Published in final edited form as:

Neuroreport. 2018 January 17; 29(2): 112–117. doi:10.1097/WNR.0000000000000919.

Effects of stressor controllability on transcriptional levels of *c-fos*, *Arc* and *BDNF* in mouse amygdala and medial prefrontal cortex

Mayumi Machida, György Lonart, and Larry D. Sanford*

Sleep Research Laboratory, Department of Pathology and Anatomy, Eastern Virginia Medical School, P.O. Box 1980, Norfolk, VA 23501, USA

Abstract

Controllability is an important factor in determining stress outcomes. Uncontrollable stress is associated with the development of psychopathology such as posttraumatic stress disorder (PTSD), whereas controllable stress is associated with adaptive stress responses and positive outcomes. In this study we investigated how controllability impacts post-stress neurobiology by assessing transcriptional levels of activity-dependent genes in medial prefrontal cortex (mPFC) and amygdala, regions important in mediating stress outcomes. Mice were subjected to either escapable shock (ES) or yoked inescapable shock (IS) as models of controllable and uncontrollable stress, respectively. Immediately (0 h) or at 2 h after shock training (20 trials; 0.5 mA, 5.0 s maximum duration; 1.0 min inter-stimulus interval), mice were sacrificed, and we interrogated expression levels of the immediate-early genes (IEGs), *c-fos* and *Arc*, and a delayed primary response gene, *BDNF*, in mPFC, amygdala, and somatosensory cortex (a control region), using real-time reverse transcription quantitative PCR (RT² qPCR). We found ES-associated up-regulation of *BDNF* in amygdala as well as in mPFC. IS suppressed *c-fos* in mPFC (0 h) but induced more *Arc* in amygdala (2 h) in comparison to ES. Freezing, an index of fear memory, and serum level corticosterone, an index of the stress response, did not differ between mice trained with ES or IS. The data are discussed with respect to the potential functional involvements of the amygdala and mPFC in mediating differential outcomes of controllable and uncontrollable stress.

Keywords

stressor controllability; amygdala; mPFC; *c-Fos*; *Arc*; *BDNF*

Introduction

Stress is associated with negative health outcomes; however, successful coping can reduce psychological distress, and thus, favorably influence health outcomes. Animal studies have repeatedly shown that controllable stress aids in the development of adaptive responses to subsequent stressors whereas uncontrollable stress does not [1, 2]. These responses likely

*Corresponding author: Fax: +1 757 446 5719., SanforLD@evms.edu.

Conflicts of interest

There are no conflicts of interest.

include differences in post-stress rapid eye movement (REM) sleep, a state hypothesized to play a role in fear memory processing [3] and adaptive role responding to stress [4]. Mice trained with controllable stress (modeled by escapable shock (ES)) show increases in REM sleep, whereas mice trained with uncontrollable stress (modeled by inescapable shock (IS)) show decreases [4].

In this study, we investigated the effects of stressor controllability and uncontrollability on neuronal changes in the medial prefrontal cortex (mPFC) and the amygdala, using an ES and IS paradigm as previously described [4]. The mPFC is involved in regulating post-stress behavioral flexibility [5, 6], whereas the amygdala plays key roles in the acquisition and consolidation of fear memory [7] and is a critical region in regulating stress-induced alterations in sleep [8]. Mice were sacrificed immediately (0 h) or at 2 h post-shock training (ST), and we profiled transcriptional levels of immediate-early genes (IEGs) - *c-fos* (FBJ osteosarcoma oncogene) and *Arc* (activity-regulated cytoskeletal-associated protein) as well as *BDNF* (brain derived neurotrophic factor) using RT² qPCR. *c-fos* is a widely-used anatomical marker of recent neural activity. *Arc* is an effector IEG that can be specifically localized to potentiated synapses [9]. *BDNF* is involved in synaptic plasticity and learning/memory and has been reported to be induced by controllable stress in the mPFC [10]. The somatosensory cortex, a region implicated in the linkage between sensory stimuli and fear memory [11] was examined as a control brain region.

