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Heat exposure in female rats elicits abnormal fear expression and cellular changes in prefrontal cortex and hippocampus

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ABSTRACT

Despite a twofold higher prevalence of fear-related disorders in women, the neurobiological factors that modulate and drive fear expression are rarely studied in female animals. Fear conditioning and extinction are useful tools for dissecting these mechanisms, and here we tested the effects of environmental manipulations – four days of exposure to 31 °C temperatures in the animal housing facility – on fear learning and memory exclusively in female rats. We found that heat exposure disrupted freezing to tone during fear conditioning, and elicited enhanced freezing during extinction and extinction retrieval. We also performed immunohistochemistry for c-fos expression in the infralimbic (IL) and prelimbic (PL) regions of the prefrontal cortex during extinction retrieval, and found that heat exposure induced a switch from IL-dominated activity to PL-dominated activity. Finally, morphological analysis of spines in hippocampal CA3 neurons revealed an increase in spine head diameter in heat-exposed animals, which may partly underlie the persistent freezing observed in these animals. Together, our data show that heat exposure can induce changes at behavioral, physiological, and structural levels, and add to a woefully lacking body of literature on fear processes in female animals.

1. Introduction

Post-traumatic stress disorder (PTSD) is twice as common in women as in men (Haskell et al., 2010), a major public health concern whose biological basis remains to be defined. For decades, research in animal models has used fear conditioning and extinction paradigms to study the neural circuitry and mechanisms involved in the pathophysiology of PTSD (Schafe, Nader, Blair, & LeDoux, 2001). But despite the twofold higher incidence of PTSD in women, less than 2% of all published fear conditioning and extinction research involves female animals (Lebron-Milad & Milad 2012). The rectification of this steep imbalance in the literature will be an important step toward more effectively treating women who suffer from PTSD, especially in light of increased participation by female troops in ongoing conflicts (DoD, 2014).

Of particular relevance to combat-related trauma is the influence of environmental stressors on fear and extinction learning. Prior exposure to stress is a known risk factor for PTSD (Breslau, Chilcoat, Kessler, Peterson, & Lucia, 1999), and there are numerous reports that stress impairs extinction retrieval in male animals (Knox, Nault, Henderson, & Liberzon, 2012; Zhang & Rosenkranz, 2013). Work in females is much more limited; only two published studies explore stress effects on extinction in adult females, with conflicting findings between them (Baran, Armstrong, Niren, Hanna, & Conrad, 2009; Hoffman, Armstrong, Hanna, & Conrad, 2010). Additionally, how stress might affect critical extinction circuitry to alter behavior has not been studied in adult females. Both the medial prefrontal cortex (mPFC) and the CA3 region of the hippocampus are integral to fear extinction (Ji & Maren, 2008; Peters, Dieppa-Perea, Melendez, & Quirk, 2010). Interestingly, these regions also undergo sexually dimorphic responses to stress exposure (Galea et al., 1997; Shansky, Hamo, Hof, McEwen, & Morrison, 2009; Shansky et al., 2010), but the functional consequences of these changes are not known.

One notable aspect to the studies mentioned above is that the stressors are distinct events that occur at least 24 h before behavioral testing begins, an experimental approach that allows analysis of the extended effects of stress exposure without directly interfering with learning processes. However, the traumatic events that lead to PTSD often occur within an environment that is itself stressful. Surprisingly, investigations into the effects of concurrent environmental stress on fear conditioning and extinction have not been done.

Our goals for this study were (1) to contribute to a sparse literature on the mechanisms of extinction in female animals, and (2) to test the validity of prolonged heat exposure as a model for

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environmental influence on fear conditioning and extinction processes. We report that heat exposure disrupted fear conditioning, extinction learning, and extinction retrieval in adult female rats. Moreover, we observed region-specific alterations in activation of the medial prefrontal cortex (mPFC) of heat-exposed animals, as well as morphological changes in hippocampal CA3 neurons.

2. Methods and materials

2.1. Animals

Adult female Long Evans rats ($n = 7$ per group) were individually housed in the Nightingale Animal Facility at Northeastern University on a 12:12 light:dark cycle with access to food and water *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Northeastern University Institutional Animal Care and Use Committee. Females were vaginally swabbed daily to ensure normal estrous cycling.

2.2. Heat exposure

One day prior to the first day of behavioral testing, animal housing room temperature was set at 31 °C, where it remained for the duration of the study.

2.3. Behavioral testing

2.3.1. Apparatus and stimuli

Rats underwent habituation, fear conditioning and fear extinction as adapted from (Sotres-Bayon, Bush, & LeDoux, 2007) in one of four identical chambers constructed of aluminum and Plexiglas walls (Rat Test Cage, Coulbourn Instruments, Allentown, PA), with metal stainless steel rod flooring that was attached to a shock generator (Model H13-15; Coulbourn Instruments). The chambers were lit with a single house light, and each chamber was enclosed within a sound-isolation cubicle (Model H10-24A; Coulbourn Instruments). An infrared digital camera, mounted on top of each chamber, allowed videotaping during behavioral procedures. The computer also controlled stimulus presentation with FreezeFrame software (Coulbourn Instruments). Chamber grid floors, trays, and walls were thoroughly cleaned with water and dried between sessions. Rats were allowed to freely explore the chamber for 4 min before tone presentation on each day began.

