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Repeated stressor exposure enhances contextual fear memory in a beta-adrenergic receptor-dependent process and increases impulsivity in a non-beta receptor-dependent fashion

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Abstract

Memory formation is promoted by stress via the release of norepinephrine and stimulation of beta-adrenergic receptors (β -ARs). Previous data demonstrate that repeated stressor exposure increases norepinephrine turnover and β -AR signaling within the amygdala, which led to the hypothesis that some stress-induced behavioral changes are likely due to facilitated associative learning. To test this, Fischer rats were exposed to chronic mild stress for four days. On day 5, subjects (including non-stressed controls) were injected with the beta-blocker propranolol or vehicle prior to conditioning in an operant box (animals receive two mild foot shocks) or passive avoidance apparatus (animals received a foot shock upon entry into the dark chamber). Twenty-four hours later, subjects were returned to the operant box for measurement of freezing or returned to the passive avoidance apparatus for measurement of latency to enter the dark chamber. Subjects were also tested in an open field to assess context-independent anxiety-like behavior. Animals exposed to chronic stress showed significantly more freezing behavior in the operant box than did controls, and this exaggerated freezing was blocked by propranolol during the conditioning trial. There was no effect of stress on behavior in the open field. Unexpectedly, retention latency was significantly reduced in subjects exposed to chronic stress. These results indicate that chronic exposure to stress results in complex behavioral changes. While repeated stress appears to enhance the formation of fearful memories, it also results in behavioral responses that resemble impulsive behaviors that result in poor decision-making.

Keywords

chronic mild stress; norepinephrine; propranolol; associative learning; contextual fear conditioning; passive avoidance

1. Introduction

Exposure to life stressors is a risk factor for development of psychopathological disorders including post-traumatic stress disorder (PTSD). One of the major diagnostic criteria for PTSD is the presence of recurrent distressing memories. In fact, the aim of behavioral treatments such as exposure therapy is to reduce the emotional and physiological responses

associated with aversive memories. Acute exposure to severe stressors, or repeated exposure to chronic mild stressors (CMSs) can both result in enhanced anxiety-like behaviors; however, it has not been well documented to what extent behavior changes are a result of enhanced associative learning processes.

Exposure to acute stress prior to classical conditioning trials facilitates learning and the formation of the memory [1], [2]. Classical learning is the process of pairing a neutral cue (conditioned stimulus) with an aversive, unconditioned stimulus such that the conditioned stimulus alone elicits a response [3]. For example, exposure to tail shock prior to the pairing of white noise with a brief periorbital shock results in significantly more conditioned eyeblink responses when the white noise is presented alone during testing [4]. Most evidence points to the release of norepinephrine (NE) and stimulation of central beta-noradrenergic receptors (β -ARs) within the amygdala as the mechanism by which stressor exposure facilitates memory formation. Evidence for this is phenomenon is quite extensive and is summarized in multiple reviews [5]–[7]. It has also been shown that administration of β -AR agonists directly into the basolateral amygdala (BLA) during training trials increases memory retention while blockade of β -ARs in the BLA reduces memory formation [8]–[12]. Exposure to restraint stress enhances subsequent long-term potentiation (LTP) in the BLA pathway induced *in vitro*, which can be prevented by prior administration of a β -AR antagonist [13]. While glucocorticoids released by the adrenal cortex during times of stress contribute to enhanced memory formation, their effects largely appear to be mediated through augmenting adrenergic signaling [10], [14]–[17]. Together, these data suggest β -ARs are involved in a common final pathway in memory consolidation.

Repeated exposure to mild stressors enhances NE turnover in the amygdala [18]; however, the effect this has on behavioral tests dependant on associative learning has not been fully determined. We previously published that some behavioral changes (e.g. decreased exploratory behavior, social withdrawal) following exposure to CMS can also be attributed to distal environmental cues (lighting, ambient temperature, and olfactory cues in the procedural room). This is significant because others have published that chronic stress does not induce anxiety-like behaviors in an open field test or elevated-plus maze [19]–[21] but does enhance freezing behavior in a contextual fear-conditioning test [1].

Here, we tested the hypothesis that exposure to CMS would alter behavioral responses in tasks that depend on classical fear conditioning. Undisturbed home cage control (HCC) and CMS animals were tested in two behavioral tests that require associative learning (e.g. contextual fear conditioning in an operant box and retention latencies in a passive avoidance test) or in a third behavioral test that is not associative learning dependant (e.g. open field). We predicted that animals exposed to CMS would show increased freezing behavior in an operant box and increased retention latencies in a passive avoidance apparatus following conditioning, but no anxiety-like behaviors in the open field test. We also tested whether administration of propranolol (a β -AR antagonist) prior to conditioning trials in the operant box and passive avoidance chamber would prevent or attenuate chronic stress-induced behavioral changes.

