May 20, 2018

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Anthony J. Santarelli, *University at Albany, State University of New York*
Arshad M. Khan
Andrew M. Poulos, *University at Albany, State University of New York*

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

Anthony J. Santarelli[a], Arshad M. Khan[b], Andrew M. Poulos[a,*,1]

[a] Department of Psychology, Center for Neuroscience, State University of New York, University at Albany, Albany, NY 12222, USA
[b] UTEP Systems Neuroscience Laboratory, Department of Biological Sciences and Border Biomedical Research Center, University of Texas at El Paso, El Paso, TX 79968, USA

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-

* Corresponding author at: Department of Psychology, University at Albany, SUNY, Life Sciences Bldg., Room 1058, 1400 Washington Avenue, Albany, NY 12222, USA.
E-mail address: apoulos@albany.edu (A.M. Poulos).
[1] AMK and AMP are co-senior authors of this study.

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

Table 1
Statistical analysis: structure and results. Illustrated is the sequence of analysis with *a-priori* tests followed by exploratory one-way ANOVAs. Bonferroni corrected multiple comparisons were completed following significant ANOVAs.

Planned comparisons dS vs. nS

	P24		P35		P90	
Region	*T-Value*	*Significance*	*T-Value*	*Significance*	*T-Value*	*Significance*
BLAa	1.2584	ns	1.1154	ns	1.1806	ns
BLAp	**4.453**	**p < 0.01**	**3.269**	**p < 0.01**	**3.783**	**p < 0.01**
CA1v	0.9198	ns	0.2687	ns	**2.8041**	**p < 0.05**
CA1d	0.4219	ns	0.0062	ns	1.1898	ns
PL	**3.447**	**p < 0.01**	–	–	–	–
IL	**2.417**	**p < 0.05**	–	–	–	–

Exploratory univariate ANOVA comparisons

	P19		P24		P35		P90	
Region	*Univariate F*	*Significance*	*Univariate F*	*Significance*	*Univariate F*	*Significance*	*Univariate F*	*Significance*
BLAa	$F_{(3,22)} = 0.199$	ns	$F_{(3,20)} = 2.191$	ns	$\mathbf{F_{(3,23)} = 4.518}$	**p < 0.05**	$F_{(3,22)} = 2.952$	p < 0.1
BLAp	$F_{(3,22)} = 0.315$	ns	$\mathbf{F_{(3,20)} = 13.609}$	**p < 0.01**	$\mathbf{F_{(3,23)} = 13.447}$	**p < 0.01**	$\mathbf{F_{(3,22)} = 8.727}$	**p < 0.01**
CA1v	$\mathbf{F_{(3,22)} = 3.382}$	**p < 0.05**	$F_{(3,20)} = 1.019$	ns	$F_{(3,23)} = 2.872$	p < 0.1	$\mathbf{F_{(3,22)} = 3.415}$	**p < 0.05**
CA1d	$F_{(3,22)} = 0.202$	ns	$F_{(3,20)} = 1.057$	ns	$F_{(3,23)} = 0.697$	ns	$F_{(3,22)} = 0.920$	ns
PL	$\mathbf{F_{(3,22)} = 3.205}$	**p < 0.05**	$\mathbf{F_{(3,20)} = 4.305}$	**p < 0.05**	$F_{(3,23)} = 0.702$	ns	$F_{(3,22)} = 0.747$	ns
IL	$F_{(3,22)} = 1.096$	ns	$F_{(3,20)} = 3.075$	p < 0.1	$F_{(3,23)} = 0.240$	ns	$F_{(3,22)} = 0.379$	ns
ACA	$F_{(3,22)} = 1.06$	ns	$F_{(3,20)} = 0.567$	ns	$F_{(3,23)} = 1.263$	ns	$F_{(3,22)} = 0.485$	ns
LA	$F_{(3,22)} = 0.051$	ns	$F_{(3,20)} = 2.451$	ns	$\mathbf{F_{(3,23)} = 4.564}$	**p < 0.05**	$F_{(3,22)} = 2.662$	p < 0.1
dCA3	$F_{(3,22)} = 0.572$	ns	$F_{(3,20)} = 0.579$	ns	$F_{(3,23)} = 0.441$	ns	$F_{(3,22)} = 0.114$	ns
dDG	$F_{(3,22)} = 2.493$	ns	$F_{(3,20)} = 0.335$	ns	$F_{(3,23)} = 0.733$	ns	$F_{(3,22)} = 1.579$	ns
rENTl	$F_{(3,22)} = 0.464$	ns	$F_{(3,20)} = 0.982$	ns	$F_{(3,23)} = 0.844$	ns	$\mathbf{F_{(3,22)} = 4.967}$	**p < 0.05**
PERI	$F_{(3,22)} = 0.831$	ns	$F_{(3,20)} = 1.609$	ns	$F_{(3,23)} = 2.303$	ns	$\mathbf{F_{(3,22)} = 5.377}$	**p < 0.01**
vCA3	$F_{(3,22)} = 3.116$	p < 0.1	$F_{(3,20)} = 0.648$	ns	$F_{(3,23)} = 0.627$	ns	$F_{(3,22)} = 1.05$	ns
cENTl	$F_{(3,22)} = 0.564$	ns	$F_{(3,20)} = 0.839$	ns	$F_{(3,23)} = 3.031$	p < 0.1	$F_{(3,22)} = 3.049$	p < 0.1

