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QUALITATIVELY DIFFERENT EFFECT OF REPEATED STRESS DURING ADOLESCENCE ON PRINCIPAL NEURON MORPHOLOGY ACROSS LATERAL AND BASAL NUCLEI OF THE RAT AMYGDALA

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Abstract—Repeated stress can elicit symptoms of depression and anxiety. The amygdala is a significant contributor to the expression of emotion and the basolateral amygdala (BLA) is a major target for the effects of stress on emotion. The adolescent time period may be particularly susceptible to the effects of stress on emotion. While repeated stress has been demonstrated to modify the morphology of BLA neurons in adult rats, little is known about its effects on BLA neurons during adolescence. This study tests the effects of repeated stress during adolescence on BLA neuronal morphology, and whether these are similar to the effects of stress during adulthood. The BLA includes the basal (BA) and lateral (LAT) nuclei, which are differentially responsive to stress in adults. Therefore, effects of stress during adolescence were compared between the BA and LAT nuclei. Morphological features of reconstructed BLA neurons were examined using Golgi–Cox-stained tissue from control or repeated restraint stress-exposed rats. We found subtle dendritic growth coupled with loss of spines after repeated stress during adolescence. The magnitude and dendritic location of these differences varied between the BA and LAT nuclei in strong contrast to the stress-induced increases in spine number seen in adults. These results demonstrate that repeated stress during adolescence has markedly different effects on BLA neuronal morphology, and the extent of these changes is BLA nucleus-dependent. Moreover, altered neuroanatomy was associated with age-dependent effects of repeated stress on generalization of fear, and may point to the necessity for different approaches to target stress-induced changes in adolescents.
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Key words: amygdala, restraint stress, Golgi, morphology, spine, dendrite.

INTRODUCTION

Stress often precipitates depression and anxiety disorders (Heim and Nemeroff, 2001; Lupien et al., 2009). A history of repeated or prolonged stress often leads to hyperactivity of the amygdala in humans (Shin et al., 1997; Armony et al., 2005; Protopopescu et al., 2005; Ganzel et al., 2007; van Wingen et al., 2011; Bogdan et al., 2012; Dannlowski et al., 2012). Depression and anxiety disorders in many patients are also associated with hyperactivity of the amygdala (Drevets et al., 1992; Breiter et al., 1996; Sheline et al., 2001; Thomas et al., 2001; Siegle et al., 2002; Davidson et al., 2003). In animal models, the basolateral complex of the amygdala (BLA) has emerged as a target for the effects of stress on a range of anxiety and depressive behaviors. Furthermore, these behavioral abnormalities after repeated stress are associated with hyperactivity of BLA neurons (Adamec et al., 2005; Correll et al., 2005; Mozhui et al., 2010; Rosenkranz et al., 2010; Zhang and Rosenkranz, 2012). A major drive of BLA neuronal activity derives from excitatory synaptic inputs to BLA neurons. Previous studies demonstrate increased excitatory drive to BLA neurons after repeated stress in adult rodents (Mozhui et al., 2010; Padival et al., 2013a; Hubert et al., 2014; Suvrathan et al., 2014), consistent with an increase of excitatory synaptic input to BLA neurons after repeated stress. Morphological correlates of increased excitatory synaptic inputs to BLA neurons have been observed in the form of increased dendritic spines and dendritic length after repeated stress (Vyas et al., 2002, 2006; Mitra et al., 2005; Hill et al., 2011, 2012; Adamec et al., 2012).

The adolescent amygdala may be differentially vulnerable to the effects of stress compared to adults, resulting in a different cascade of changes in BLA physiology in adolescent rats (Zhang and Rosenkranz, 2012; Hetzel and Rosenkranz, 2014). Repeated stress increases the firing rate of BLA neurons in adult rats, but increases the number of active neurons in adolescent rats (Zhang and Rosenkranz, 2012). Moreover, increases in membrane excitability are seen across principal neurons of the BLA following stress exposure, but more selective effects are seen in the adult BLA (Hetzel and Rosenkranz, 2014). This parallels with distinct behavioral effects of stress across age (Stone and Quartermain, 1997; Spear, 2000; Luine et al., 2007; Toledo-Rodriguez and Sandi, 2007; Eiland et al., 2012; Zhang and Rosenkranz, 2013). One possible cause for this age-dependency is different effects

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Abbreviations: ANOVA, analysis of variance; BA, basal nucleus of the amygdala; BLA, basolateral area of the amygdala; EPSP, excitatory postsynaptic potential; LAT, lateral nucleus of the amygdala; P, postnatal day.

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of repeated stress on morphology of BLA neurons. The purpose of this study was to determine whether repeated stress causes morphological changes in the adolescent BLA that are distinct from changes in the adult BLA.

