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Stressor Controllability and Fos Expression in Stress Regulatory Regions in Mice

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Abstract

Controllability is an important determinant of the effects of stress on behavior. We trained mice with escapable (ES) and inescapable (IS) shock and examined behavioral freezing and Fos expression in brain regions involved in stress to determine whether stressor controllability produced differential activation of these regions. Mice (C57BL/6J) were trained to escape footshock by moving to a safe chamber in a shuttlebox. This terminated shock for both ES mice (n=5) and yoked-control mice receiving IS (n=5). Handling control (HC) mice (n=5) experienced the shuttlebox, but never received footshock. Training took place on three days (20 trials per day, 0.2 mA, 5.0 sec maximum duration, 1.0 min interstimulus interval). On day 3, the animals were killed two h after training and the brains were processed for Fos expression in the amygdala, hypothalamic paraventricular nucleus (PVN), laterodorsal tegmental nucleus, locus coeruleus and dorsal raphe nucleus. Fos expression after IS was greater than after ES and HC in all regions ($p < .05$). Fos expression after ES was greater than HC only in PVN ($p < .05$). Freezing in ES mice was equal to or greater than in IS mice whereas HC mice showed minimal freezing. Differential activation of brain regions implicated in stress may, in part, account for differences in behavior in the aftermath of uncontrollable and controllable stress.

Keywords

amygdala; dorsal raphe nucleus; Fos; hypothalamic paraventricular nucleus; laterodorsal tegmental nucleus; locus coeruleus; stress

1. Introduction

Stress can have a significant, persisting negative impact on health and can result in lasting changes in behavior. However, stressors are often encountered without producing permanent or pathological changes. The difference between successful and unsuccessful coping with stress may involve the resilience of the organism [1] as well as characteristics of the stressor. Stressor controllability, along with intensity and predictability, has been found to be important factors in the effects of stress [2]. For example, lack of stressor controllability has been suggested to be a factor in the development of posttraumatic stress disorder (PTSD) [3,4].

Conditioned fear training is conducted with a fear-inducing stressor (usually footshock) [5,6] presented in an experimental paradigm in which the animal receiving training has no control over the stressor. Through association to the footshock, initially neutral environmental cues and contexts acquire the capacity to elicit behavioral and physiological responses indicative of fear and anxiety including behavioral freezing (e.g. [7-9]), autonomic responses (e.g. [10-12]) and fear-potentiated startle (e.g. [5,6]). Disturbances in sleep also often follow a

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stressful or traumatic event (reviewed in [13]) and our work and that of others has demonstrated that footshock and shock-associated fearful cues and contexts produce similar alterations in sleep, including a prominent reduction in rapid eye movement sleep (REM) [14-17]. By comparison, training with avoidable [18-21] and escapable (Unpublished Results) footshock (controllable stressors) is followed by significant increases in REM even though the same stressor (footshock) is initially experienced before the avoidance responses is acquired. This suggests that there may be significant differences in regional brain activation after experience with controllable and uncontrollable stressors.

The immediate early gene, *c-fos*, and its protein product, Fos, have been widely used to detect neuronal populations that are “activated” during a variety of behavioral and stressful paradigms (e.g. [22-31]. *C-fos* has low basal expression levels in most neural systems and is rapidly up-regulated in response to stimuli. *C-fos* is detectable in neurons within 20 min of stimulation and it peaks around 30 min whereas Fos takes 90 to 120 minutes to develop. Thereafter, *c-fos* and Fos decline rapidly [32]. Both are readily detectable and give cellular level resolution in various areas of the brain making them good markers for identifying functional involvement of specific brain regions [33,34]. Fos has been used to identify regions involved in escapable and inescapable shock in rats [30,31], but to our knowledge, no studies have been conducted in mice.

In this study, we trained mice with inescapable (uncontrollable) footshock (IS) and escapable (controllable) footshock (ES) and examined Fos expression as a measure of neural activation in a number of brain regions involved in the stress response and/or in the regulation of arousal. These included the paraventricular nucleus of the hypothalamus (PVN), the amygdala, and in the brainstem, the locus coeruleus (LC), the dorsal raphe nucleus (DRN) and the laterodorsal tegmental nucleus (LDTg). We also examined freezing, a common behavioral index of fear [7,35,36], during shock training. This enabled us to compare immediate emotional reactivity across conditions and to examine the relationship of initial emotionality to subsequent determination of Fos expression.