Bonferroni corrected multiple comparisons

		dS vs. HMC	*dS vs. nS*	*dS vs. iS*	*iS vs. HMC*	*iS vs. nS*	*nS vs. HMC*
P19	*CA1v*	ns	ns	ns	ns	ns	ns
	PL	ns	ns	ns	ns	ns	ns
P24	*BLAp*	**p < 0.01**	–	**p < 0.01**	ns	ns	ns
	PL	ns	–	ns	ns	ns	ns
P35	*BLAa*	**P < 0.05**	–	ns	ns	ns	ns
	BLAp	**p < 0.01**	–	**p < 0.05**	ns	ns	ns
	LA	**p < 0.05**	ns	ns	ns	ns	ns
	cENTl	ns	ns	ns	ns	ns	ns
P90	*BLAp*	**p < 0.01**	–	ns	**p < 0.05**	ns	ns
	CA1v	**p < 0.05**	–	ns	ns	ns	ns
	rENTl	**p < 0.05**	ns	ns	**p < 0.05**	ns	**p < 0.05**
	PERI	**p < 0.01**	ns	ns	ns	ns	**p < 0.05**

Correlations

	BLAa-Freezing	*BLAp-Freezing*	*LA-Freezing*	*PL-BLAp*	*IL-BLAp*	*vCA1-BLAp*
P19	r = 0.153	**r = 0.606**	r = 0.424	r = 0.060	r = −0.041	r = 0.093
	ns	**p < 0.01**	ns	ns	ns	ns
P24	r = 0.031	**r = 0.636**	r = 0.041	**r = 0.500**	r = 0.272	r = -0.273
	ns	**p < 0.05**	ns	**p < 0.1**	ns	ns
P35	r = 0.179	**r = 0.662**	r = 0.341	**r = 0.504**	r = 0.490	r = 0.222
	ns	**p < 0.01**	ns	**p < 0.05**	p < 0.1	ns
P90	r = 0.012	**r = .528**	r = −0.024	r = 0.154	r = 0.358	r = 0.459
	ns	**p < 0.05**	ns	ns	ns	p < 0.1

