Neurobiology of Learning and Memory xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Hippocampal cytosolic estrogen receptors regulate fear generalization in females

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ARTICLE INFO

Article history:
Received 7 October 2015
Revised 27 December 2015
Accepted 25 January 2016
Available online xxxx

Keywords:
Contextual memory precision
Fear generalization
Estradiol
Hippocampus
Contextual fear
Memory retrieval

ABSTRACT

Generalization of fear responses is a symptom of many anxiety disorders and we have previously demonstrated that female rats generalize fear to a neutral context at a faster rate compared to males. This effect is due in part, to activation of ER and modulation of memory retrieval mechanisms resulting in fear generalization. Given that the effects of estradiol on fear generalization required approximately 24 h, our data suggested possible genomic actions on fear generalization. To determine whether these actions were due to cytosolic versus membrane bound receptors, female rats were given infusions of ICI 182,780, a cytosolic estrogen receptor antagonist, into the lateral ventricle or dorsal hippocampus simultaneously with estradiol treatment or with an ER agonist (DPN). Infusions of ICI into the lateral ventricle or the dorsal hippocampus blocked fear generalization induced by peripheral or central treatment with estradiol or DPN, suggesting that estradiol acts through cytosolic ERβ receptors. In further support of these findings, intracerebroventricular or intra-hippocampal infusions of bovine serum conjugated estradiol (E2-BSA), activating membrane-bound estrogen receptors only, did not induce fear generalization. Moreover, rats receiving intra-hippocampal infusions of the ERK/MAPK inhibitor, U0126, continued to display estradiol-induced generalization, again suggesting that membrane-bound estrogen receptors do not contribute to fear generalization. Overall, these data suggest that estradiol-induced enhancements in fear generalization are mediated through activation of cytosolic/nuclear ER within the dorsal hippocampus. This region seems to be an important locus for the effects of estradiol on fear generalization although additional neuroanatomical regions have yet to be identified.

1. Introduction

Considerable research indicates that contextual fear generalization—the inability to discriminate between different contexts and, thus, recalling a fear memory in neutral contexts—increases over time (For review, see Jasnow, Cullen, & Riccio, 2012). Fear generalization can also be interpreted as a loss of memory precision for contextual cues; thus, mechanisms contributing to the establishment, maintenance, and recall of contextual memory are implicated in this process. Despite the importance of fear generalization as a fundamental component underlying many anxiety disorders, including PTSD (Brewin, 2001; Grillon & Morgan, 1999; Jovanovic et al., 2009), we do not fully understand how this phenomenon occurs. Moreover, females are 60% more likely than males to be diagnosed with an anxiety disorder such as PTSD (Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Kessler et al., 1994; Wang et al., 2005), and the exact cause of this sex difference remains unknown. Estrogens influence fear and anxiety behavior in rodents and humans (Díaz-Véliz, Alarcón, Espinoza, Dussaubat, & Mora, 1997; Frye, Petralia, & Rhodes, 2000; Frye & Walf, 2004; Morgan & Pfaff, 2001, 2002; Morgan, Schulkin, & Pfaff, 2004; Nofrey, Ben-Shahar, & Brake, 2008; Toufexis, Myers, Bowser, & Davis, 2007; Zuluaga et al., 2005), yet the contribution of estrogens to fear generalization has only recently been examined (Lynch, Cullen, Jasnow, & Riccio, 2013; Lynch et al., 2014).

Our previous research demonstrated that female rats displayed a faster rate of fear generalization to a neutral context after passive avoidance training compared to male rats; an effect driven, in part, by estradiol (Lynch et al., 2013). These findings were the first to show enhanced context fear generalization driven by estradiol and suggested a novel modulatory role for the steroid hormone on generalization mechanisms. Additionally, we utilized injections of estradiol during the learning and memory process and found that systemic injections of 17β-estradiol resulted in increased fear generalization only at a time point thought to effect memory

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http://dx.doi.org/10.1016/j.nlm.2016.01.010
1074-7427/

retrieval (Lynch et al., 2014). However, the specific brain regions and downstream mechanisms through which estrogens modulate the precision of contextual memory retrieval and generalization have not been characterized.

One brain region implicated in memory generalization is the hippocampal formation. In addition, the hippocampus contains an abundance of estrogen receptors (ERs), and estrogens have effects on hippocampal neuronal morphology across the estrous cycle (Beltrán-Campos et al., 2011; Gould, Woolley, Frankfurt, & McEwen, 1990; Shors, Chua, & Falduto, 2001; Wallace, Luine, Arellanos, & Frankfurt, 2006; Woolley & McEwen, 1992). Considerable data indicate that estrogens have *classical* genomic effects occurring within a time frame of hours to days that are driven by cytosolic estrogen receptors (Couse & Korach, 1999; Etgen, 1984; Falkenstein, Tillmann, Christ, Feuring, & Wehling, 2000; McKenna & O'Malley, 2002; O'Malley & Means, 1974). In addition to *classical activation*, estrogens can also have rapid signaling through membrane-bound receptors with effects occurring within a time frame of seconds to minutes (Vasudevan & Pfaff, 2007). A number of recent studies have suggested that estradiol enhances object recognition through activation of membrane bound ERs within the hippocampus and through subsequent ERK/MPK and metabotropic glutamate receptor signaling (Fan et al., 2010; Fernandez et al., 2008; Gresack & Frick, 2006; Lewis, Kerr, Orr, & Frick, 2008; Zhao, Fan, & Frick, 2010). However, given the time frame in which estradiol induces fear generalization in our studies (i.e. between 6 and 24 h) (Lynch et al., 2013, 2014), we hypothesized that estradiol-induced generalization occurs via a genomic effect on retrieval, requiring the activation of cytosolic ERs within the hippocampus.