2.3.2. Fear conditioning procedure

Conditioning was conducted in groups of four rats at a time, each in a different chamber (see above). All rats were exposed to five tone (CS) presentations (habituation), followed by seven conditioning trials (CS–US pairings) on day 1. The CS was a 30-s, 5 kHz, 80 dB SPL sine wave tone, which co-terminated with a 0.5-s, 0.7 mA footshock US during fear conditioning. Mean intertrial interval was 4 min (2–6 min range) throughout habituation and fear conditioning. Fear behavior was measured using freezing, defined as the cessation of all movement with the exception of respiration-related movement and non-awake or rest body posture. Freezing was continuously recorded during the conditioning session and analyzed using FreezeFrame Software. Minimum bout was set at 2 s. After conditioning, rats were returned to their home cages and to the colony room.

2.3.3. Extinction procedures

Freezing was recorded continuously during the extinction training (20 tone alone trials, day 2) and retrieval sessions (3 tone alone trials, day 3), presented as 2-trial blocks (extinction) or 3-tone average (extinction retrieval) in Fig. 1. Both extinction training and testing took place in the same chamber as fear conditioning, but with different contextual cues (floor, light, and odor). Consistent with the fear conditioning procedure, throughout extinction sessions (training and test) mean inter-trial interval was 4 min (2–6 min range). Extinction training occurred during low-estrogen estrous phases (estrus, metaestrus, diestrus) for all animals, as in (Zeidan et al., 2011).

2.4. Euthanasia

Twenty-five minutes after the completion of Extinction Retrieval testing, animals were anesthetized and sacrificed by transcardial perfusion of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PBS, pH 7.4). Brains were extracted and post-fixed in PFA for 6 h, and then placed in 0.1% sodium azide in PBS at 4 °C for storage.

2.5. Immunohistochemistry

All IHC procedures were conducted by an experimenter blind to the treatment group of the animals. 50 μm prefrontal cortex-containing sections were collected using a vibrating microtome (Leica). Three IL and PL-containing sections (approximate Bregma range +3.0–3.5) from each animal were isolated and washed six times for 10 min in 0.1% Triton-X 100 in PBS (PBS-T), and then incubated in a blocking buffer containing 5% normal donkey serum in PBS-T for 1 h at room temperature. Sections were then incubated with polyclonal goat anti-c-fos 1:2000 (sc-52; Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. Sections were rinsed six times for 10 min in PBS-T and incubated for 1.5 h at room temperature in a biotinylated donkey anti-goat (1:500, 705-065-147, Jackson ImmunoResearch, West Grove, PA). After 6 × 10 min PBS-T washes, sections were incubated with ABC (1:200; Vector Labs, Burlingame CA) for 1.5 h at room temperature. Sections were then washed 6 times for 10 min in PBS-T and washed once in 0.05 M Tris–HCl buffer (pH = 7.6) and visualized in DAB with nickel enhancement. After labeling, sections were rinsed in water, mounted, dried, and coverslipped using Permount mounting media (SP15-100, Fisher Scientific Fairlawn, NJ). Light images of IL and PL were captured with a 20× objective and a zoom of 1.5 on an Olympus (BX51) equipped with a Qimaging Retiga 1300 digital camera (Olympus America, Center Valley, PA). Four 423 μm^2 images per hemisphere (two in each of IL and PL) were collected from each section. Images were imported to ImageJ software and c-fos+ neurons were counted manually by an experimenter blind to experimental conditions.

2.6. Morphology analysis

250 μm, dorsal hippocampus-containing sections were collected using a vibrating microtome (Leica). Iontophoretic microinjections of fluorescent dye Lucifer Yellow were done into hippocampal CA3 neurons using a DC current of 1–6 nA for 5–10 min, until distal processes are filled with dye and no further loading could be observed. Sections were then mounted and coverslipped in Vectashield (Vector Laboratories, Burlingame CA). Dendritic segments were imaged using an Olympus Fluoview 1000 confocal microscope fitted with a 100× microscope and a digital zoom of 3. The selection of a particular branch for optical imaging had to satisfy the following criteria: (1) the entire segment had to fall within a depth of 80 μm, owing to the working distance of the lens; (2) it had to be either parallel or at an acute angle to the coronal surface of the section; and (3) it did not show overlap with other branches that would obscure visualization of spines. Segments from second, third and fourth order branches of the apical tree were selected at random. Approximately 8 segments per neuron were selected. After gain and offset settings were opti-

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