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Graded Fear Generalization Enhances the Level of cfos-Positive Neurons Specifically in the Basolateral Amygdala

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Fear is an important emotional reaction in response to threatening stimuli and is important for survival. However, when fear occurs in inappropriate circumstances, it can lead to pathological conditions including an increased vulnerability for developing anxiety disorders such as posttraumatic stress disorder (PTSD). Patients with PTSD generalize fear to contexts or to environments that are not associated with the trauma. We sought to explore if increasing the level of dissimilarity relative to the context in which mice learn fear results in changes in the level of fear responding to the new context. We also determined with this procedure if the number of cells expressing the immediate early gene cfos changes with the corresponding level of expressed fear within brain regions known to be important in modulating fear, including the basolateral amygdala (BLA) and hippocampus. Our results indicate that mice that were tested in increasingly different contexts showed significantly different levels of fear responses. Freezing level was higher in the context most similar to the acquisition context than the one that was highly different. The level of cfos within the BLA, but not hippocampus, was also significantly different between the test contexts, with higher levels in the somewhat similar compared with the most different context. Overall, these results highlight the BLA as a critical region in the node of fear circuitry for modulating fear generalization. © 2016 Wiley Periodicals, Inc.

Key words: fear conditioning; fear generalization; amygdala

INTRODUCTION

Fear is a natural emotional reaction to any threatening stimulus and is important for survival and to protect us from imminent danger. Disproportionate levels of fear can, however, lead to pathological conditions and increased vulnerability for developing anxiety-related disorders such as posttraumatic stress disorder (PTSD) (Orr et al., 2002; Pitman et al., 2012). Fear generalization, a phenomenon through which fear is transferred from a stimulus associated with an aversive event to a similar stimulus, is a characteristic of many anxiety disorders (Grillon and Morgan, 1999; Rothbaum and Davis, 2003; Milad et al., 2006; Jovanovic et al., 2010; Norrholm et al., 2011; Sijbrandij et al., 2013; Lissek et al., 2014; Bowers and Ressler, 2015). Hypervigilance, reexperiencing, and avoidance are relevant to fear generalization especially in PTSD (Kessler et al., 1995; APA, 2000, 2013; Yehuda, 2001; Yehuda and LeDoux, 2007; Lissek and Grillon, 2010).

Fear becomes associated with stimuli through Pavlovian fear conditioning. To study this associative process in laboratory animals, a neutral stimulus (conditional stimulus), such as a tone, is paired with an aversive stimulus (unconditional stimulus), such as a foot shock. After this occurs, the conditional stimulus elicits defensive responses such as freezing (Fanselow et al., 1988; Rosen and Schulkin, 1998; Fanselow and Wassum, 2016). Therefore, the time spent freezing provides a good indication of fear in rodents (Fanselow, 1980). Inappropriately high levels

SIGNIFICANCE

As fear generalization occurs in individuals suffering from anxiety disorders including posttraumatic stress disorder, understanding the neurobiology of this phenomenon could help develop effective therapeutics for such disorders. Our results show that the basolateral portion of the amygdala is an important region in which enhanced fear generalization leads to increased neuronal activity.

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of freezing suggest levels of fear that could interfere with normal adaptive functions. In mice, fear generalization can be measured by training animals to fear one context and then testing them in a different context by measuring freezing levels in that context. As mice are exploratory by nature in a novel environment, a higher level of freezing in the novel context indicates higher fear. Given similarities in the fear circuitry in rodents and humans, investigation of the neural circuitry involved in fear generalization in mice is likely to have translational significance (Fendt and Fanselow, 1999; Milad et al., 2006; Mineka and Oehlberg, 2008).

Fear can trigger multiple cellular and molecular cascades including expression of the immediate early gene, cfos. Changes in levels of cfos are extensively used as a proxy for neuronal activity, and analyzing cfos levels within a brain region provides an indirect measure of cellular activity in that region. Processing of context memory requires both emotional and context components, with the amygdala and hippocampus being critical nodes in the respective neural circuits (Davis, 1992; Kim and Fanselow, 1992; Bechara et al., 1995; Maren and Fanselow, 1996; Fanselow and Gale, 2003; LeDoux, 2003; Kim and Jung, 2006; Shin and Liberzon, 2010).

