Neuropharmacology xxx (2014) 1–8

Contents lists available at ScienceDirect

Neuropharmacology

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

Glutamatergic mechanisms associated with stress-induced amygdala excitability and anxiety-related behavior

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ARTICLE INFO

Article history:
Received 26 September 2013
Received in revised form
16 April 2014
Accepted 21 April 2014

Keywords:
Stress
Basolateral amygdala
GluN1
GluK1
Glutamate
C57BL/6J

ABSTRACT

The neural factors underlying individual differences in susceptibility to chronic stress remain poorly understood. Preclinical studies demonstrate that mouse strains vary greatly in anxiety-related responses to chronic stress in a manner paralleled by differential stress-induced changes in glutamatergic signaling in the basolateral amygdala (BLA). Previous work has also shown that alterations in the amygdala gene expression of the GluN1 NMDA and the GluK1 kainate receptors are associated with stress-induced alterations in anxiety-like behavior in the C57BL/6J mouse strain. Using in vivo behavioral pharmacological and ex vivo physiological approaches, the aim of the current study was to further elucidate changes in glutamate neurotransmission in the BLA caused by stress and to test the functional roles of GluN1 and GluK1 in mediating stress-related changes in behavior. Results showed that stress-induced alterations in anxiety-like behavior (light/dark exploration test) were absent following bilateral infusion of the GluK1 agonist ATPA into the BLA. Intra-BLA infusion of the competitive NMDA antagonist AP5 produced a generalized behavioral disinhibition/locomotor hyperactivity, irrespective of stress. Slice electrophysiological recordings showed that ATPA augmented BLA GABAergic neurotransmission and that stress increased the amplitude of network-dependent spontaneous excitatory postsynaptic currents and amplitude of GABAergic miniature inhibitory postsynaptic currents in BLA. These findings could indicate stress-induced BLA glutamatergic neuronal network hyperexcitability and a compensatory increase in GABAergic neurotransmission, suggesting that GluK1 agonism augmented GABAergic inhibition to prevent behavioral sequelae of stress. Current data could have implications for developing novel therapeutic approaches, including GluK1 agonists, for stress-related anxiety disorders.

1. Introduction

Psychological stress is a major risk factor for various neuropsychiatric conditions and individuals differ in their sensitivity to stress, partly due to predisposing genetic and neurobiological factors (Caspi et al., 2010). Rodent models offer an experimentally tractable strategy for studying the effects of stress on behavior, and then delineating the mediating neural factors (Holmes and Singewald, 2013). We have previously found that following stress C57BL/6J mice showed a pattern ostensibly indicative of decreased anxiety-like behavior (i.e., more time in the light compartment of the light/dark exploration test and open arms of the elevated plus-maze). However, further analysis suggests that this stress-induced response in C57BL/6J mice can be interpreted as an anxiety-related drive to escape the apparatus or an 'active-coping' response (Mozhui et al., 2010; Uchida et al., 2011) – a hypothesis supported by the finding that the response was effectively reversed by chronic treatment with the anxiolytic fluoxetine (Ihne et al., 2012).

The neural and molecular basis of these stress-induced behavioral abnormalities currently remains unclear. The basolateral amygdala (BLA) is activated by stressors (Singewald, 2007; Whittle et al., 2010) and is a major target of chronic stress (Duvarci and Pare, 2007; Kavushansky and Richter-Levin, 2006; Maroun et al., 2013; Reznikov et al., 2009; Roozendaal et al., 2009). Gene expression analysis of multiple corticolimbic regions, including the BLA, from

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http://dx.doi.org/10.1016/j.neuropharm.2014.04.015
0028-3908/

C57BL/6J before and after chronic stress exposure revealed differential expression of multiple genes (Mozhui et al., 2010). Intriguingly, there was increased gene expression of *Grin1* (GluN1 subunit NMDA receptor) and decreased expression of *Grik1* mRNA (GluK1, formerly GluR5, kainate receptor subtype) in the BLA of C57BL/6J mice following stress.

These prior findings are of particular interest given GluN1 and GluK1 are important regulators of amygdala synaptic transmission and plasticity (Aroniadou-Anderjaska et al., 2012; Lack et al., 2008; Li and Rogawski, 1998; Wu et al., 2007), and more generally, in the context of a growing literature implicating amygdala glutamate signaling in stress-related anxiety (Cryan and Dev, 2007; Griebel and Holmes, 2013; Krishnan et al., 2007; Surget et al., 2009). The functional roles of amygdala GluK1 and GluN1 in mediating stress-induced anxiety in C57BL/6J mice are unclear, as is the precise nature of the stress-induced changes in amygdala glutamate-mediated synaptic transmission. The major aim of the current study was to address these questions using a combination of in vivo behavioral pharmacological and ex vivo electrophysiological approaches.