The BLA is composed of several nuclei, including the basal (BA) and lateral (LAT) nuclei. The BA and LAT have complementary, but distinct roles in affective behavior. Previous studies demonstrate spinogenesis and dendritic hypertrophy of BA and LAT neurons in adult rats exposed to repeated stress. However, the pattern of these morphological changes is different between the BA and LAT neurons (Nietzer et al., 2011; Padival et al., 2013b). Little is known about the effects of repeated stress on the morphology of BLA neurons in adolescence. Therefore, the current study examines and contrasts the pattern of changes in the BA and LAT nuclei of the BLA in adolescents. Changes in dendritic structure and spine number are associated with changes of excitatory drive, and were the focus of this study. Neuronal morphology was reconstructed after Golgi–Cox staining, and BA and LAT principal neuronal dendrites and spines were quantified. These morphological indices were compared between control and repeated restraint stress-exposed adolescent male rats. A change in the number of spines may impact the selectivity of the response to an afferent glutamatergic signal. Previous studies demonstrate that acuity of excitatory input to the BLA determines specificity of fear behavior (Shaban et al., 2006; Antunes and Moita, 2010). Therefore, the effects of stress on fear generalization were also compared between adolescent and adult male rats.

EXPERIMENTAL PROCEDURES

All experiments were approved by the Institutional Animal Care and Use Committee of the Rosalind Franklin University (protocol #11–47 and 13–10), and followed The Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Efforts were made to minimize animal suffering and to reduce the number of animals used. A portion of the data from adult rats from a previously published study (Padival et al., 2013b) was collected in a parallel manner with the current study, and was submitted to new analysis for comparison to adolescent rats in this study.

Subjects

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were group housed (2–3/cage) in a controlled climate animal facility. The housing room had a 12:12 light:dark schedule and food and water were available *ad libitum*. After 1 week of habituation to the animal facility [postnatal day (P) 25–31], restraint stress was performed in a procedure room by placement of a rat in a restraint hemi-cylinder for 20 min per session. A control group was handled in the same manner as the restraint group, except they remained in a transparent transport cage with bedding in the housing room, instead of placement in a restraint cylinder. Rats were stressed or control handled for seven out of nine consecutive days (P32–40). This pattern has been shown to maximize the effects of repeated restraint on adrenal gland weight, anxiety-like behavior, and electrophysiological changes in the BLA (Zhang and Rosenkranz, 2012; Zhang et al., 2014). In addition, this pattern was chosen to conform to previous morphological studies in adult rats. One day after the final treatment (P41), rats were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and decapitated. The brains were rapidly removed for Golgi–Cox staining. The adult rats in this study were exposed to control or repeated restraint procedures in the exact same manner as the adolescent rats, between P63 and P75.

Golgi–Cox stain

Golgi–Cox staining of the brain tissue was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Columbia, MD, USA), following the protocol suggested by the manufacturer. After removal of the brain, the brain was blocked and placed immediately into Golgi impregnation solution in an opaque container, protected from light. The impregnation solution was changed after 24 h and brains were stored in the dark for 15–18 days at room temperature. Brain tissue was then transferred to solution C of the FD Rapid GolgiStain kit for 24 h at 4 °C. After 24 h the solution was replaced with fresh solution C, and the brain was stored at 4 °C for 7 days. Brains were sectioned (100-μm thickness, Leica SM 2000 R microtome), and slices were collected in 20% sucrose in 0.1 M phosphate buffer (pH 7.4) at room temperature. Slices were mounted on gelatinized slides and air dried (25 min–1 h), then rinsed in double-distilled H_2O (two times, 4 min each rinse). Slides were dehydrated in 50%, 75% and 95% ethanol for 4 min each, then in 100% ethanol four times for 3 min each. Slides were cleared with xylene (three times, 4 min each), then coverslipped with Permount and were allowed to dry overnight.

Neuronal reconstruction

Golgi-stained neurons from LAT and BA were reconstructed using Neurolucida software (MBF Bioscience, Williston, VT, USA) under bright field illumination with the 100× objective of a Nikon Eclipse E400 microscope. LAT and BA were defined based on previously established borders (Swanson and Petrovich, 1998; Paxinos, 2004). Some sections of Golgi-stained tissue were lightly counterstained with Nissl to determine LAT and BA borders based on fiber tracts and structural landmarks. Only neurons that appeared to be completely filled were utilized. Secondary and tertiary dendrites, as well as spines, had to be visible. In addition, there could be no breaks (> 5 μm) in the dendrites. Neurons were selected based on morphology consistent with BLA principal neurons (e.g., obvious primary dendrites and spines; McDonald, 1982). Reconstructions were performed by an individual that was blind to treatment conditions. Aspiny neurons that displayed small somata with few dendrites or large somata with bipolar primary dendrites were not included in this analysis. Dendritic branching was

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