2. Experimental Procedures

2.1. Subjects

The subjects were 7- to 9-week-old male, C57BL/6J (B6) mice (n=15) obtained from Jackson Laboratories, Bar Harbor, ME. The mice were acclimatized to individual housing for 1 week prior to being subjected to any experimental procedures. The colony room was set on a 12:12 light/dark cycle with lights on from 0700 to 1900 h, and room temperature was maintained at 24.5±0.5°C. Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by Eastern Virginia Medical School’s Animal Care and Use Committee (Protocol # 05-017). All possible efforts were made to minimize the number of animals used and their suffering.

2.2. Training Procedures

After habituation to the colony conditions, the mice were randomly assigned to three conditions: ES (n=5), IS (n=5) and handling control (HC, n=5). Mice in the ES condition were able to learn to escape a footshock by moving to the non-occupied chamber in a shuttlebox (Coulbourn Instruments, Model E10-15SC). This terminated shock in the ES condition and also terminated shock delivery to yoked-control mice receiving IS in a separate shuttlebox. Thus, both groups received identical amounts of footshock, but the IS mice could not influence the amount of shock they received. Mice in the HC condition were allowed to freely explore the shuttlebox for same duration as the ES and IS mice, but never received footshock.

Shock training took place at the same time on 3 consecutive days (S1, S2, and S3). Three days of training were used to maximize the probability that mice in the ES group had fully learned the escape response and that mice in the IS group had learned the shock was inescapable. The mice were allowed to freely explore the shuttlebox for 5 min after which they were presented with 20 footshocks (0.2 mA, 5.0 sec maximum duration) at 1.0 min intervals. Five min after the last shock, the mice were returned to their home cages. The entire procedure was of approximately 30 min duration. The chamber was thoroughly cleaned with diluted alcohol before each training session. On training days S1 and S2, the animals were left undisturbed in their home cages until the training session on the following day. On day S3, the animals were killed 2 h after training and the brains processed for Fos immunohistochemistry.

Coulbourn Graphic State software (ver. 2.1) running on a Pentium-class computer was used to control the administration and timing of footshock to ensure that mice in the ES and IS conditions receive the same duration of footshock. Footshock was produced via Coulbourn Precision Regulated Animal Shockers (Model E13-14) and administered via grid floors in the shuttleboxes. The software also tracked the duration of the shock to enable determination of the amount of shock each yoked pair received across training trials and across days.

Each training session was videotaped for subsequent scoring of freezing, defined as the absence of body movement except for respiration [7,35,36]. Freezing was scored by a trained observer, blind to condition, in 5-sec intervals during 1.0-min observation periods over the course of the 30 min the mice were in the shock chamber. The percentage time spent in freezing was calculated (FT%: freezing time/observed time $\times$ 100) for each animal for each observation period.

2.3. Perfusion and fixation

At sacrifice, the mice were anesthetized with isoflurane (inhalation: 5% induction, 2% maintenance) and then transcardially perfused with 50 ml ice cold saline, followed by 30 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). Brains were immediately removed and post-fixed in the same fixative at 4° C for 24-48 h and then immersed in 30% sucrose in 0.1 M PB for 48 h at 4° C.

2.4. Immunohistochemistry

Sections were made through the amygdala, PVN, LDTg, LC and DRN. Thirty- μ m thick coronal sections were cut from frozen blocks from AP 3.46 to -2.08 mm (5.54 mm=184 sections) of the brain. Every fourth section was collected as a sample (46 sections per sample). One sample was used for Fos staining. A second sample was used as a blank control which was processed identically but did not contain the Fos antibody. Free floating methods were used for immunohistochemical staining as described previously [37]. The sections were washed in 0.01 M phosphate buffered saline (PBS, pH 7.4) and incubated in 0.3% hydrogen peroxide (H_2O_2)-2% normal goat serum in 0.01 M PBS for 30 min at room temperature to eliminate endogenous peroxidase activity and to block nonspecific binding sites. Then sections were washed 3 times for 10 min in PBS and were then incubated for 48 h at 4° C with the Fos antibody (1:20,000, Ab-5, Oncogene Research Products, Cat# PC38) in PBS containing 0.3% Triton X-100 and 2% normal goat serum. After washing with PBS, sections were incubated for 2 h at room temperature with biotinylated goat anti-rabbit secondary antibody (1:600, Sigma, Product No.B8895) in PBS containing 2% normal goat serum. Subsequently, the sections were washed and incubated for 1 h at room temperature with horseradish peroxidase avidin-biotin complex (1: 100 ABC reagent in PBS-TX, Avidin- Biotin Complex, Vector ABC kit). After washing, sections were reacted with DAB, mounted to slides and allowed to dry on the slides for 48h. Then the sections were dehydrated using graded alcohol, cleared by xylene and protected with cover slides.