2. Methods

2.1. Subjects

Thirty-two Fischer-344 rats were purchased from Harlan Laboratories (Indianapolis, IN), weighing approximately 200–262g ($\bar{x}$ = 230.1g ; σ = 14.5g) at baseline. Animals were housed individually in clear Plexiglas habitats (40×20×20cm), with food and water available *ad libitum*, and kept on a 12:12 light/dark cycle, with lights on at 07:00h. Upon delivery, rats spent the first seven days becoming acclimated to the environment and were then handled for five additional days prior to the commencement of the study. Studies were conducted in accordance with the guidelines of the PHS Guide to the Care and Use of Laboratory Animals, and approved by the Kent State University Institutional Animal Care and Use Committee.

2.2. Repeated stress protocol

Subjects were exposed to a series of stressors following an established protocol [23], or were left undisturbed as HCC animals. This four day repeated stress paradigm was chosen since we previously demonstrated that it results in increased NE turnover in the amygdala, sensitized β -AR mediated responses, and enhanced fear conditioning to distal contextual cues [23]. On the morning (08:00 – 10:00) of day 1, CMS rats were placed in DecapiCone rodent restrainers (Braintree Scientific, Inc., Braintree, MA) for 60min, before being returned to their home cage. At 15:00h, food was removed from CMS rat cages for 18h. On day 2, CMS subjects were placed in novel habitats, each with a piece of filter paper containing 35 μ L trimethylthiazoline (a component of fox feces) to simulate predator odor [22] (Contech Enterprises, Victoria, BC). Rats were then placed back in their home cages but were housed in constant light conditions overnight. On the morning of day 3, animals were exposed to restraint stress again for 60min, then at 15:00h their bedding material was dampened with approximately 1500 mL tap water. On day 4, subjects were exposed to forced swim for 5min in glass cylinders measuring 49×18.7cm (inner height and diameter, respectively) filled approximately to the 37.5cm line with water at a temperature of 21°C. Following this task, subjects were placed in cages containing dry bedding. At 15:00h, water bottles were removed from CMS home cages for 18h and returned on the following morning. Following CMS, and prior to behavioral and physiological observations, CMS group weights were recorded.

2.3. Behavioral testing

Behavioral assays were performed to test associative learning (e.g. contextual fear conditioning and passive avoidance) and generalized anxiety (e.g. open field), with tests occurring 2h apart. The order of tests was counterbalanced to protect against possible order effects. A second cohort of animals was tested in a passive avoidance task only.

2.3.1. Conditioned fear—One day following the stress protocol (day 5), subjects (n =32) were placed in an 8.5"×8.5"×11" (21.59×21.59×27.94cm) operant box (Lafayette Instrument Company, Lafayette, IN) with a floor consisting of a series of electrically conductive steel bars; after two minutes, animals received two foot shocks (1.5mA for two seconds), the second shock administered two minutes after the first. One minute following the second foot

shock, animals were removed from the box and placed back into their home cages. Twenty-four hours later, subjects were placed back into the operant box and behavior was recorded for 15min and freezing behavior was evaluated. Freezing behavior was defined as complete immobility, save for movements necessary for respiration. Scoring was performed by a trained researcher blind to group assignment. Scores were obtained by checking the video every ten seconds for fifteen minutes, and one point was assigned for each instance of freezing behavior, with a maximum possible score of 90pts. Points were not assigned if at any time point within the ten second interval a subject showed any additional voluntary movement beyond what was required for respiration.

2.3.2. Open field—Subjects were placed in a 60×60×60cm Plexiglas box and spontaneous motor activity was recorded using a ceiling mounted video camera for 10min. Movements within the open field were evaluated using EthoVision XT tracking software (v8.0, Noldus Information Technology, Wageningen, NL). Total time spent in the center section of the open field was measured with lower values indicating an increase in anxiety-like behavior; measurements of locomotion -- distance traveled and time spent in motion -- were also recorded. The parameters of the open field were defined by creating a 10×10 grid (100 subdivisions) on the space within the viewport in the tracking software; the inner 64% of these subdivisions (8×8) was designated as the center section.

2.3.3. Passive avoidance—A second group of animals ($n=32$) were tested in a passive avoidance apparatus. The apparatus consisted of two chambers: an illuminated, transparent “safe” compartment and a darkened, opaque “shock” compartment. The compartments were divided by a wall with a door that closed upon entry into the opposing chamber. Both chambers sat atop a floor constructed of electrically conductive steel bars; the floor was tilted in the center, which triggered the closing of the door between the chambers when the subject had fully cross the threshold. On day 5, subjects were placed in the “safe” compartment of the apparatus. After a 20s delay, the door to the dark chamber was opened, and the subject was allowed to freely explore the apparatus. Once the subject fully entered the dark chamber (four paws and tail inside the compartment), the door closed and after a two-second delay, the subject received a 1.5mA foot shock for two seconds. One minute following the foot shock, subjects were removed from the apparatus and placed back into their home cages. Twenty-four hours later, subjects were returned to the apparatus and once again placed in the safe compartment. The latency for the subject to enter the dark chamber with all four paws was recorded. If the subject did not cross into the dark chamber after a maximum time of 538s (8'58"), the door closed automatically and the test was terminated.