In the present study, we first sought to investigate whether mice demonstrate different levels of freezing, as indicative of different levels of fear generalization, in contexts that are increasingly different from the one in which they acquired the fear learning. Secondly, we wanted to determine whether differences in graded fear generalization lead to changes in expression levels of cfos within the basolateral amygdala (BLA) and hippocampus in a manner that corresponds to the level of freezing. We hypothesized that the level of freezing would be linearly graded across the three increasingly dissimilar contexts and that levels of cfos within the BLA and hippocampus would match the level of freezing.

MATERIALS AND METHODS

Subjects

A total of 20 male PACAP-EGFP mice ($n = 20$) (3–4 months) expressing enhanced green fluorescent protein in PACAP-containing neurons were housed in plastic clear cages in the vivarium with lights on at 7 AM and lights off at 7 PM (Condro et al., 2016). These mice, originally generated on an FVB/NTac background, were serially backcrossed to C57BL/6 for at least five generations. Previous studies have shown dysregulation in the neuropeptide PACAP in patients with PTSD (Ressler et al., 2011). Although it was not a current focus of the experiments presented here, experiments in the future will explore whether there are changes in levels of cfos specifically within PACAP-containing cells in the fear circuitry. Experiments were performed between 10 AM and 3 PM. The mice were kept on ad libitum access to food and water in a light- and temperature-controlled vivarium. All experimental procedures were in accordance with the Animal Research Committee at the University of California, Los Angeles.

Behavioral Procedure

The conditioning apparatus consisted of four sound- and light-attenuated conditioning boxes (Med Associates Inc., Georgia, VT), and mice were run individually in the conditioning boxes (Fig. 1). The conditioning boxes were equipped with a Near Infra-Red (NIR) Video Fear Conditioning System and could be configured to represent different contexts by changing the internal structure, floor texture, illumination, and odor. We used Context A ($28 \times 21 \times 21$ cm) with a clear Plexiglas back wall, ceiling, and front door with aluminum sidewalls. Context A consisted of a grid floor with evenly spaced and stainless steel rods and cleaned and scented with 50% Windex. The floor in context A was connected to a shocking apparatus, which delivered a scrambled foot shock. Context B had a clear Plexiglas back wall, ceiling, and door with aluminum sidewalls. The inner structure of the chamber was altered by adding a white curved sidewall that extended across the back wall. The floors of context B consisted of grid floor with stainless steel rods that were evenly spaced but at alternating heights and cleaned and scented with 1% acetic acid solution. Context C consisted of triangular opaque black Plexiglas sidewalls at an angle of 60° to the floor, with an acrylic white board floor. The context was cleaned and scented with 50% Simple Green and illuminated with just red light. Context D consisted of white acrylic flooring, a white curved sidewall that extended to only one side of the wall. This context was cleaned and odored with 70% isopropyl alcohol and illuminated with just red lights.

Measure of Freezing

Freezing is defined as the lack of movement except for respiration (Fanselow, 1980). The software (VideoFreeze, Med-Associates Inc.) performed real-time video recordings at 30 frames per second using a set threshold level that has been previously validated to match human-scored freezing (Anagnostaras et al., 2001). Each frame has an "activity unit" score, and based on previously validated hand-scoring measures, freezing was defined as a subthreshold activity—that is, when the motion threshold held at 50 activity units for longer than 1 sec. Percentage freezing = time freezing/total time × 100. Data are presented as mean percentages (± standard error of the mean [SEM]).

Fear Generalization

The behavioral testing procedure was divided into two parts. The experimental design is shown in Figure 1. The first part involved fear acquisition, in which mice ($n = 20$) were placed in context A every day and subjected to a 0.65-mA, 1-second foot shock after 4 minutes. Freezing in this context was measured every day for 5 days until all the mice displayed an asymptotic level of freezing. Postshock reactivity to shocks or activity bursts was also measured by analyzing activity bursts after the shock (Fanselow, 1982). After subjects acquired fear, they were divided randomly into three groups (first group, $n = 6$; second group, $n = 7$; and third group, $n = 7$), and each group went to one of the three generalization contexts a day after fear acquisition, and their freezing was measured for 8 minutes. After all groups acquired an asymptotic level of fear, 24 hr later each group was tested in a different generalization

