Neurobiology of Learning and Memory 155 (2018) 42–49

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

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

Short communication

Contextual fear retrieval-induced Fos expression across early development in the rat: An analysis using established nervous system nomenclature ontology

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ARTICLE INFO

Keywords:
Contextfear conditioning
Development
Amygdala
Prefrontal cortex
Brain atlas
Fos

ABSTRACT

The neural circuits underlying the acquisition, retention and retrieval of contextual fear conditioning have been well characterized in the adult animal. A growing body of work in younger rodents indicates that context-mediated fear expression may vary across development. However, it remains unclear how this expression may be defined across the full range of key developmental ages. Nor is it fully clear whether the structure of the adult context fear network generalizes to earlier ages. In this study, we compared context fear retrieval-induced behavior and neuroanatomically constrained immediate early-gene expression across infant (P19), early and late juvenile (P24 and P35), and adult (P90) male Long-Evans rats. We focused our analysis on neuroanatomically defined subregions and nuclei of the basolateral complex of the amygdala (BLA complex), dorsal and ventral portions of the hippocampus and the subregions of the medial prefrontal cortex as defined by the nomenclature of the Swanson (2004) adult rat brain atlas. Relative to controls and across all ages tested, there were greater numbers of Fos immunoreactive (Fos-ir) neurons in the posterior part of the basolateral amygdalar nuclei (BLAp) following context fear retrieval that correlated statistically with the expression of freezing. However, Fos-ir within regions having known connections with the BLA complex was differentially constrained by developmental age: early juvenile, but not adult rats exhibited an increase of context fear-dependent Fos-ir neurons in prelimbic and infralimbic areas, while adult, but not juvenile rats displayed increases in Fos-ir neurons within the ventral CA1 hippocampus. These results suggest that juvenile and adult rodents may recruit developmentally unique pathways in the acquisition and retrieval of contextual fear. This study extends prior work by providing a broader set of developmental ages and a rigorously defined neuroanatomical ontology within which the contextual fear network can be studied further.

1. Introduction

A putative neural circuit underlying contextual fear conditioning has been described in young adult animals, 60–120 days of age (Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014; Orsini, Yan, & Maren, 2013; Poulos, Ponnusamy, Dong, & Fanselow, 2010). In adult rat and mouse, the basolateral complex of the amygdala (BLA complex), dorsal and ventral parts of the hippocampus (dH, vH) and subregions of the medial prefrontal cortex (mPFC) are involved in the acquisition, retention, retrieval and extinction of context mediated fear learning (Fanselow & Poulos, 2005; Kim & Jung, 2006; Rozeske, Valerio, Chaudun, & Herry, 2015; McCullough, Morrison, & Ressler, 2016). The extent to which neural activity in this network serves the developing organism remains to be fully established, but evidence suggests that context-mediated fear behaviors may recruit and utilize distinct neural pathways during development (Kim & Richardson, 2009; Shechner, Hong, Britton, Pine, & Fox, 2014; Baker, Den, Graham, & Richardson, 2014; Baker, Bisby, & Richardson, 2016; Callaghan & Richardson, 2014; Jones & Monfils, 2016). Accordingly, we sought to quantify Fos-immunoreactive (Fos-ir) neurons in this context fear neural network in 19-, 24-, 35- and 90-day old male rats using a defined ontology of brain regions to generate novel hypotheses concerning developing context fear neural circuits and to maximize intra- and inter-laboratory reliability of these experimental findings.

In adult mammals, memories of contextual stimuli are established and formed within subfields of the hippocampus (Conejo, Gonzalez-

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https://doi.org/10.1016/j.nlm.2018.05.015
Received 12 January 2018; Received in revised form 7 May 2018; Accepted 19 May 2018
Available online 25 May 2018
1074-7427/

Pardo, Lopez, Cantora, & Arias, 2007), which project directly (vH) and indirectly (dH) to nuclei of the BLA complex. Concurrent activation of these and footshock-responsive BLA neurons form the basis of an associative context-fear memory (Schauz & Koch, 2000; Alvarez, Biggs, Chen, Pine, & Grillon, 2008; Johansen, Tarpley, LeDoux, & Blair, 2010). Subsequent retrieval of context fear depends upon sufficient reactivation of hippocampal (Raybuck & Lattal, 2014) and BLA neurons, followed by further activation of defensive motor pathways (Amano, Duvarci, Popa, & Paré, 2011).

In the developing rat, the expression of context fear emerges around postnatal day (P) 24 (Pugh & Rudy, 1996; Raineki et al., 2010 but see Foster & Burman, 2010; Callaghan & Richardson, 2014). This expression has been largely attributed to the functional emergence of the dH. At these ages as in adult rats, pharmacological inactivation or ablation of the dH has been shown to produce anterograde amnesia for context fear memories, while the protein expression of the immediate early gene (IEG), *c-fos,* within the dH reveals a conditioning dependent increase (Raineki et al., 2010; Foster & Burman, 2010; Schiffino, Murawski, Rosen, & Stanton, 2011; Robinson-Drummer, Dokovna, Heroux, & Stanton, 2016). However, other reports examining IEG mRNA of c-Fos and Early growth response 1 (EGR1) in the dH of juvenile rats have did not identify similar conditioning related effects, which may due to different behavioral procedures employed and/or the ages tested (Asok, Schreiber, Jablonski, Rosen, & Stanton, 2013; Schreiber, Asok, Jablonski, Rosen, & Stanton, 2014; Deal, Erickson, Shiers, & Burman, 2016; Heroux et al., 2018). Recent work in adult animals has also implicated a compensatory role of medial prefrontal cortical (mPFC) regions in contextual fear conditioning in animals with pre-training damage to the dH (Zelikowsky et al., 2013). In juvenile rats, pharmacological antagonism of muscarinic and glutamatergic neurotransmitter systems within the mPFC alone can disrupt context fear learning (Heroux, Robinson-Drummer, Rosen, & Stanton, 2016; Robinson-Drummer, Heroux, & Stanton, 2017). This function of mPFC may extend to early adulthood (~P60), however its timeline is largely unknown (Chakraborty, Asok, Stanton, & Rosen, 2016). Although these observations have been established in juvenile and adult rats independently, the extent to which these observations under conserved conditioning procedures are age-dependent remains to be fully anatomically characterized.