2.4. Beta-adrenergic receptor antagonist administration

1h prior to contextual fear training, subjects were given an intraperitoneal (i.p.) injection of either a β -adrenergic receptor antagonist, propranolol (Sigma-Aldrich, St, Louis, MO) dissolved in sterile endotoxin-free saline at a dose of 2mL kg^{-1} or an equivalent volume of 0.9% saline vehicle. We have previously used propranolol to block stress-induced brain cytokines and behavioral responses since it readily crosses the blood-brain barrier [23], [24].

2.5. Statistical Analysis

Comparisons between groups were made using a two-way analyses of variance (ANOVA) followed by a Tukey HSD *post hoc* test when necessary; $\alpha=0.05$ for all measures, results are expressed as means $\pm$ SEM. Statistical analysis was performed using RStudio.

2.6. Experimental design

To test the effects of chronic stress exposure on contextual fear conditioning, subjects were divided into two groups ($n=16$ /group), one experimental group being exposed to CMS and a non-stressed HCC group. A second cohort was similarly divided into CMS and HCC control groups ($n=16$ /group), and tested in the passive avoidance apparatus. To examine the role of β -ARs in stress-induced fear learning, the aforementioned CMS and HCC groups were subdivided further into drug and vehicle groups, resulting in a total of four groups, with eight subjects per group for each portion of the experiment.

3. Results

3.1. Effect of repeated stress on freezing behavior

Control animals previously exposed to foot shocks in an operant box showed freezing behavior approximately 45% of the time when returned to the same operant box (without shock) 24h later. Animals exposed to CMS prior to conditioning showed significantly more freezing behavior, approximately 70% of the time, when tested 24h after conditioning (Fig. 1). To examine if enhanced β -AR activation during conditioning contributes to altered behavioral changes in stressed animals, animals were given a 2mg kg⁻¹ i.p. injection of propranolol prior to conditioning. Propranolol had no effect on freezing behavior in control animals, but significantly decreased freezing behavior in stressed animals. A two-way ANOVA revealed a significant stress-by-drug interaction ($F[1,28]=5.507$, $p=0.026$); using a Tukey HSD *post hoc*, a significant effect of stress was observed when comparing vehicle-injected CMS versus HCC animals ($p=0.037$), while there was no difference in freezing behavior between control and stressed animals receiving propranolol ($p=0.969$).

3.2. Effect of CMS on passive avoidance behavior

In the passive avoidance apparatus, control animals exposed to foot shock upon entering the dark chamber in the training trial showed a nearly 400s latency to enter the dark chamber when tested 24h later. Animals exposed to CMS prior to training had significantly shorter latencies (approximately 100s) to enter the dark chamber compared to HCC rats (Fig. 2). This finding was supported by a two-way ANOVA that revealed a significant main effect of stress ($F[1,28]=44.039$, $p<0.001$). Propranolol treatment prior to the training trial reduced the latency to enter the dark chamber when tested 24h later regardless of stress condition. A two-way ANOVA revealed a significant main effect of drug treatment ($F[1, 28]=14.935$, $p<0.001$). There was also a significant interaction between stress and drug ($F[1, 28]=6.822$, $p=0.014$) with *post-hoc* analyses revealing propranolol significantly reduced latencies to enter the dark chamber in control animals ($p<0.001$) but did not result in further reductions in latency in stressed animals ($p=0.812$).

3.3. Effect of CMS on non-contextual anxiety-like behavior

In the open field there was no significant effect of CMS ($F[1, 26]=0.015$, $p=0.903$) on the amount of time spent in the center zone, nor was there an effect of propranolol treatment ($F[1, 26]=1.409$, $p=0.246$) in either condition (Fig. 3). Additionally, there were no significant effects of stress or receptor antagonism, respectively, on overall locomotor activity -- either by distance traveled ($F[1, 28]=1.755$, $p=0.196$; $F[1, 26]=1.448$, $p=0.239$) or by time spent in motion ($F[1, 28]=0.052$, $p=0.822$; $F[1, 26]=3.414$, $p=0.075$).