2. Material and methods

2.1. Subjects

Subjects were 2–3 month old male C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Following shipping, mice were acclimated to the vivarium for at least 1 week prior to experimental manipulation in a temperature- (22 ± 3 °C) and humidity- (45 ± 15%) controlled vivarium under a 12-h light/dark cycle (lights on at 06:00). Food and water were provided *ad libitum*. Separate cohorts of mice were used for the behavioral and electrophysiological experiments. All experimental procedures were approved by the NIAAA and UNC Animal Care and Use Committees, and followed the National Institutes of Health guidelines outlined in "Using Animals in Intramural Research."

2.2. Implantation of amygdala-targeting guide cannula

Mice were anesthetized with isoflurane and bilaterally implanted via stereotaxic surgery (Kopf Instruments, Tujunga, CA, USA) with 26-gauge indwelling cannulae (Plastics One, Roanoke, VA, USA) targeting the BLA, as previously described (Gunduz-Cinar et al., 2013). The coordinates were AP −1.4, ML ±3.3, DV −3.8 to −4.1. Mice were single-housed following surgery and given a 1-week recovery period before stress during which dummy cannulae were intermittently manipulated to habituate mice to handling and prevent cannula blocking.

To verify cannula placements at the completion of testing (see Fig. 1B), mice were perfused with 4% paraformaldehyde. Fixed brains were sectioned (50 μm thickness) on a vibrating microtome (American Optical 860, San Marcos, USA) and stained with cresyl violet to aid localization of cannula tips. Cases in which cannula were not bilaterally localized in the BLA were excluded. To further confirm the localization of infusion, some mice were infused with a fluorophore (BODIPY® TMR-X, SE, 5 mM in PBS, DMSO 40%, Invitrogen, Eugene, OR, USA) and brain (50 μm-thick) sections visualized with a SteREO Lumar V12, Fluorescent Microscope (Carl Zeiss Microscopy LLC, Thornwood, USA) equipped with a CY3 filter (545/25 nm excitation, 565 lpxr dichroic, and 605/70 nm emission) (see Fig. 1C).

2.3. Repeated restraint stress

Mice were placed in ventilated 50 mL Falcon tubes for 2 h per day (10:00–12:00 h) for 10 consecutive days, while non-restrained mice remained in their home cage, as previously described (Ihne et al., 2012; Mozhui et al., 2010). Body weight was measured before and after the 10-day stress period to confirm that stress led to a loss of body weight (Ihne et al., 2012; Mozhui et al., 2010).

2.4. Drug infusion and behavioral testing

One day after the final stress exposure, mice were tested on the light/dark exploration test (LDE) for anxiety-related behavior (Crawley, 1981; Yang et al., 2008) (for schematic of experimental design, see Fig. 1A). The apparatus and procedure was as previously described (Ihne et al., 2012; Mozhui et al., 2010). The apparatus was an opaque black Plexiglas shelter (39 × 13 × 16 cm) with a 13 × 8 cm aperture at floor level that opened onto a large white Plexiglas square arena (39 × 39 × 35 cm) illuminated to ~90 lux. Mice were placed halfway, head-forward, in the shelter to begin a 15-min session. The latency to first exit the shelter and enter the light compartment, the number of entries into the light compartment, the accumulated time spent in the light compartment, and the accumulated distance traveled in the light and dark compartments during the first 5 min of the session was measured by the Ethovision videotracking system (Noldus Information Technology Inc., Leesburg, VA, USA).

Mice were randomly assigned to 1 of 3 drug groups: 2.5 μg AP5 sodium salt (NMDAR antagonist), 125 pmol ATPA (GluK1 agonist), or 0.9% saline vehicle. Drug concentrations were chosen based on previous studies in rats or mice (Holmes et al., 2012; Lack et al., 2008; Walker et al., 2002). Fifteen minutes prior to testing, bilateral 33-gauge injectors (Plastics One, Roanoke, VA, USA) with 1 mm projection were lowered into the indwelling guide cannulae. A volume of 0.3 μl per hemisphere was infused via a syringe pump (Harvard Apparatus PHD 22/2000, Holliston, MA, USA) at a rate of 0.25 μl per minute. To ensure diffusion into the brain and minimize reflux, custom-modified injector needles with a 1.2–1.3 mm projection beyond the cannula tip were inserted prior to infusion to produce a small space in the tissue, and injectors were left in place for 5 min to allow for complete diffusion and then removed. Mice remained in the homecage until testing.