Given the emergence of connectome-based neuroanatomical mapping of neural pathways (Swanson & Lichtman, 2016), a significant and emerging challenge in behavioral neuroscience is the mapping – within a formally defined spatial model of the brain – of molecular expression patterns associated with the functional activation of brain regions during specific behaviors (Watts, Khan, Sanchez-Watts, Salter, & Neuner, 2006; also see Bota, Sporns, & Swanson, 2012). Recently, this challenge has been addressed for certain aspects of feeding behavior by Zséli et al., (2016), who mapped expression patterns of Fos, using the formal nervous system nomenclature ontology proposed by Swanson (1992, 1999, 2004, 2018). In the present study, we have begun applying this strategy toward the postnatal development of contextual fear conditioning to examine in infant, juvenile and adult rats the activation of anatomically heterogeneous regions associated with the retrieval of context fear memory. These regions include the hippocampus, mPFC, and BLA complex (including the lateral amygdalar nucleus (LA) and the anterior and posterior portions (BLAa, BLAp) of the basolateral amygdalar nuclei. We hypothesize that delayed but not immediate footshock will result in context fear retrieval in juvenile and adult (Blanchard, Fukunaga, & Blanchard, 1976; Fanselow, 1986; Landeira-Fernandez, DeCola, Kim, & Fanselow, 2006), but not in infant rats. Furthermore, we hypothesize that this retrieval would correspond to elevations in the numbers of Fos-ir neurons among subregions/nuclei of the hippocampus and basolateral portions (anterior and posterior) of the BLA complex. Finally, we also hypothesize that there is an age-dependent recruitment of the mPFC in the retrieval of context fear, evident by elevated numbers of Fos-ir neurons within juvenile, but not adult animals.

To test these hypotheses, we performed a detailed analysis of contextual fear retrieval and associated neural activation in infant (P19), juvenile (P24 & P35), and adult (P90) rats matched to specific rodent brain atlas plates. Retrieval of context fear was assessed in subjects that received an un-signaled delayed (3 min) or immediate (< 3 s) footshock. Ninety minutes following context fear testing, subjects were sacrificed, and their brain tissue processed for Fos immunohistochemistry. Nissl-counterstained tissue sections containing the Fos-ir patterns, selected on the basis of their correspondence to specific atlas maps in the Swanson (2004) rat brain atlas, were used to define and parcellate nuclei of the BLA complex, hippocampal formation and prefrontal cortex. As previously described by Zséli et al (2016), we sought to ensure that our semi-quantitative analysis of Fos-IR were performed from comparable tissue representations of each subnuclei, so as to decrease sampling error across subjects (Simmons & Swanson, 2009) of distinct ages. Fos-ir neurons within a single representation of each subnuclei were quantified across training condition and age. Portions of these data have been presented in preliminary form (Santarelli et al., 2016).

2. Materials and methods

Two-day old male Long-Evans rats were cross fostered among dams and randomly assigned to specific testing age groups that were weaned at 21 days of age. For future analysis of neuroanatomical tract tracing not presented in this report, subjects were each anesthetized (3–5% isoflurane) and iontophoretically infused (three days prior to conditioning) with the retrogradely transportable tract tracer, Fluorogold, using a 20-μm diameter glass micropipette targeting the right BLA complex. Prior to experimental conditioning procedures, subjects on three consecutive days were habituated to handling and transportation procedures. Four groups of animals were used in this experiment: two trained groups and two control groups. The trained groups received a single 1.5 mA footshock, which occurred after a 3-min delay (dS group) or immediately (iS group) upon placement into the conditioning apparatus. The control groups were either exposed to conditioning apparatus, with no footshock (nS group) or remained in the home cage (HMC group). Twenty-four hours later, subjects were returned to the conditioning apparatus for a 4-min test of context fear (dS, iS, nS) or remained in the home cage (HMC). Freezing, defined as the absence of any movement except that related to respiration (Fanselow, 1980), was visually scored by a trained observer blind to the experimental conditions. Ninety minutes after the testing period ended, all subjects were deeply anesthetized and transcardially perfused with 4% *p*-formaldehyde. Brains were extracted, post-fixed in the same fixative for 72 h, and cut into 50 μm-thick coronal sections. Selected tissue sections were immunostained for Fos protein and counterstained with a fluorescent Nissl stain (see Supplemental Materials for details, including Research Resource Identifiers (RRIDs) for all antibody and counterstaining reagents, per the guidelines of Bandrowski et al., 2016). Brain sections were mounted onto glass slides and scanned at ×20 magnification using a fluorescent microscope to examine Nissl and Fos labeling patterns. For each image, the regions and their constituent nuclei were parcellated and analyzed for Fos-ir neurons by trained experimenters blind to the experimental conditions of each subject (for details, see Supplemental Materials). The numbers of Fos-ir neurons within a single subnuclei/region representation (50 μM) for each age group were analyzed by univariate ANOVAs followed by either *post-hoc (Bonferroni correction)* or *planned comparisons as* detailed in Table 1.

3. Results

3.1. Behavioral analysis

Overall, freezing resulted from a significant interaction between age