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Research report

Repeated restraint stress enhances cue-elicited conditioned freezing and impairs acquisition of extinction in an age-dependent manner

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HIGHLIGHTS

- Repeated restraint enhanced conditioned freezing in both adolescent and adult rats.
- Repeated restraint impaired extinction of fear conditioning in adolescent rats.
- Repeated restraint has age-dependent effects on auditory fear conditioning.

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ABSTRACT

Affective disorders are believed to involve dysfunction within the amygdala, a key structure for processing emotional information. Chronic stress may contribute to affective disorders such as depression and anxiety via its effects on the amygdala. Previous research has shown that chronic stress increases amygdala neuronal activity in an age-dependent manner. However, whether these distinct changes in amgydala neuronal activity are accompanied by age-dependent changes in amygdala-dependent affective behavior is unclear. In this study, we investigated how chronic stress impacts amgydala-dependent auditory fear conditioning in adolescent and adult rats in a repeated restraint model. We found that repeated restraint enhanced conditioned freezing in both adolescent and adult rats. But repeated restraint led to impaired acquisition of fear extinction only in adolescent rats. Along with previous findings, these results suggest that chronic stress may precipitate affective disorders via differential mechanisms, with different outcomes at different ages.

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1. Introduction

Changes in mood and impaired cognition are characteristic of affective disorders. Chronic stress contributes to the recurrence and exacerbation of affective disorders such as depression and post-traumatic stress disorder (PTSD) [1–3]. The amygdala plays an important role in interpretation and expression of affect, especially those related to fear [4–7]. The amygdala is particularly vulnerable to the effects of stress [8,9] and contributes to stress-related affective disorders. Animal studies shown that chronic stress results in amygdala hypertrophy [10,11] and hyperactivity [12,13], which are accompanied by enhanced anxiety state measured in the elevated plus maze (EPM) [13–15] and fear behavior measured by conditioned freezing [16,17]. Human studies reported similar amygdala hypertrophy and hyperactivity in patients with affective disorders [18–21]. These findings all suggest that chronic stress precipitates affective disorders via modification of amygdala function. Therefore, understanding how chronic stress impacts amygdala physiology and amygdala-dependent behaviors is important for uncovering the pathophysiology of stress-related affective disorders. However, stress during adolescence may exert unique and particularly disruptive effects.

Adolescence is characterized by emergence of specific social and cognitive behaviors [22–24], and is therefore difficult to pinpoint. However, puberty occurs during adolescence, and is associated with distinct measurable biological changes. Puberty in male Sprague-Dawley rats is typically near postnatal day (PND) 42, and this was used as a conservative point to limit the study of adolescence in our study. The emergence of adolescence has been estimated as early as weaning (typically PND 20–21). Here, we used a conservative point of PND 28 [23] when elevation of gonadal hormones begins [25]. Adulthood is defined as sexual maturity, and typically occurs by PND 60 in male rats [23,26]. Similar to these

Abbreviations: ANOVA, Analysis of Variance; BLA, basolateral amygdala; CRF, corticotropin-releasing factor; EPM, elevated plus maze; HPA axis, hypothalamic-pituitary-adrenal axis; Mpfc, medial prefrontal cortex; PND, postnatal day; PTSD, post-traumatic stress disorder.

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studies, adult rats were PND 65 at the beginning of experiments, several days after sexual maturity in our study.

Our previous study has shown that repeated restraint stress leads to increased number of spontaneously active BLA neurons in adolescent rats but results in increased firing rate of individual BLA neurons in adult rats [27]. It is unclear if this age-dependency of the effects of repeated restraint on BLA neuronal activity occurs in parallel with differences in BLA-dependent behaviors. One classic behavior that reflects BLA function is cued fear conditioning [7,28,29]. A short (3 days) course of stress during adolescence had no effect on acquisition of conditioned fear, but increased conditioned freezing during testing [30]. However, the purpose of that study did not include comparison between adolescent and adult rats, nor to examine extinction of conditioned freezing. To test whether repeated stress exerts different effects on fear conditioning in different age groups, we examined the impact of repeated restraint on amygdala-dependent auditory fear conditioning in adolescent and adult rats. Freezing during acquisition was measured, as well as conditioned freezing and acquisition of extinction one day after acquisition to determine if repeated restraint stress exerts age-dependent effects on fear conditioning.

2. Material and methods

2.1. Animals

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. Adolescent and adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used in this study. 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 (light cycle from 7:00 a.m. to 7:00 p.m.). Adolescent rats arrived at the animal facility at PND 25 with an approximate body weight of 60–70 g. They were habituated in the facility before starting the restraint or control handling protocol which began at PND 29 (approximate body weight 70–80 g), and included the subsequent 9 days. Adolescent rats were PND 39 for fear conditioning with an approximate body weight of 130–160 g. Adult rats arrived at PND 58 with an approximate body weight of 260–280 g. They were PND 65 at the initiation of restraint or control procedures. Adult rats were PND 76 with an approximate body weight of 320–350 g on the day of fear conditioning.

2.2. Repeated restraint protocol

To model the effects of chronic stress, a 7-day repeated restraint protocol was used [13]. Age-matched animals were randomly assigned into non-restraint and repeated restraint groups. After habituating to the animal facility for at least 4 days, rats were subjected to restraint 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 [13]. This specific design (Fig. 1) reduces habituation to restraint, which would otherwise be significant [31,32]. The restraint tube was an acrylic cylinder with flattened bottom (3 different sizes of restraint tubes were used, depending on the size of the rat). All rats that were placed into restraint cylinders were securely immobilized, as determined by inability to turn around, but retaining restricted movement of head and limbs. If any rat displayed evidence of being too loosely secured (as demonstrated by ability to turn lower or upper body) or too tightly secured (as demonstrated by inability to move head), the position of the securing door was changed. 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 (Fig. 1). All the procedures were performed between 8:00 a.m and 3:00 p.m., during the light phase of the light/dark cycle. To assess the additive nature of repeated restraint, two control 1-Day restraint groups were added. 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. Rats were run in a manner that counterbalanced age and stress groups over the course of the study.