Materials and methods

Subjects

BALB/cJ mice (8–10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Upon arrival, the animals were individually housed and given *ad libitum* access to food and water. The colony room was kept on a 12:12 light/dark cycle. Room temperature was maintained at $24.5 \pm 0.5^{\circ}\text{C}$. Throughout the experimental procedures, measures were taken to minimize unnecessary pain and discomfort of the animals. 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.

Training procedures

Mice were randomly divided into ES, IS training, or handling control groups ($n = 10\text{--}11$ for each). Control mice freely explored a shuttlebox, but never received shock (NS). ES mice received shock but were able to terminate it by moving to a non-occupied chamber in the shuttlebox (Model E10–15SC, Coulbourn Instruments, Whitehall, PA). This escape movement also terminated shock delivery to yoked IS mice in a separate shuttlebox, which did not have control over shock termination. Thus, a pair of mice received identical shock, but it was characterized as either controllable or uncontrollable. Shock training (ST) was conducted during the 3rd h after lights on and lasted 30 min. Presentation of 20 footshocks (0.5 mA, 5.0 s max. duration, 1 min intervals) began 5 min after the mice were placed in the shuttleboxes and was controlled by a Coulbourn Graphic State V2.1 software via Coulbourn Precision Regulated Animal Shockers (Model E13–14). The mice were undisturbed during 5

min pre- and post-ST periods. Escape latency was estimated using duration of shock mice experienced on each shock. ST was videotaped for scoring of freezing, defined as the absence of body movement except for respiration. Immediately after ST (0 h), half of the animals were decapitated for blood and brain tissue collection after being deeply anesthetized with 15% halothane in mineral oil. The other half were killed 2 h after ST and processed identically.

Quantitative measurement of corticosterone (CORT)

Blood samples were collected in tubes containing EDTA, and were centrifuged at $1500 \times g$ at 4°C for 10 minutes. CORT concentrations were determined by an EIA kit (Cayman Chemical, Ann Arbor, MI).

Total RNA isolation

Brains were placed in ice-cold phosphate buffered saline (PBS), pH 7.4, and 1-mm thick coronal slices were made by the aid of a mouse brain matrix (Brain Research Laboratories, Waban, MA). Amygdala (approximately around: -3.3 to -0.3 mm AP), mPFC (3.0 to 1.5 mm AP) and somatosensory cortex (-3.3 to -0.3 mm AP) were collected using visual landmarks with the aid of the Mouse Brain Atlas (2nd edition, Academic Press) into TRIzol® (Invitrogen, Carlsbad, CA). Total RNA was extracted using the guanidine thiocyanate method. The samples were further purified using a RNA isolation kit (#PA-001, SABiosciences, Frederic, MD). Concentration and quality of samples were determined by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). A 260/280 ratio 2.0 and a 260/230 ratio 1.7 was considered as suitable for further analysis.

Reverse Transcription Real-Time quantitative Polymerase Chain Reaction (RT² qPCR)

Total RNA (120 ng) was converted to complementary DNA (cDNA) using a 1st strand cDNA synthesis kit (#C-03, SABiosciences). After eliminating genomic DNA (gDNA) using a GM mixture (SABiosciences) at 42°C for 5 min, the RNA strand was reverse transcribed into cDNA at 42°C for 15 min, followed by 5 min heating at 95°C to stop the reaction. The quality of the cDNA was assessed by a quality control kit (#PAMM-999A, SABiosciences). Intra- and inter-assay results for housekeeping genes showed consistency among replicates and reproducibility among PCR runs. PCR reaction containing cDNA template (1~8 ng) combined with SYBR Green dye (# PA-111, SABiosciences) was amplified in triplicates by a Bio-Rad iCycler Thermocycler & iQ Real-Time PCR Systems (Bio-Rad, Hercules, CA) using two step PCR conditions: step 1: 10 min at 95°C; step 2: 40 cycles of 15 s at 95°C following 60 s at 60°C. At the end of each PCR run, the specificity of the PCR amplification was determined by melting curve analysis. Dissociation curves for each target gene confirmed that all primers showed a single peak. Prior to experimentation, serial dilutions of cDNA templates obtained from NS mice were subjected to generate a linear standard curve for each gene by region. We strived for an amplification efficiency of 90 ~ 105 %. We evaluated β -actin as a reference gene. Ct value of β -actin was similar between samples in each region and was resistant to experimental conditions at both time points, thus was used as an internal control in this study. We reported the relative change in each gene expression applying the 2^{-Ct} method. After normalization by calculating target