and condition (Fig. 1C). ($F_{(9,87)} = 5.042$, $p < 0.01$) indicating that contextual fear conditioning increased with developmental age (P19 [dS n = 6, iS n = 5, nS n = 7, HMC n = 4], P24 [dS n = 5, iS n = 4, nS n = 6, HMC, n = 5], P35 [dS n = 5, iS n = 5, nS n = 6, HMC n = 7] and P90 [dS n = 7, iS n = 6, nS n = 4, HMC n = 5]). In support of our behavioral hypothesis, delayed, but not immediate footshock resulted in a significant increase in context elicited freezing relative to non-shock controls in P24 ($t_{(9)} = 1.995$, $p < 0.05$), 35 ($t_{(9)} = 10.619$, $p < 0.01$), and 90 ($t_{(9)} = 11.760$, $p < 0.01$) rats. Moreover, as predicted, neither dS or iS exhibited significant freezing in P19 rats. In addition, freezing was significantly greater in dS than iS groups in P35 ($t_{(8)} = 7.877$, $p < 0.01$) and P90 ($t_{(11)} = 4.717$, $p < 0.01$), but not P24 rats. Post hoc comparisons of dS freezing between age groups revealed that P90 rats froze to a greater extent than P19 ($p < 0.01$) and

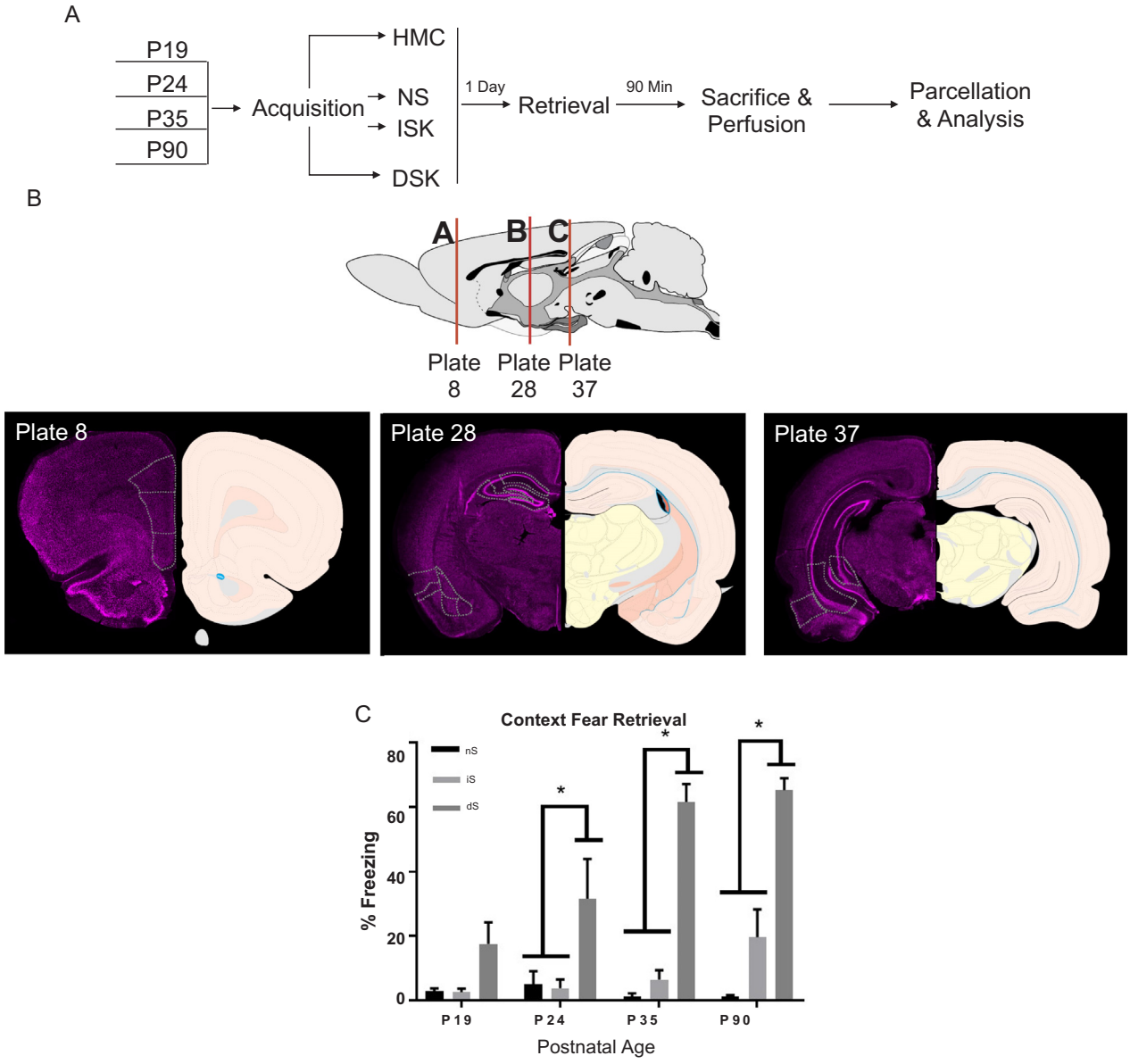

Fig. 1. Study design and behavioral testing results: A: Subjects were randomly assigned into either the HMC (home cage), nS (no shock), iS (immediate-shock), or dS (delayed-shock) groups. Context fear retrieval was assessed 24 h post-conditioning and was followed by sacrifice and perfusion of the experimental animals. B: Representative examples of the sections mapped to corresponding Swanson (2004) atlas levels 8, 28 and 38. C: Context fear retrieval represented as a total mean percentage of time spent freezing (+SEM) during a 4-min context fear test. Asterisks (*) denote significance at the $p < 0.05$ threshold. The number sign (#) denotes a trend toward significance at the $p < 0.1$ threshold. The open access maps in Panel B are reproduced from Swanson (2004) *Brain maps: structure of the rat brain*, 3rd edition; under the terms of a Creative Commons Attribution-NonCommercial 4.0 International License (https://creativecommons.org/licenses/by-nc/4.0/) and are available for download at larrywswanson.com.