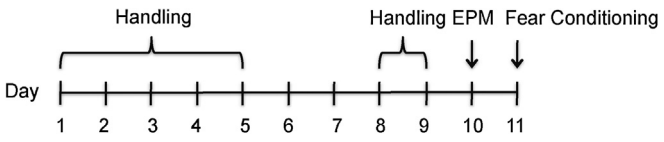

Fig. 1. Schematic of experimental design. Rats were exposed to control handling (or restraint stress) for 5 days. This was followed by 2 days with no manipulation, and another 2 days of handling (or restraint stress). This design decreases habituation to restraint stress.

2.3. Elevated plus maze

To verify 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 used for adolescent rats was a scaled-down version of the EPM used for adults. The EPM was scaled down based on average crown-to-rump body length. This approach was used by other labs, and confirmed in those labs by measurement of gait width [e.g. 33]. The EPM (Scientific Designs, Pittsburgh, PA) consisted of four arms: two open arms (width × length: small maze 3.25 in. × 14.75 in.; big maze 4.25 in. × 19.75 in.) and two closed arms (width × length × wall height: small maze 3.25 in. × 14.75 in. × 14 in.; big maze 4.25 in. × 19.75 in. × 18 in.). Each arm was attached to a sturdy leg, elevated 32 in. from the ground. The EPM test was conducted as described previously [13,27]. 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 an index of anxiety-like behavior. In addition, the total number of arm entries was measured and used as an indicator of locomotor activity

2.4. Fear conditioning

Fear conditioning in this study was a two-day procedure. Conditioning and testing were performed in different plexiglass chambers with distinct contexts (wall pattern and color, odors, and flooring) to minimize contextual freezing. Each chamber was enclosed by a sound-attenuating cabinet (UGO Basile, VA, Italy). Two sound attenuated cabinets were used, one for conditioning and one for testing (same dimensions: 21 in. × 17.5 in. × 21.3 in. height). The two cabinets were in the same room. The conditioning chamber measured 10.6 in. × 10.6 in. × 14.1 in. height. The testing chamber measured 13.5. in. × 10 in. × 12 in. height. Mounted inside each cabinet were an audio speaker (UGO Basile, VA, Italy), a house light, an infrared LED light and a ceiling mounted digital camera that was sensitive to light in the IR range (Fire-i, Unibrain, San Ramon, CA) which was connected to a personal computer (Dell E6500) running video-tracking software (Any-Maze, Stoelting, WI) that detects and records behavior. Conditioning consisted of 2 min habituation followed by 5 pairings of a neutral tone (10 s, 1500 Hz, 85 dB) with a footshock (1 s, threshold intensity; see below) that co-terminated with the tone. Conditioning trials were presented at 60 s intertrial intervals. Rats remained in the chamber for 1 min after the end of last conditioning trial, and were then returned to their home cage. The next day, conditioned freezing and its within session extinction were tested in a contextually distinct chamber. The testing consisted of a 2 min habituation followed by 15 trials of tone presentation (20 s, 1500 Hz, 85 dB) at a 60 s intertrial interval. No footshock was presented during testing trials. After testing, animals were returned to their home cage.

To determine threshold intensity of footshock for fear conditioning, footshock was delivered in 0.1 mA increments from 0.2 mA (0.2 mA, 0.3 mA, 0.4 mA) to each animal immediately before the fear conditioning procedure. The same individual animal experienced this threshold procedure, followed by fear conditioning using its threshold footshock intensity. In this study, threshold intensity was defined as the footshock intensity which leads to forepaw withdrawal (adolescent rats typically 0.4 mA; adult rats typically 0.3 mA; see Results for further detail). A previous study demonstrated that most rats had no obvious response to 0.1 mA footshock on our apparatus [17]. Therefore in the current study, we tested intensities starting at 0.2 mA, and increased the intensity in 0.1 mA increments until forepaw withdrawal was observed. Thus, each rat received only one footshock at/near the forepaw withdrawal threshold, and a total of 1–3 footshocks at subthreshold intensities. Freezing was quantified by the software based on a threshold of change in video image pixels. A freezing episode had to last a minimum of 2 s to be included in the software analysis. These criteria were compared against visually-confirmed freezing (behavioral immobility except for movement associated with respiration). Total freezing during each trial (entire 60 s) was used as an index of conditioned fear and converted to a percentage ([time of freezing/60 s] × 100) for analysis. The first trial was planned for comparison. However, in these experiments the freezing during the first trial was sub-maximal. There was no significant difference in the freezing response in the first trial in both age groups (adolescent: non-restraint 55.73 ± 6.42%, $n = 14$ rats; repeated restraint 62.22 ± 6.49%, $n = 15$ rats; $t = 0.71$, df = 27, $p = 0.48$; adult: non-restraint 31.89 ± 4.48%, $n = 15$ rats; repeated restraint 38.57 ± 5.72%, $n = 14$ rats; $t = 0.93$, df = 27, $p = 0.36$, unpaired t test). In addition to the first trial, the majority of rats in all groups still displayed significant amount of freezing response (more than 30%) in trial 2–5. Therefore, the initial 5 trials were used to confirm the initial conditioned freezing during extinction. The last 5 trials were used to assess the later freezing during the extinction phase.

To examine whether increased footshock intensity could lead to resistance to acquisition of fear extinction in adult rats, a separate non-restraint and repeated