The primary antibody was absent in blank control sections, which were otherwise processed identically. Omission of the primary antibody resulted in complete loss of nuclear staining.

2.5. Data Quantification and Analyses

The data for average shock duration for pairs of yoked control mice were analyzed in two blocks of ten trials for each training day. The analysis was conducted with a 3 (Day) $\times$ 2 (Time Block: first 10 min, second 10 min) ANOVA. The data for freezing in individual mice were analyzed with a 3 (Group: ES, IS and HC) $\times$ 3 (Day: S1, S2 and S3) $\times$ 3 (Shock Period: pre-shock, shock and post-shock) ANOVA. When appropriate, post hoc comparisons were conducted with Tukey tests.

Fos expression was visualized in brain sections using a Nikon Eclipse E800 microscope. The sections were standardized as much as possible using a mouse brain atlas [38]. Digital photographs of the selected regions were taken with a Spot digital camera attached to the microscope at 10x magnification. Fos positive cells were analyzed in the selected regions with the Metamorph Image Analysis program. Fos positive cells in the selected regions were analyzed in 3 sequential sections and were summarized and expressed as number per region.

Fos counts across treatment group (ES, IS and HC) were compared with one-way ANOVAs. When appropriate, post hoc comparisons were conducted using Tukey Tests.

3. Results

3.1. Shock Duration

Figure 1 shows the mean shock duration experienced by the pairs of yoked control mice in the ES and IS conditions. The ANOVA revealed a significant Day main effect [$F(2,24) = 10.87$, $p < .001$] and a significant Time Block main effect [$F(1,24) = 14.30$, $p < .001$]. The Day $\times$ Time Block interaction was not significant. Post hoc comparisons across days found that shock duration was decreased on S2 and S3 compared to S1 (Figure 1 A). Shock duration did not significantly differ between S2 and S3. There was also a significant decrease in shock duration from the first 10 min to the second 10 min of training (Figure 1 B). The difference was greatest on S1 (not shown).

3.2. Freezing

Figure 2 shows FT% for the ES, IS and HC plotted across training days and across Shock Periods. The ANOVA revealed significant main effects for Group [$F(2, 108) = 170.77$, $p < .001$], Day [$F(2, 108) = 18.30$, $p < .001$], and Shock Period [$F(2, 108) = 14.96$, $p < .001$]. There were also significant interaction effects for Group $\times$ Day [$F(4,108) = 5.43$, $p < .001$], Group $\times$ Shock Period [$F(4, 108) = 3.64$, $p < .01$] and Day $\times$ Shock Period [$F(4, 108) = 5.95$, $p < .001$].

Post hoc comparisons revealed that ES mice showed greater overall FT% than did IS mice. Across Days (Figure 2 A), both ES and IS mice showed enhanced freezing compared to the HC mice on all days. ES mice showed greater FT% than IS mice on S2 and S3, but not S1. Across Shock Periods (Figure 2B), ES and IS mice showed greater FT% than did HC mice in all analyses. ES mice showed greater FT% than did IS mice during the post-shock period, but not during the pre-shock or shock period.

3.3. Fos Expression Across Conditions

Figure 3 presents sample immunohistochemical sections showing Fos expression across conditions in PVN and Figure 4 shows quantified Fos expression across conditions in each of the brain regions we examined. The ANOVAs were significant for comparisons across groups in all brain regions that we examined (PVN [$F(2,12) = 25.08$, $p < .001$]; Amygdala [$F(2,12) =$

13.41, $p < .001$]; LC [$F(2,12) = 19.63$, $p < .001$]; DRN [$F(2,12) = 10.02$, $p < .01$]; LDTg [$F(2,12) = 6.08$, $p < .05$].

Post hoc comparisons (see Figure 4) revealed that Fos expression in PVN in the IS group was significantly higher than in the ES and HC groups, and that Fos expression in PVN in the ES group was significantly greater than in the HC group. However, in other regions (amygdala, LC and DRN), the pattern of Fos expression across conditions was similar with significantly elevated Fos expression in the IS group compared to both ES and HC groups. In LDTg, Fos expression was elevated in the IS group compared to HC, but not compared to ES. Other than in PVN, Fos expression in the ES and HC groups did not significantly differ.