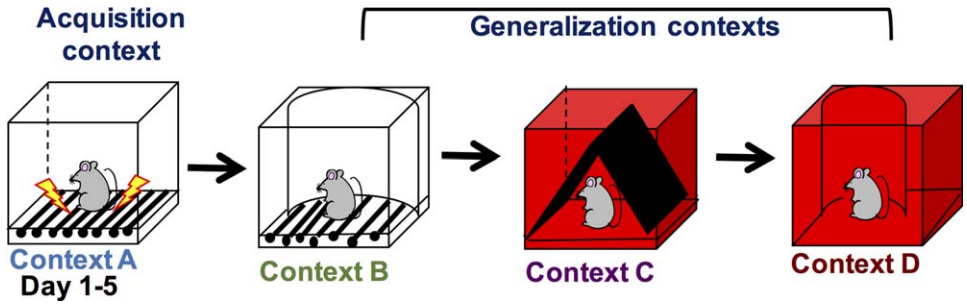

Fig. 1. Behavioral testing procedures for fear acquisition and generalization. Three groups of animals went through fear acquisition first in context A, and then were separately tested in increasingly different contexts B, C, or D.

test context once (Fig. 1). The first group ($n = 6$) went through a fear generalization context, which consisted of different grid floors, scent used, and transport, and their freezing was measured for 8 minutes. The second group ($n = 7$) was placed in a context C that was quite different from the acquisition context, with the grid floor covered with a white plastic board. The last group ($n = 7$) was placed in another context D, which was most different. Ninety minutes after the generalization test, all the animals were anesthetized and perfused with phosphate-buffered saline containing 4% paraformaldehyde, and their brains were kept in the paraformaldehyde for a day at 4°C and transferred into 30% sucrose solution before slicing.

Immunohistochemistry

For immunohistochemistry, brains were cryoprotected, and 40-μm coronal sections were collected serially containing the hippocampus and the BLA. Positive cfos immunolabeling was analyzed and quantified in brain sections containing the BLA and hippocampus. We chose three representative slices from the anterior (Bregma + 2.46), middle (Bregma + 1.94), and posterior (Bregma + 1.62) (Paxinos, 1998) regions. On day 1, tissue sections were washed in 1x Tris-buffered saline (TBS) three times for 5 minutes, then blocked in 1 mL of 1x TBS with 5% normal donkey serum, 0.1% bovine serum albumin, and 0.3% Triton-X for 1 hr. Then the tissue sections were incubated overnight at 4°C with the primary goat polyclonal to cfos (1:500, 24 h, abcam; RRID: SCR_012931) primary antibody. According to the manufacturer, this antibody is a "synthetic peptide conjugated to Blue Carrier Protein by a Cysteine residue linker corresponding to the internal sequence amino acids 283-295 of Human c-Fos (NP_005243.1)." On the second day, the sections were washed in 1x TBS three times for 5 minutes each and then incubated in the Alexa 594 donkey anti-goat secondary antibody (1:200, 2 H, Life Technologies) for 2 hr at room temperature. After washing with 1x TBS three times for 5 minutes each, tissue sections were mounted on glass slides and cover-slipped using Prolong Gold (Thermo Fisher Scientific) with 4′,6-diamidino-2-phenylindole, and the edges were sealed with clear nail polish.

The tissue sections were analyzed using a Keyence BZ-X700 All-in-One Fluorescence Microscope. Images were analyzed with Fiji image processing software (NIH, Bethesda, MD; RRID: SCR_002285). Images were converted to binary mode (black and white image). Two experimenters blind to the experimental conditions counted the cells. Number of positively cfos-labeled neurons within a defined square region (320 × 320 μm) that was held constant within a brain region of interest. The signal density was calculated by setting the density threshold to 80, and an acceptable particle size was set at 0.5 to 80 for immunoreactivity. The pixel sizes and circularity of the cfos-labeled cells were set at a level that allowed automatic subtraction of background. For each animal, cfos cells were counted from both hemispheres and averaged over three sections. Consistency in counting was also verified by another investigator.

Statistical Analysis

Separate groups of mice were used for each generalization test, and their percent freezing levels were analyzed with a two-factor analysis of variance (ANOVA) for the acquisition and with a between-subjects ANOVA for the generalization data. Significant effects indicated by the ANOVA were further analyzed with a post hoc Bonferroni correction. The level of significance used for all analyses was $P < 0.05$.

RESULTS

All Groups of Animals Acquired an Asymptotic Level of Freezing

As shown in Figure 2, all three randomly divided groups of animals acquired asymptotic levels of freezing. A two-factor ANOVA revealed that there was no main effect of group but a main effect of day on the acquired fear in all three groups ($P < 0.05$). Post hoc analysis revealed that day 1 was significantly different from day 5 freezing in all three groups ($P < 0.05$). We also analyzed postshock reactivity in all the groups (Fig. 2B), which revealed that the animals did not differ from each other in their reactivity to shock.