genes vs. β -actin Ct differences, the data were then compared with levels of the NS group at 0 h and were presented as fold change by each region.

Data analyses

Data were analyzed with ANOVAs using SigmaStat (version 2.03, SPSS Inc., Chicago, IL). When appropriate, post hoc comparisons were conducted using Tukey tests. In cases where the normality test failed, a Kruskal-Wallis ANOVA on ranks was performed. Differences with a p value < 0.05 were considered significant.

Results

Escape latency decreased over ES trials

Fig. 1A shows typical changes in escape latency from an ES mouse, indicating improvement in escape performance over trials. Mean shock duration was analyzed by 4 blocks of 5 trials (B1–B4) and expressed as percentage changes relative to B1 (Fig. 1B). There was a significant time effect ($F_{[3, 40]} = 2.97$, $p = 0.048$), and duration significantly decreased in B4 by 38% ($\pm 10\%$) relative to B1, indicating that ES mice learned shock termination.

Fear behavior and the peripheral stress response did not differ in ES and IS mice

Freezing was assessed as a measure of fear behavior. Significant group ($F_{[2, 61]} = 16.62$, $p < 0.001$) and time ($F_{[1, 62]} = 63.00$, $p < 0.001$) effects, and a group $\times$ time interaction ($F_{[5, 58]} = 16.62$, $p < 0.001$) were found (Fig. 2). No freezing was observed in any of the animals during pre-ST, and their behavior during the period did not differ across groups. In contrast, during post-ST, both ES and IS mice showed significantly greater freezing in comparison to NS mice, which did not freeze. There was no statistically significant difference between ES and IS in freezing. Fig. 3 shows plasma CORT levels for each group at 0 h and 2 h. There were significant group ($F_{[2, 29]} = 10.19$, $p < 0.001$) and time ($F_{[1, 30]} = 334.57$, $p < 0.001$) effects, and group $\times$ time interaction ($F_{[5, 26]} = 13.45$, $p < 0.001$). At 0 h, both ES and IS mice showed an increase in CORT that was almost double that seen in NS mice ($F_{[2, 12]} = 12.37$, $p = 0.001$: NS = 133.7 ± 5.83 ng/ml, ES = 261.9 ± 22.10 , IS = 253.5 ± 10.22). At 2 h, CORT levels were diminished in all groups. The levels did not differ between groups. There were no differences in CORT between ES and IS mice at either time point.

ES and IS differentiated expressions of *c-fos*, *Arc*, and *BDNF*

Responses in *c-fos* were observed immediately after training, but levels were generally diminished at 2 h (time effect, marked by &) (Fig. 4A–C). There was no group effect found in the amygdala (Fig. 4A). In contrast, in the mPFC (Fig. 4B), IS training suppressed *c-fos* at 0 h ($F_{[2, 10]} = 8.433$, $p = 0.007$) with levels decreased by 56% in comparison to NS, whereas ES training induced *c-fos* at a similar level to that in NS mice. In the somatosensory cortex, *c-fos* was greater in the shock trained groups (ES and IS) than in non-shocked mice (NS), but there was no effect of controllability (Fig. 4C). *Arc* was upregulated immediately at 0 h under all treatment conditions in all the three regions (Fig. 4D–F). In the amygdala expression of *Arc* in the IS group remained upregulated at 2 h and was significantly greater than in the NS or ES groups ($F_{[2, 12]} = 5.766$, $p = 0.018$: NS = 0.36, ES = 0.44, IS = 0.74) (Fig. 4D). ES training significantly increased the level of *BDNF* at 2 h in the amygdala

