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REPEATED RESTRAINT STRESS INCREASES BASOLATERAL AMYGDALA NEURONAL ACTIVITY IN AN AGE-DEPENDENT MANNER

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Abstract—Chronic stress is a precipitating factor for affective disorders such as depression and anxiety. This is associated with the effects of chronic stress on the amygdala. Adolescents may be more vulnerable to the effects of chronic stress, which may be related to its impact on amygdala function. However, the stress-induced changes in amygdala neuronal activity, and the age-dependent impact of chronic stress on amygdala neuronal activity have not been studied in depth. In this study, we investigated how repeated restraint impacts basolateral amygdala (BLA) projection neuron activity in both adolescent and adult rats. Using *in vivo* extracellular recordings from anesthetized rats, we found that repeated restraint increased the number of spontaneously firing neurons in the BLA of adolescent rats, but did not significantly increase the firing rate. In contrast, repeated restraint increased the firing rate of BLA neurons in adult rats, but did not change the number of spontaneously firing neurons. This is the first direct evidence of how stress differently impacts amygdala physiology in adolescent and adult rats. These findings may shed light on the mechanism by which chronic stress may age-dependently precipitate psychiatric disorders. Published by Elsevier Ltd. on behalf of IBRO.

Key words: chronic stress, repeated restraint, adolescent, amygdala, extracellular electrophysiology.

INTRODUCTION

Chronic stress can induce changes in affective behaviors, including emotion and associative learning (Shors, 2004; Teicher et al., 2006). Chronic stress is associated with the development of affective disorders such as anxiety and depression (Heim and Nemeroff, 2001; Hammen, 2005). The amygdala, in addition to playing a pivotal role in processing emotional information, modulates the stress responses (Davis et al., 1994; Feldman et al., 1995; Herman and Cullinan, 1997; Van de Kar and Blair, 1999). However, the amygdala itself undergoes structural changes when subjected to chronic stress, such as increased dendritic branching and increased number of spines in BLA projection neurons (Vyas et al., 2002; Mitra et al., 2005). This modification of amygdala structural properties is accompanied by increased emotional reactivity (Wood et al., 2008) and enhanced amygdala-dependent affective behaviors, such as fear conditioning to discrete cues (Conrad et al., 1999; Toledo-Rodriguez and Sandi, 2007) as well as enhanced anxiety-like behaviors (Conrad et al., 1999) in experimental animals. Human imaging studies indicate amygdala hyperactivity and hyper-responsiveness in people that experienced chronic stress, such as combat veterans and abused women and children (Rauch et al., 2000; Protopopescu et al., 2005; Bremner et al., 2008), as well as patients with major depression (Drevets et al., 1992; Frodl et al., 2002). All this evidence suggests that chronic stress contributes to abnormal affective behaviors and possibly psychiatric disorders via its effect on the amygdala. The enhanced emotional reactivity after chronic stress may be driven by increased neuronal activity of projection neurons, the main efferent neurons of the amygdala. While there is much known about the impact of acute stressors on BLA physiology (e.g. Shors, 1999; Vouimba et al., 2004, 2006; Pelletier et al., 2005; Kavushansky and Richter-Levin, 2006; Isoardi et al., 2007; Karst et al., 2010), much less is known about the effects of chronic stressors on BLA physiology. Several studies indicate that chronic stress, stress exposure repeated at least 3 times, or chronic treatments that may mimic the effects of stress, lead to increased amygdala neuronal activity through mechanisms that include increased excitability, reduced inhibition, and inappropriate modulation by monoamines (Braga et al., 2004; Correll et al., 2005; Buffalari and Grace, 2009; Jiang et al., 2009; Patel et al., 2009; Mozhui et al., 2010; Rosenkranz et al., 2010). Understanding how chronic stress affects amygdala neuronal physiology will shed light on the pathophysiology of stress-induced psychiatric disorders.

Adolescence is a critical period for brain development, characterized by neuro-anatomical rearrangements (Spear, 2000; Sisk and Foster, 2004; Romeo et al., 2006). In humans, adolescence is accompanied by an increased incidence of affective disorders (Kessler and Avenevoli, 2001; Merikangas et al., 2010). Given the

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Abbreviations: ANOVA, Analysis of Variance; BL, basal nucleus; BLA, basolateral amygdala; CRF, corticotropin-releasing factor; EEG, electroencephalogram; EPM, elevated plus maze; HPA axis, hypothalamic–pituitary–adrenal axis; K–S, Kolmogorov–Smirnov; LAT, lateral nucleus; mPFC, medial prefrontal cortex; PND, postnatal day.

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large effect of stress hormones on brain development, it is not surprising that brain regions undergoing maturation, such as the amygdala, are susceptible to stress during adolescence. Adolescent rodents display greater body weight loss and reduction of open arm exploration in the elevated plus maze (EPM) as well as greater cue specific fear conditioning in response to different stressors compared to adult rodents (Stone and Quartermain, 1997; Toledo-Rodriguez and Sandi, 2007). Stress exposure during adolescence may cause greater physiological disturbances than exposure during adulthood, which may lead to age-dependent differences in affective behaviors. In this study, repeated restraint was used to model the effects of chronic stress. We hypothesized that chronic stress exerts greater effect on amygdala neuronal activity in adolescent rats compared to adult rats. Uncovering the age-dependent effect of chronic stress on amygdala neuronal activity will increase our understanding of the age-dependent impact of stress on psychiatric conditions that involve abnormal amygdala function, and may produce age-appropriate preventative and curative measures for stress-induced psychiatric disorders.

