ANOVA (SigmaPlot, Systat Software Inc, San Jose, CA), one-way ANOVA, or *t*-tests (GraphPad, GraphPad Software Inc, La Jolla, CA) depending on the experimental design. A value of $p < 0.05$ was considered statistically significant. Significant between-group differences in the ANOVAs were measured using Newman–Keuls post hoc tests.

3. Results

3.1. Anatomically distinct glutamatergic inputs maintain functional independence independent of treatment

Previous studies indicate EC–BLA and IC–BLA afferents are functionally independent using *in vitro* recording techniques (Tsvetkov et al., 2004). Using paired stimuli (500 ms) at mixed (IC_1–EC_1; EC_2–IC_2) or single afferents (EC_1–EC_2; IC_1–IC_2) we tested if this independence was maintained across CIE and WD (Fig. 1). A two-way ANOVA (treatment condition vs. stimulation site) indicated significant main effects for stimulation site [$F(3,60) = 48.748$, $p < 0.05$] but not treatment condition [$F(2,60) = 1.84$, $p > 0.05$] (Fig. 1). These data coupled with no significant interaction [$F(6,60) = 1.781$, $p > 0.05$] suggests CIE and WD exposure do not impact the functional independence of EC and IC afferents.

3.2. WD increases presynaptic release probability at IC–BLA afferents

We utilized paired electrical stimulations across a range of interpulse intervals (25–100 ms) to characterize treatment effects on presynaptic function at IC–BLA inputs (Fig. 2). Previous reports