4. Discussion

The current results demonstrate that even though virtually identical amounts of shock was received, Fos expression after IS was greater than after ES in the regions that we examined. In addition, Fos expression after ES was greater than after HC only in PVN, but not in amygdala, LC, DRN or LDTg. Thus, mice in the IS group showed significantly greater Fos expression compared to mice in the ES and HC groups. Differential Fos expression across regions after ES and IS suggests that stressor controllability was an important factor in the relative activation in these regions in association with the stress response.

Behavioral freezing has been used to evaluate fear, with greater FT% being interpreted as indicating stronger fear reactions [7,35,36]. FT% did not differ across groups in the pre-shock period of day 1 (not shown) and HC mice showed minimal FT% in all trials and appeared to freely explore the chamber with no noticeable differences across or within days. However, FT% in the ES and IS mice was greater than in the HC mice during the pre-shock period when all days were considered, indicating that the intervening shock presentations had induced fearful behavior in both ES and IS mice.

FT% in the ES mice was equal to or greater to that in the IS mice even though the ES mice learned the escape response and showed reduced Fos activation in brain regions involved in the stress response compared to IS mice. A factor that should be considered is that freezing and increased vigilance are adaptive responses (along with startle, heart rate and blood pressure changes) that occur during danger and that can facilitate defensive responses such as escape and avoidance [39]. Thus, though exposure to the shock chamber would have been a fearful experience for both ES and IS groups, fear, as indicated by freezing, did not prevent the ES mice from learning the adaptive response and reducing the duration of the shock they received.

The PVN is a critical component of stress circuitry in the brain [40-43] and is the final common pathway for information influencing the hypothalamo-pituitary-adrenal (HPA) axis [44,45]. Fos activation in PVN has been interpreted as an indicator of the amplitude of the stress response and to indicate the degree of activation of the HPA axis [28,46,47]. Thus, less Fos in PVN in ES mice compared to IS mice may indicate reduced intensity in the stress response of mice that could behaviorally influence the termination of shock though Fos expression in PVN of ES mice was significantly greater than that of HC mice. Handling and exposure to novel environments also may have evoked a stress response in HC mice [48] though the intensity may have been decreased with three repetitions across days. It is also possible that Fos expression in the ES and IS animals could have varied across days of training.

Brainstem serotonergic [31,49-53] and noradrenergic [54] regions appear to play important roles in stressor controllability, and have roles in regulating PVN [44]. Both DRN and LC in mice were activated more in response to IS than to ES in this study, findings consistent with much of the work on controllable and uncontrollable stress in rats. For example, yoked control rats showed higher Fos expression in DRN than did rats that were able to terminate shock via

turning a wheel [31]. IS also activates 5-HT DRN neurons to a greater degree than does ES thereby increasing 5-HT in DRN and in target areas [49-52]. IS in rats produced sustained increases in NA turnover in various brain regions regardless of stress duration, whereas with ES, NA utilization was reduced after the coping response was learned [54].

There is less information regarding the potential role of the brainstem cholinergic structure we examined, LDTg, in the stress response. In a previous study in mice, we found increased Fos in LDTg after shock training in a cued fear paradigm, but not after presentation of the fearful cue alone [37]. Thus, Fos activation in LDTg may be more related to the physical stress induced by the shock.

The amygdala has a demonstrated role in regulating physiological responses to stressors, the regulation of adaptive behaviors and the regulation of homeostasis (For recent review see [55]). The amygdala mediates behaviors associated with conditioned fear [56,57] and it influences PVN through outputs via the central nucleus of the amygdala and the bed nucleus of the stria terminalis [44]. It also projects prominently to brainstem regions we examined including LC, DRN and LDTg [58-62]. Previous work in our lab using counter staining for corticotropin releasing hormone (CRH) to delineate the central amygdala found enhanced Fos expression after IS training in the basal amygdala, lateral amygdala and the amygdalostriatal region, but not in the central amygdala [37]. We did not distinguish nuclei in this study, but there well may have been regional differences in Fos expression.