Percent Freezing was Significantly Higher in the Group Tested in Context B versus the Group Tested in Context D

Between-subjects ANOVA revealed that there was a main effect of test context on percent freezing (Fig. 2C; $F = 3.87$, $P < 0.05$). Post hoc test revealed that percent freezing of the group in context B was not significantly

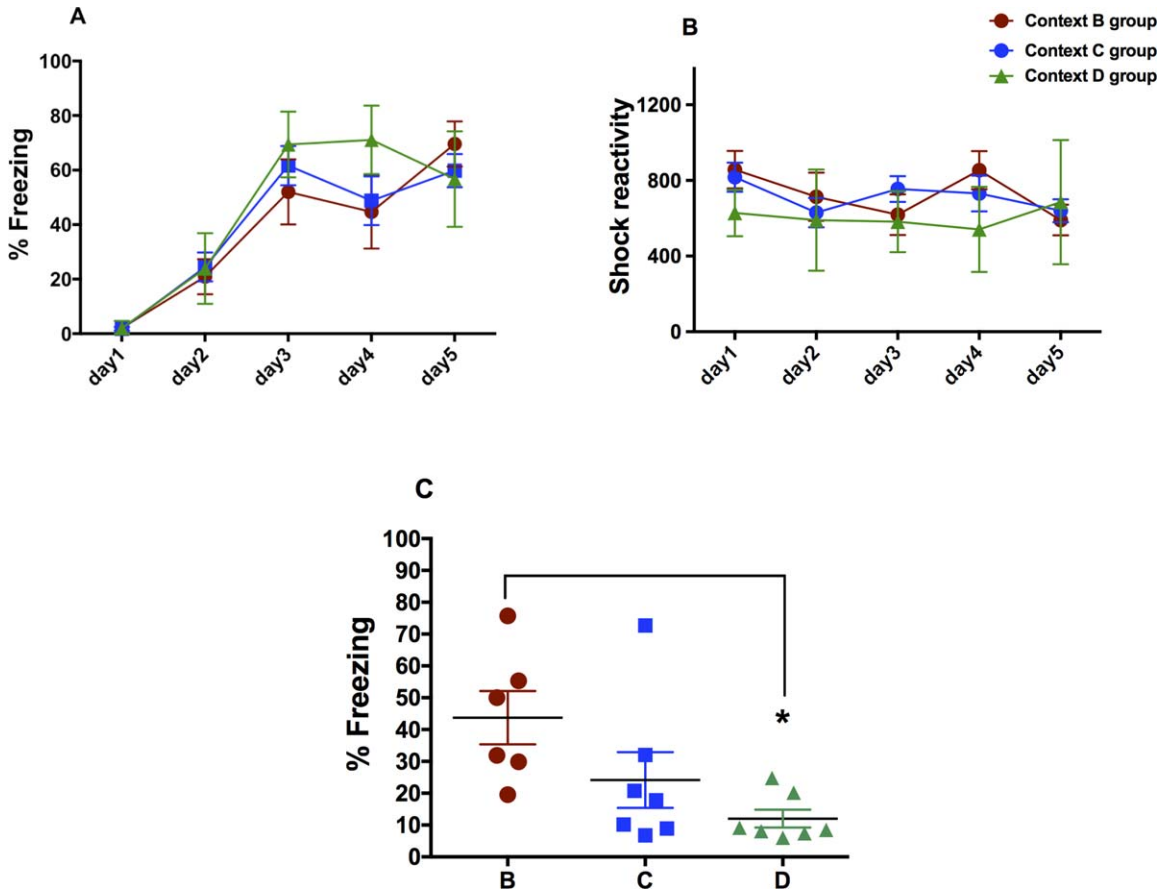

Fig. 2. Mean percent freezing during acquisition, shock reactivity, and mean percent freezing during generalization tests. **A**: Mean percent freezing (± standard error of the mean [SEM]) during the first 4 minutes of the acquisition before delivery of shock in three groups (context B group, $n = 6$; context C group, $n = 7$; and context D group, $n = 7$). Between-subjects analysis of variance revealed that the three groups did not differ from each other in fear acquisition. **B**: Postshock reactivity to shock during acquisition in the three groups was not different. **C**: Mean percent freezing (± SEM) of the three groups of mice during the 8-minute context test in three varying contexts B, C, and D. *Post hoc analysis revealed that mean percent freezing in context B was significantly different from context D ($P < 0.05$).

different from the group in context C; however, it was significantly different from the group in context D ($P < 0.05$). Groups in contexts C and D did not differ from each other in their percent freezing.