Within the amygdala, BLA is the major afferent interface that receives information from all sensory modalities (Turner and Herkenham, 1991; McDonald, 1998). Previous work done in our lab has shown that repeated restraint resulted in BLA projection neuron hyper-excitability in adult rats, which may lead to increased activity of projection neurons (Rosenkranz et al., 2010). In this study, we used *in vivo* extracellular electrophysiological recordings to test if repeated restraint exerts a greater impact on BLA projection neuron activity in adolescent rats compared to adult rats.

EXPERIMENTAL PROCEDURES

Ethical approval

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science.

Materials

Urethane, Cresyl Violet and sucrose were purchased from Sigma (St. Louis, MO). Pontamine Sky Blue was purchased from Alfa Aesar (Ward Hill, MA). NaCl and formaldehyde were purchased from Fisher Scientific (Pittsburgh, PA).

Animals and restraint protocol

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) arrived at postnatal day (PND) 21 and PND 53–58 for adolescent and adult rats respectively. They were housed 2 or 3 per cage in the Rosalind Franklin University animal facility with free access to food and water, and maintained on a 12-h light/dark cycle. To model the effects of chronic stress, a 7-day repeated restraint protocol was used. Animals of the same age were randomly assigned into 4 groups: non-restraint group, two different 1-day restraint groups, and the repeated restraint group. After habituating to the animal facility for at least 4 days, rats were subjected to stress or control handling. Rats in the repeated restraint group were placed into a hemi-cylinder restraint tube 20 min/session, 1 session/day for 7 out of 9 days in the procedure room (Rosenkranz et al., 2010). The restraint tube was an acrylic cylinder with flattened bottom (dimensions dependent on animal size: rats 30–125 g were placed in a cylinder 5 in. × 2 in., rat 125–250 g in a cylinder 6 in. × 2.5 in., and rats 250–500 g in a cylinder 8 in. × 3.25 in.). Rats in the non-restraint group were placed into a clear Plexiglas transportation cage 20 min/session, 1 session/day for 7 out of 9 days. All the procedures were performed between 8:00 am and 3:00 pm, during the lights on cycle. To assess the additive nature of repeated restraint, we also included two control 1-day restraint groups. Rats in 1-day restraint B group (B ∼ 1 day *B*efore the behavior test) were handled the same way as non-restraint rats except they were subjected to restraint on the last day of this procedure. Rats in 1-day restraint F group (F ∼ *F*irst day of the restraint protocol) were subjected to restraint on the first day of the procedure and then handled identically to non-restraint rats during the remaining 8 days.

Elevated plus maze

To validate the effectiveness of our repeated restraint protocol, we tested animals in the EPM one day after the final restraint/control handling session. Two sets of EPMs designed specifically for animals of different ages were used in this study. The EPM (Scientific Designs, Pittsburgh, PA) consisted of four arms: two open arms (width × length: small maze 4 in. × 15 in.; big maze 5 in. × 20 in.) and two closed arms (width × length × wall height: small maze 4 in. × 15 in. × 14 in.; big maze 5 in. × 20 in. × 18 in.). Each arm was attached to a sturdy leg, elevated 32 in. from the ground. Animals were placed at the junction of four arms, facing the open arm opposite the experimenter. Animal behavior was recorded for 5 min and analyzed by a personal computer (Dell E6500) running video-tracking software (Any-Maze, Stoelting, Wood Dale, IL). The time spent on open arms was measured and used as index of anxiety-like behavior. In addition, the number of closed arm entries was measured and used as an indicator of locomotor activity.

In vivo extracellular recording

To examine the neuronal activity of BLA projection neuron, we used *in vivo* extracellular electrophysiological recording. One day after the EPM behavioral test, rats were anesthetized with urethane (1.5 g/kg dissolved in 0.9% saline, i.p.) and placed on a stereotaxic device (Stoelting, Wood Dale, IL). Their body temperature was monitored via a rectal temperature probe, and maintained at 36–37 °C using a heating pad with a temperature controller (Model TC-1000, CWE Inc, Ardmore, PA). The amygdala was localized using a stereotaxic atlas (Paxinos and Watson, 1998). The coordinates used for amygdala centered on 4.8–5.5 mm lateral from midline, 2.5–3.8 mm caudal from bregma for adult rats. Coordinates were adjusted for adolescent rats according to the measured distance between bregma and lambda. Burr holes were drilled on the skull bilaterally at locations overlying the BLA. The left hole was used for fixing a screw for electroencephalogram (EEG) recording. The dura from the right hole was removed. Single-barrel electrodes were constructed from glass pipettes (World Precision Instruments, Sarasota, FL), and pulled using a vertical microelectrode puller (PE-2; Narishige, Tokyo, Japan), and broken under a microscope to produce a tip 1–2 μm in diameter. The electrode was filled with 2% Pontamine Sky Blue in 2 M NaCl and then slowly lowered into the amygdala via a hydraulic microdrive (Model MO-10, Narishige, East Meadow, NY). Recordings began no earlier than 45 min after surgery.

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