($F_{[1, 7]} = 9.662$, $p = 0.017$: NS = 1.79, ES = 4.46, IS = 2.17) (Fig. 4G), and in the mPFC ($F_{[2, 26]} = 7.080$, $p = 0.004$: NS = 0.71, ES = 1.59, IS = 0.64) (Fig. 4H), but not in the somatosensory cortex (Fig. 4I).

Discussion

We found that controllable and uncontrollable stress, modeled by ES and IS, differentially induced transcriptional activity in a region-specific manner. Although there could be some contribution by microglia, at least theoretically, in production of cortical BDNF [12], we assume mRNA changes we found mostly reflect neuronal origin. Under physiological conditions, immediate early genes *c-fos* and *Arc* are activated by strong synaptic activity and are believed to be critical in conversion of short-term plasticity into long-term plasticity [13]. Importantly, our data suggested functional involvement of the amygdala in mediating effects of controllability, although previous research on controllability have predominantly focused on the mPFC. In amygdala, as well as in mPFC, *BDNF* was significantly increased after ES, but not IS. BDNF has been widely linked to variety of behavior paradigms including stressor controllability [10], fear consolidation [14] and fear extinction [15]. Recent studies have also linked BDNF to sleep regulation. For example, increased BDNF has been associated with increased sleep after sleep deprivation in rats [16]. In humans, correlation between decreased serum BDNF and insomnia has been reported [17]. More recently, a positive correlation between REM sleep and increases of BDNF selectively in REM-sleep-regulating regions has been found [18]. Given that the amygdala regulates stress-induced alterations in sleep [3], our finding of ES-induced *BDNF* in the amygdala may be associated with post-ES increases in REM sleep documented previously [4], although further work will be required to ascertain the precise relationship.

We found that levels of post-IS *c-fos* were significantly lower in the mPFC at 0 h compared to ES and NS, whereas post-ES *c-fos* was almost identical to NS. The results, i.e., uncontrollable-stress-associated downregulation in mPFC, are consistent with functional studies of mPFC on responses to uncontrollable stress, e.g., blocking mPFC neuronal activity with muscimol did not alter escape behavior in rats presented with IS [2], whereas activation of mPFC with picrotoxin prior to IS promoted later escape learning in rats trained to escape shock in a shuttlebox [19]. Controllability also impacted neural activity in the amygdala as *Arc* remained upregulated at 2 h in IS trained mice, whereas upregulation was attenuated in ES trained mice. *Arc* has been extensively used in studies of associative learning and long-term potentiation (LTP) [9, 20, 21]. *Arc* is localized to recently potentiated synapses through interaction with proteins critical to synaptic plasticity [9, 21]. As such, the gene is known to play important roles in fear conditioning, a type of associative learning. *Arc* knock-out mice exhibit impaired fear memory [22], and upregulation of *Arc* in the amygdala is followed by successful consolidation of fear memory [23, 24]. Since the paradigm we used is a variant of fear conditioning, the sustained increase of *Arc* in the amygdala at 2 h after IS, but not ES, may indicate that aspects of fear memory consolidation were different in association with stressor controllability and uncontrollability, even though a classical index of fear memory (freezing) was similar in both groups. This observation was consistent with our previous studies in mice trained with ES and IS [4, 25] which

demonstrate disassociation of behavioral freezing and neurobiological/physiological consequences.

We found significantly greater *c-fos* expression in somatosensory cortex in shocked animals (ES/IS) than non-shocked (NS), however, no genes tested in this area showed a differential response to controllability. These results are consistent with our hypothesis that the level of sensory input (shock) experienced by the ES and IS animals was not significantly different, and also consistent with the CORT results; both ES/IS mice showed similar levels in CORT indicating that the HPA axis response was not different regardless of whether or not shock was controllable. These results are in agreement with studies of others [26].