Increased Levels of Freezing in the Different Generalization Contexts Led to Increased Number of cfos-Expressing Cells in the BLA but Not in the Hippocampus

To investigate neuronal activity in the BLA and hippocampus in the fear generalization paradigm, we analyzed number of cfos-expressing cells in these brain regions. Between-subjects ANOVA revealed that there was a main effect of context in number of cfos-positive cells in the BLA (Fig. 3A) but not in the hippocampus (Fig. 3B). An individual-sample *t*-test revealed that the number of cfos-positive cells in the group with highest freezing—that is, the group tested in context B—was significantly higher than the group tested in context D, but not different from the group tested in context C. In contrast, ANOVA revealed that there was no main effect of context in the number of cfos-positive cells in the hippocampus (Fig. 3A, B). *t*-Test revealed that cfos expression levels in the BLA were significantly higher than in context D ($P < 0.05$). Again, this was not the case in the hippocampus. The number of cfos-positive cells in the group in context B (highest freezing, Fig. 1C) was significantly higher than the group tested in context D, but not different from the group tested in context C.

DISCUSSION

The first goal of the experiments described here was to determine whether level of contextual fear is graded when tested in three contexts that are increasingly dissimilar to the one in which the initial conditioning or learning took place. The second goal of the experiments was

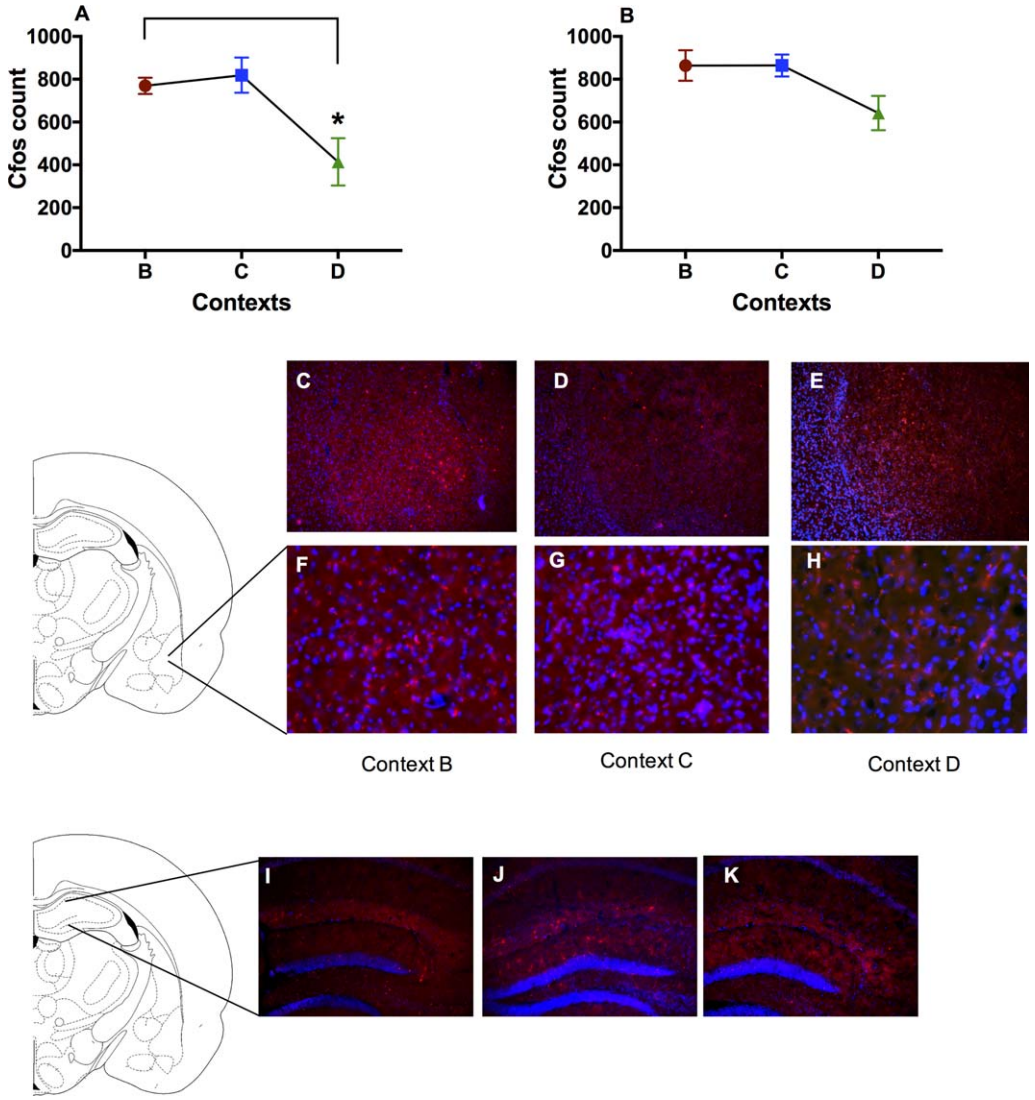

Fig. 3. Level of immunoreactivity of cfos-positive cells in the basolateral amygdala (BLA) and hippocampus of the groups tested in contexts B, C, and D in BLA and hippocampus. **A**: *Post hoc analysis revealed that cfos level in context B was significantly higher than in context D in the BLA ($P < 0.05$). **B**: No difference was found in the level of cfos in the hippocampus. **C–H**: Representative photomicrographs showing cfos immunoreactivity in the BLA. Top panel (**C, D, E**) showing BLA at a 10X magnification and bottom insets showing higher magnification images. **I–K**: Representative photomicrographs showing cfos in the hippocampus.

to determine whether the level of cfos within the BLA and hippocampus matches the level of expressed fear in the test contexts. Our findings revealed that when the mice were conditioned in one context and tested in three contexts that were increasingly dissimilar to the acquisition context, the level of freezing was significantly higher in the context most similar to the conditioning context compared with the one that was completely dissimilar. Moreover, the level of cfos within the BLA was also significantly different between those two contexts.