2. Methods

2.1. Animals and housing conditions

Adult female ovariectomized (OVX) Long Evans rats approximately 90 days old were used for all experiments. Eleven days prior to behavioral manipulations, animals were ovariectomized, cannulated, and then individually housed and maintained on a 14/10 h light/dark cycle (Lynch et al., 2013, 2014). Food and water were available *ad libtum* throughout the experiment. All animal procedures were carried out in accordance with Kent State University Institutional Animal Care and Use (IACUC) guidelines.

2.2. Passive avoidance procedure

Behavior was conducted in a black/white passive avoidance chamber (52 × 30 × 35 cm, Passive Avoidance Apparatus 7550, Ugo Basil, Comerio, Italy). Female rats were trained in passive avoidance 11 days after ovariectomy. For training, animals were brought to Context A (training context), held on the experimenter's hand for 30 s, and placed on the white side of the shuttle box. The door was raised after 20 s and the initial latency to cross into the black compartment (all four paws) was recorded. Upon crossing, the sliding door closed and 5 s after closing, a 2-s, 1.0 mA scrambled footshock was delivered. Ten seconds after receiving the footshock, the animal was removed from the chamber and returned to the main colony.

For testing, rats were brought back into the experimental room at the specific retention interval. Half of the rats were tested in Context A (training) and half in Context B (neutral). Context A was a 1.6 × 2.33 m room with house fluorescent lights and contained bare white walls and no artificial scents or sounds and was cleaned with 70% Ethanol; Context B was a 1.83 × 2.74 m room that was lit by a 25-w red light bulb with posters on the walls. Context B had White noise (70 db) and was cleaned with 60% quatricide. In each context, the experimenter wore different gloves (Rubber dish glove in A; vinyl lab glove in B) to handle the rat. The test procedure was identical to training except the sliding door remained open for a maximum of 540 s and no shocks were delivered. The initial latency to cross was recorded as the dependent measure of fear behavior. Any animal that did not cross was given a score of 540 s. Upon crossing or at 540 s, the animal was removed and returned to the main colony.

2.3. Surgical procedures

For ovarectomies, adult female rats were anesthetized with isoflurane vapors and received a bilateral ovariectomy through a dorsal incision (Lynch et al., 2013, 2014). Immediately after ovariectomy, rats were placed in a stereotaxic instrument for implantation of guide cannulas aimed at the lateral ventricle or dorsal CA1. Stereotaxic coordinates were derived from (Paxinos and Watson, 1986). The head was positioned in the stereotaxic instrument so that the skull was level between lambda and bregma before implantation of the guide cannulas. Rats were implanted with a unilateral cannula (Plastics One) aimed at the lateral ventricle (D/V: −3.4; A/P: −0.9; M/L: +1.6) or bilateral cannula aimed at the dorsal CA1 hippocampus (14°, D/V: −3.1; A/P: −4.0; M/L: +3.3). Dummy cannula were screwed in place to keep them patent. Animals were allowed to recover for 9 days and then were handled for 5 min a day for 2 consecutive days before passive avoidance training.

2.4. Site verification

Cannula placement was verified using 0.5 μl infusions of xylene cyanol FF at 0.25% in saline followed by rapid decapitation. Brains were fresh frozen and sliced on a cryostat and slices were mounted and observed for correct placement using an inverted microscope. Any animal with a misplaced cannula was not included in the final analysis (3% of animals).

2.5. Drug administration

Estradiol benzoate (E2) (Caymen Chemical) was diluted in 50% dimethylsulfoxide (DMSO) at a 10 mM concentration for intracranial infusions (Kuroki, Fukushima, Kanda, Mizuno, & Watanabe, 2000). E2 for peripheral administration was dissolved in sesame oil (15 μg/0.1 mL) (Chang et al., 2009; Zeidan et al., 2011). Estradiol benzoate conjugated to BSA (E2-BSA) was centrifuged in a centrifugal filter unit with a molecular weight cut-off of 3000 kDa (Millipore) and spun at 16,110*g* for 10 min. Filters were washed with 5% DMSO, spun for another 10 min at 16,110g, and washed again with 5% DMSO before being spun for 30 min at 16,110*g* (Santollo, Marshall, & Daniels, 2012; Taguchi, Koslowski, & Bodenner, 2004). The MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene; Sigma Aldrich) was dissolved in 50% DMSO to a concentration of 1 μg/μl for a final dose of 0.5 μg per hemisphere (Fernandez et al., 2008; Fortress, Fan, Orr, Zhao, & Frick, 2013; Zhao, Fan, Fortress, Boulware, & Frick, 2012). The cytosolic ER antagonist, ICI 182,780 was dissolved in DMSO at a concentration of 50 μg/μl for ICV and intrahippocampal infusions. The ERα specific agonist, PPT (4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*-phenol; Caymen Chemical) was dissolved in DMSO at a concentration of 0.2 pg/μl and infused at a dose of 0.1 pg per hemisphere. The ERβ specific agonist, DPN (2,3-*bis*(4-hydroxyphenyl)-propionitrile, Caymen Chemical) was dissolved in DMSO at a concentration of 40 pg/μl (Boulware, Heisler, & Frick, 2013). At these low doses, PPT and DPN are specific for ERα and ERβ, respectively (Stauffer et al., 2000).

Please cite this article in press as: Lynch III, J. F., et al. Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of Learning and Memory* (2016), http://dx.doi.org/10.1016/j.nlm.2016.01.010