Conclusion

Our data demonstrate that controllability can differentially impact neural markers of activation and synaptic plasticity independently of HPA activation. ES training led to pronounced upregulation of *BDNF* in the amygdala as well as in the mPFC, and also distinct *c-fos* and *Arc* expression patterns. These differences may delineate processes that distinguish adaptive and maladaptive responses to stress.

Acknowledgments

Source of funding

This work was supported by NIH research grant MH64827.

References

1. Korte SM, De Boer SF, Bohus B. Fear-potential in the elevated plus-maze test depends on stressor controllability and fear conditioning. *Stress*. 1999; 3(1):27–40. [PubMed: 19016191]
2. Amat J, et al. Previous experience with behavioral control over stress blocks the behavioral and dorsal raphe nucleus activating effects of later uncontrollable stress: role of the ventral medial prefrontal cortex. *J Neurosci*. 2006; 26(51):13264–72. [PubMed: 17182776]
3. Sanford LD, Suchecki D, Meerlo P. Stress, arousal, and sleep. *Curr Top Behav Neurosci*. 2015; 25:379–410. [PubMed: 24852799]
4. Sanford LD, Yang L. Differential Effects of Controllable and Uncontrollable Footshock Stress on Sleep in Mice. *Sleep*. 2010
5. Amat J, et al. Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. *Nat Neurosci*. 2005; 8(3):365–71. [PubMed: 15696163]
6. Yehuda R, LeDoux J. Response variation following trauma: a translational neuroscience approach to understanding PTSD. *Neuron*. 2007; 56(1):19–32. [PubMed: 17920012]
7. LeDoux JE. Emotion circuits in the brain. *Annu Rev Neurosci*. 2000; 23:155–84. [PubMed: 10845062]
8. Wellman LL, et al. The basolateral amygdala determines the effects of fear memory on sleep in an animal model of PTSD. *Exp Brain Res*. 2014; 232(5):1555–65. [PubMed: 24519098]
9. Steward O, et al. Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron*. 1998; 21(4):741–51. [PubMed: 9808461]
10. Bland ST, et al. Expression of fibroblast growth factor-2 and brain-derived neurotrophic factor mRNA in the medial prefrontal cortex and hippocampus after uncontrollable or controllable stress. *Neuroscience*. 2007; 144(4):1219–28. [PubMed: 17197100]
11. Sacco T, Sacchetti B. Role of secondary sensory cortices in emotional memory storage and retrieval in rats. *Science*. 2010; 329(5992):649–56. [PubMed: 20689011]