4. Discussion

The data presented here partially support our hypothesis that repeated stress exposure alters behavioral responses due to enhanced classical fear conditioning. Exposure to four days of variable stressors prior to contextual fear conditioning in an operant box significantly enhanced freezing behavior tested 24h later. The enhanced freezing was not due to overall increased anxiety since no differences in behaviors were observed when animals were exposed to the open field, a test of anxiety that does not depend on associative learning. These findings support previous reports that chronic stress enhances contextual fear conditioning [1], and demonstrates this is likely not due to generalized anxiety. The lack of a generalized anxiety-like behavioral phenotype is consistent with our previous observation in the open-field test [23]. There is a degree of variability in the expression of anxiety-like behaviors after repeated exposure to stress, with some stating that, while chronic stress does consistently contribute to development of depressive behaviors, it is not necessarily anxiogenic [25]–[27]. We think that this variability in the expression of “classical” anxiety-like behaviors (e.g. elevated plus-maze, open field) can perhaps be attributed to the context in which the observations were made [23], [28]. Furthermore, for the first time we demonstrated that the enhanced freezing in chronically stressed animals is dependent on β -AR activation during the aversive experience since administration of propranolol during the conditioning trial reduced freezing behavior in stressed animals to near ‘normal’ levels observed in control animals.

Contrary to our initial hypothesis, repeated stressor exposure decreased, rather than increased latencies of animals to enter a dark chamber previously paired with foot shock. We had predicted that enhanced association between the dark chamber and foot shock during conditioning would increase latencies to enter the dark chamber in stressed animals tested 24h later. Previous work has shown that direct infusion of NE into the BLA following passive avoidance training significantly increases latency to enter a shock chamber [11]. The findings of the present study are contrary to this result when tested in animals subjected to repeated stressor exposure. This presents a clear dissociation between the effects of NE infusion and stress exposure. It is likely that NE infusion into the BLA enhances associative learning but does so without causing widespread neural changes that occur following repeated stressor exposure that result in more complex behavioral changes observed in the present study. We do not believe that animals exposed to CMS fail to associate the dark chamber with an aversive stimulus since animals displayed no impairment, but rather an enhancement, in contextual fear conditioning. Given that the stress protocols were identical for all CMS rats and that the time interval between conditioning and testing for the

contextual fear conditioning and passive avoidance tests were also identical, we believe these differences are due to the different neural pathways that regulate the behavioral responses to each test. In the contextual fear conditioning task, animals simply need to recognize the context that was previously paired with foot shock and evoke a behavioral (freezing) response, which is largely mediated by projections from the amygdala to the periaqueductal grey [29]. Alternatively, in the passive avoidance test animals need to recall the environment in which they were shocked and inhibit their natural tendency to move into the dark chamber. Avoidance behavior involves interconnections between the amygdala and areas of the prefrontal cortex that mediate behavioral control [30]. Stressor exposure can impair prefrontal cortex function, thus, even though stressed animals demonstrate increased contextual fear learning, their responses to specific behavioral tasks reflect the effects of stress on multiple brain areas, not simply associative learning within the amygdala. We propose the lower latencies to enter the dark chamber indicate increased impulsivity in stressed animals. It is well-known that stress can increase impulsive behaviors such as drug abuse [31]–[34], risk taking behavior [35]–[38], and food intake [39]. In fact, enhanced formation of fearful memories and increased impulsivity (including risk taking behaviors, drug abuse, and emotional outburst) are both commonly observed in veterans repeatedly exposed to stressful environments [37].

Another interesting observation was the fact that propranolol did not affect contextual fear memory, but did affect passive avoidance behavior, in control animals suggesting that the level of norepinephrine release and β -AR stimulation (likely within the amygdala) in control animals was not sufficient to facilitate contextual fear memory formation during conditioning. Alternatively, in the passive avoidance test, which requires both contextual fear memory and behavioral inhibition (suppression of an animals' natural desire to move into the dark chamber), norepinephrine release in control animals was sufficient to alter behavior since propranolol administration reduced latency to escape in control animals. Since there was no effect of propranolol on contextual fear memory, it suggests norepinephrine more likely plays a role in normal behavioral inhibition (mediated by the prefrontal cortex). Low-to-moderate levels of norepinephrine help stimulate prefrontal cortex and aid in behavioral control, too little or too much norepinephrine release in the prefrontal cortex results in poor behavioral inhibition – often described as impulsivity [40]–[42]. Our data suggest β -AR stimulation during conditioning is necessary for normal behavioral inhibition in the passive avoidance test.

In conclusion, repeated stress exposure results in complex behavioral responses. While we provide evidence that stressor exposure facilitates associative learning processes via β -ARs in a contextual fear conditioning test, it failed to do so in a passive avoidance task that also requires associative learning. These data demonstrate that stressor exposure affects multiple brain areas resulting in complex behavioral responses, and researchers should be cautious when trying to extrapolate the effects of specific stress hormones on behavioral responses to those observed following stressor exposure.

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Fig. 1.

Fig. 2.

Fig. 3.