Our finding that enhanced fear generalization led to increases in cfos within the BLA fits with the findings that show heightened activity in the amygdala in anxiety-related disorders including PTSD and in animal models as a result of fear generalization or decreases in fear extinction (Fendt and Fanselow, 1999; Milad et al., 2006; Shin and Liberzon, 2010). Others have shown that this brain region is important for modulating fear and safety signals by working in concert with other regions such as the medial prefrontal cortex (Likhtik and Paz, 2015). BLA is an anatomically situated hub for providing emotional valence to sensory information via its glutamatergic-auditory, taste, visual, and somatosensory inputs/outputs from and to cortical/subcortical regions, and expresses some GABAergic interneurons. Fear information is processed by the BLA and sent to the central amygdala, which

in turn plays an important role in fear expression via its projections to autonomic and endocrine systems including the hypothalamus, midbrain, and brainstem to elicit behaviors like freezing (LeDoux et al., 1988, 1990; Davis, 1992; Fanselow and LeDoux, 1999; LeDoux, 2000; Jovanovic and Ressler, 2010). While studies have shown the importance of the amygdala in fear acquisition, our study adds to our previous findings that showed that context fear generalization in rats enhances levels of activity regulated cytoskeletal protein, another neuronal activity marker within the BLA specific to context fear but not specifically in the hippocampus (Zelikowsky et al., 2014).

Differential freezing in the different contexts did not lead to a significantly different level of cfos expression in the hippocampus. A plethora of research shows the importance of the dorsal hippocampus (DH) for processing contextual information and the integration of that information into a unified representation of the context that supports differentiation from other contexts (Fanselow, 2000; McHugh et al., 2007; Nakashiba et al., 2012; Krasne et al., 2015). However, this research also shows that the DH does not create the context–fear association (Fanselow, 2000; Barrientos et al., 2002; Stote and Fanselow, 2004). These data for cfos are consistent with data from another immediate early gene, ARC within the DH (Zelikowsky et al., 2014), in supporting these theoretical notions. If the DH supports contextual but not emotional processing, then one would expect similar cfos expression in this region irrespective of what context was being processed. On the other hand, if the BLA is processing the emotional value of the context one would expect, as was found here, that BLA would track emotional responding.

CONFLICT OF INTEREST STATEMENT

None of the authors report any conflicts of interest or financial arrangements pertaining to this work.

ROLE OF AUTHORS

All the authors of this paper had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MSF, AKR, JW. Acquisition of data: AKR. Analysis and interpretation of data: AKR. Drafting of the manuscript: AKR. Critical revision of the manuscript for important intellectual content: AKR, MSF, JW. Statistical analysis: AKR. Obtained funding: JW, MSF. Administrative, technical, and material support: MSF, JW. Study supervision: JW, MSF

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