12. Rauskolb S, et al. Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2010; 30(5):1739–49. [PubMed: 20130183]
13. Kawashima T, Okuno H, Bito H. A new era for functional labeling of neurons: activity-dependent promoters have come of age. *Frontiers in neural circuits*. 2014; 8:37. [PubMed: 24795570]
14. Monfils MH, Cowansage KK, LeDoux JE. Brain-derived neurotrophic factor: linking fear learning to memory consolidation. *Mol Pharmacol*. 2007; 72(2):235–7. [PubMed: 17522182]
15. Radiske A, et al. Requirement for BDNF in the reconsolidation of fear extinction. *J Neurosci*. 2015; 35(16):6570–4. [PubMed: 25904806]
16. Faraguna U, et al. A causal role for brain-derived neurotrophic factor in the homeostatic regulation of sleep. *J Neurosci*. 2008; 28(15):4088–95. [PubMed: 18400908]
17. Giese M, et al. BDNF: an indicator of insomnia? *Mol Psychiatry*. 2014; 19(2):151–2. [PubMed: 23399916]
18. Datta S, et al. The homeostatic regulation of REM sleep: A role for localized expression of brain-derived neurotrophic factor in the brainstem. *Behav Brain Res*. 2015; 292:381–92. [PubMed: 26146031]
19. Amat J, et al. Activation of the ventral medial prefrontal cortex during an uncontrollable stressor reproduces both the immediate and long-term protective effects of behavioral control. *Neuroscience*. 2008; 154(4):1178–86. [PubMed: 18515010]
20. Guzowski JF, et al. Environment-specific expression of the immediate-early gene *Arc* in hippocampal neuronal ensembles. *Nat Neurosci*. 1999; 2(12):1120–4. [PubMed: 10570490]
21. Steward O, Worley PF. A cellular mechanism for targeting newly synthesized mRNAs to synaptic sites on dendrites. *Proc Natl Acad Sci U S A*. 2001; 98(13):7062–8. [PubMed: 11416188]
22. Plath N, et al. *Arc/Arg3.1* is essential for the consolidation of synaptic plasticity and memories. *Neuron*. 2006; 52(3):437–44. [PubMed: 17088210]
23. Ploski JE, et al. The activity-regulated cytoskeletal-associated protein (*Arc/Arg3.1*) is required for memory consolidation of pavlovian fear conditioning in the lateral amygdala. *J Neurosci*. 2008; 28(47):12383–95. [PubMed: 19020031]
24. Barot SK, et al. Functional imaging of stimulus convergence in amygdalar neurons during Pavlovian fear conditioning. *PLoS One*. 2009; 4(7):e6156. [PubMed: 19582153]
25. Liu X, Tang X, Sanford LD. Stressor controllability and Fos expression in stress regulatory regions in mice. *Physiol Behav*. 2009; 97(3–4):321–6. [PubMed: 19275908]
26. Drugan RC, et al. Impact of water temperature and stressor controllability on swim stress-induced changes in body temperature, serum corticosterone, and immobility in rats. *Pharmacol Biochem Behav*. 2005; 82(2):397–403. [PubMed: 16236352]

Fig. 1.

Escape latencies and shock durations of mice trained with ES (n=11). (A) Latency in ms from a representative animal plotted over 20 shock trials. The solid line demonstrates the variability of individual trials. The dotted trend line indicates improved escape behavior across trials. (B) Mean shock duration was presented in 4 blocks of 5 trials (trial 1–5 (B1), 6–10 (B2), 11–15 (B3), and 16–20 (B4)). Data are presented as percentage of B1 (set to 100%). Graph shows mean $\pm$ SEM. Significant difference compared to B1 is indicated by asterisk (*, $p < 0.05$).

Fig. 2.

Percent freezing plotted for NS (value too small to show, $n=10$), ES (filled, $n=11$), and IS (open, $n=11$) groups during the 5 min pre- and post-ST in the shuttlebox. The percentage time spent in freezing was calculated as FT%: freezing time/5 min. Graph shows mean $\pm$ SEM values. Significant differences in comparisons to NS are indicated by asterisk (*, $p < 0.05$). Significance differences between pre- and post-ST are indicated by ampersand (&).

Fig. 3. Corticosterone levels in NS (shaded), ES (filled), and IS (open) groups at 0 h and 2 h post-ST ($n=4\sim6$ for each group). Graph shows mean $\pm$ SEM values. Significant difference in comparison to NS is indicated by asterisk (*, $p < 0.05$). Significant time effect between 0 h and 2 h is indicated by ampersand (&).

Fig. 4.

RT² qPCR analysis of *c-fos*, *Arc*, and *BDNF* gene expressions at 0 h and 2 h in amygdala, mPFC, and somatosensory cortex. NS (shaded), ES (filled), and IS (open) (n=4~6 for each group). After β -actin normalization, mRNA fold changes of *c-fos* (A, B, C), *Arc* (D, E, F), and *BDNF* (G, H, I) in the ES/IS groups relative to the control 0 h NS (set to 1) was determined and expressed by 2^{-Ct} methods. Graphs show mean $\pm$ SEM values. Significant differences between ES and IS groups are indicated by pound signs (#, $p < 0.05$). Significant differences in comparisons to NS are indicated by asterisks (*), and significant time effects between 0 h and 2 h are indicated by ampersands (&).