Arousal is a component of the stress response [43] and it is likely that there was stress-induced activation in regions that we did not examine. It also should be noted that evaluation of stressor controllability likely requires processing at higher cortical levels than those we examined and the medial prefrontal cortex (mPFC) has been found to be a critical region in the perception of control and in mediating the consequences of stress [63-65]. Part of the influence of the mPFC is enacted through its effects on the DRN and possibly LC [63,65] and mPFC may influence the HPA axis through actions on PVN [63]. mPFC-amygdala circuitry also appears important for mediating the effects of stress, in particular, those related to learning and memory and the extinction of fearful responses [64].

While our results are consistent with much of the work on controllable and uncontrollable stress in rats, they differ from those in a recent report by Coco and Weiss [30] that surveyed brain Fos expression in rats trained with controllable and uncontrollable tail shock. Coco and Weiss [30] included the structures we examined, as well as others, and found that stress increased Fos activation compared to home cage controls and/or apparatus control animals that received all handling procedures but never experienced tail shock. Their study did not find significant differences in amount of Fos activation in the controllable stress and yoked uncontrollable stress conditions in PVN, the amygdala, LC or LDTg. Coco and Weiss [30] also reported increased Fos in the rostral DRN of the controllable stress group, a finding that differs from other studies in rats using a similar wheel-turn paradigm [31]. However, the rats were trained to turn a wheel to produce a 1 min delay until the onset of the next shock whereas in our study, mice simply had to move to the safe side of a shuttlebox at the onset of shock. In addition, training on the task in the Coco and Weiss [30] study took place every two to three days over the course of four weeks, shock intensity was increased every 30 minutes across trials and the last session before the animals were killed was 1.5 h in duration. Thus, the longer training sessions, increasing shock levels across trials as well as greater task complexity may have made the controllable stress condition in the Coco and Weiss [30] study significantly more stressful than the simpler response and much shorter training sessions that we used, and could have accounted for the differences between studies. Other studies that used more complex learning paradigms than ours have also failed to find significant differences between the controllable and uncontrollable stress conditions. For example, Helmreich et al. [66] did not find differences

in CRH, enkephalin, neurotensin or vasopressin mRNA levels within the medial parvocellular region of the PVN in ES and yoked control rats trained in a tailshock paradigm that used more intense shock and required increasing amounts of wheel turn to terminate tailshock across trials. Thus, differences in the type and number of behavioral responses required to “control” the stressor may be important factors in comparing results across experiments.

In summary, our study demonstrated differences in Fos activation in brain regions linked to stress and to the control of arousal in mice trained with ES or IS. Mice trained with ES showed reduced Fos activation even though they received identical amounts of footshock and exhibited similar or greater levels of behavioral freezing. This suggests that fear, as indicated by freezing, in the controllable stress group did not prevent adaptive responses as the ES mice readily learned the escape response.

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Figure 1.

Mean shock duration experienced by the pairs of mice in the escapable shock (ES) and yoked control inescapable shock (IS) conditions. S1, S2, and S3 indicate three successive days of shock training (A). The data for the 20 min shock training session are plotted as two 10 min blocks (B). Comparisons between groups that were significant are indicated by brackets. **, $p < .01$; ***, $p < 0.001$ (Tukey test).

Figure 2.

FT% plotted for escapable shock (ES), inescapable shock (IS) and handling control (HC) groups across days of training (A) and across shock period (B). S1, S2, and S3 indicate three successive days of shock training. Pre-shock, shock, and post-shock refer to observation periods before, during and after shock administration. Comparisons between groups that were significant are indicated by brackets. **, $p < .01$; ***, $p < .001$ (Tukey test).

Figure 3. Example sections showing Fos expression in the hypothalamic paraventricular nucleus (PVN) across conditions. HC-Handling control, IS-inescapable shock, ES-escapable shock, 3V-3rd ventricle.

Figure 4.

Fos expression examined two h after the session of shock training on day 3. Five animals were in each of three groups: escapable shock (ES), inescapable shock (IS) and handling control (HC). Sections were made through the hypothalamic paraventricular nucleus (PVN), amygdala, laterodorsal tegmental nucleus (LDTg), locus coeruleus (LC) and dorsal raphe nucleus (DRN). Panels in the left column show statistical comparisons among groups for each region, panels in the middle column shows example section taken from the IS group and panels in the right column illustrates the location of the section where counts were performed. 3V, third ventricle; 4 V, fourth ventricle; AH, anterior hypothalamus; Aq, aqueduct; CNA, central

nucleus of the amygdala; LA, lateral amygdala;. Comparisons between groups that were significant are indicated by brackets. *, $p < .05$; **, $p < .01$; ***, $p < .001$ (Tukey test).