2.5. Slice electrophysiology

One day following the final exposure to stress, mice were sacrificed via deep isoflurane anesthesia and decapitation, and brain slices containing the BLA were prepared as previously described (Li et al., 2012; Mozhui et al., 2010). Briefly, following sacrifice, brains were rapidly removed and 300 μm slices were cut on a Leica VT1000S vibratome (Leica Biosystems, Buffalo Grove, IL, USA) in a cold (1–4 °C), sucrose-based external solution. Slices were then immediately placed in normal ACSF at 30 °C and allowed to recover for at least 1 h. Following recovery, individual slices were placed in a holding chamber and continuously perfused with normal ACSF maintained at 30 °C at a rate of 2 mL per minute. Neurons were visualized using infrared video microscopy (Olympus, Center Valley, PA, USA). Recording electrodes (3–5 MΩ) were pulled with a Flaming-Brown Micropipette Puller (Sutter Instruments, Novato, CA, USA), using thin-walled borosilicate glass capillaries. Signals were acquired by a Multiclamp 700 B amplifier (Molecular Devices, Sunnyvale, CA, USA), digitized at 10 kHz, and analyzed using Clampfit 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Input resistance and access resistance were continuously monitored throughout all experiments, and those in which changes in access resistance exceeded 20% were excluded from all data analyses.

Whole-cell voltage clamp recordings were performed to test for alterations in synaptic plasticity, as measured by AMPAR/NMDAR ratio, a putative marker for synaptic potentiation (Humeau et al., 2007), using a cesium gluconate-based internal solution [(in mM) 117 gluconic acid, 20 HEPES, 0.4 EGTA, 5 TEA, 2 $MgCl_2$, 2 QX-314, 4 Na_2ATP, 0.4 Na_2GTP, pH 7.3, 285–290 mOsmol]. Evoked NMDAR-mediated synaptic transmission was examined by placing a stimulating electrode in the dorsal part of the BLA and then measuring the relationship between stimulus intensity and peak amplitude of evoked NMDAR mediated currents. Fast synaptic transmission, which is primarily AMPAR-mediated (Christian et al., 2013), was assessed from recording pharmacologically isolated spontaneous synaptic events at a membrane potential of −70 mV, in the presence of picrotoxin.

To simultaneously record both spontaneous excitatory and inhibitory events from individual neurons, the recording configuration was altered by adjusting the intracellular solution, and then recording at the reversal potential for the opposing current, as previously described for other brain regions (Kim et al., 2013). Specifically, inhibitory postsynaptic currents (IPSCs) were recorded at +10 mV, the reversal potential for AMPAR-mediated currents, and excitatory postsynaptic currents (EPSCs) were recorded at −55 mV, the reversal potential for GABAR-mediated events, using a cesium methanesulfonate-based intracellular solution [(in mM) 135 cesium methanesulfonate, 10 KCl, 1 $MgCl_2$, 0.2 EGTA, 2 QX-314, 4 MgATP, 0.3 GTP, 20 phosphocreatine, pH 7.3, 285–290 mOsmol). To determine network-independent synaptic transmission, recordings were repeated in the presence of the sodium channel blocker tetrodotoxin, which blocks action potential firing and thereby recurrent network-driven transmission.

GluK1 signaling in the BLA has been suggested to effect anxiety-like behavior by modulating the excitability of GABAergic interneurons, based on the observation that intra-BLA injection of the GluK1 blockade increases anxiety-like behavior while application of the GluK1 agonist, ATPA, depolarizes interneurons and increases spontaneous GABAergic transmission in this region (Wu et al., 2007). To test for alterations in GluK1 modulation of BLA function, recordings were made after pharmacologically isolating GABAergic transmission by including 50 μM APV (NMDAR antagonist) and 100 μM GYKI53655 (AMPAR antagonist) in the extracellular solution. IPSCs were recorded at +10 mV using a cesium gluconate-based internal solution. Following the establishment of a stable baseline, the GluK1 agonist, ATPA, was bath applied at a concentration of 3 μM for 10 min followed by a 10 min washout period.

2.6. Statistical analysis

The effect of stress exposure and drug treatment on body weight and behavioral measures was analyzed using 2-factor analysis of variance (ANOVA). Bonferonni-adjusted post-hoc tests were used to probe significant ANOVA terms. As effects of stress and drug treatment were predicted a priori, planned t-tests were conducted to compare groups in the absence of significant ANOVA terms. Outliers were identified as cases with behavioral scores >2 standard deviations from each overall group mean, resulting in the exclusion of one case from each of the following groups: non-

Please cite this article in press as: Masneuf, S., et al., Glutamatergic mechanisms associated with stress-induced amygdala excitability and anxiety-related behavior, Neuropharmacology (2014), http://dx.doi.org/10.1016/j.neuropharm.2014.04.015