P24 rats ($p < 0.05$). This was further supported by a significant linear trend analysis, which found that freezing in the dS, but not iS or nS conditions increased with age ($F_{(3,19)} = 8.940$, $p < .01$).

3.2. BLA complex activation

In order to determine context fear retrieval-induced BLA complex neural activation, we assessed Fos-ir within BLAp (Fig. 2A). LA, and BLAa (Table 1, Supplemental Fig. S2). Separate one-way ANOVAs for the numbers of Fos-ir neurons within the BLAp identified a main effect of condition for each age group [P24 ($F_{(3,20)} = 13.609$, $p < .01$); P35 ($F_{(3,19)} = 13.447$, $p < 0.01$); P90 ($F_{(3,23)} = 8.727$, $p < 0.01$)], except P19 rats ($F_{(3,22)} = 0.315$, $p > .05$). In support of our hypothesis, P24, P35, and P90 rats – which for the most part all demonstrated context freezing under delayed shock – displayed numbers of Fos-ir neurons within the BLAp that were significantly elevated in dS compared to nS [P24($t_{(9)} = 4.453$, $p < 0.01$); P35($t_{(9)} = 3.269$, $p < 0.01$); P90($t_{(9)} = 3.783$, $p < 0.01$)] conditions. Post-hoc tests revealed significant elevations in the numbers of Fos-ir neurons in dS compared to HMC [P24($t_{(10)} = 4.164$, $p < 0.01$); P35($t_{(10)} = 10.497$, $p < 0.01$); P90($t_{(10)} = 4.1636$, $p < 0.01$) and iS [P24 ($t_{(7)} = 3.448$, $p < 0.05$); P35($t_{(8)} = 4.707$, $p < 0.01$)] conditions after correction for multiple comparisons. However, the dS and iS comparison of Fos-ir neuron counts for the P90 rats was not significantly different [P90($t_{(10)} = 4.164$, $p < 0.01$)] nS]. In P19 rats, which showed no significant freezing, the numbers of Fos-ir neurons within the BLAp did not differ among any experimental conditions. For each age group, we detected a positive correlation between the numbers of BLAp Fos-ir neurons and freezing (P19 ($r_{(18)} = 0.606$, $p < 0.01$); P24 ($r_{(15)} = 0.636$, $p < 0.05$); P35 ($r_{(16)} = 0.662$, $p < 0.01$); P90

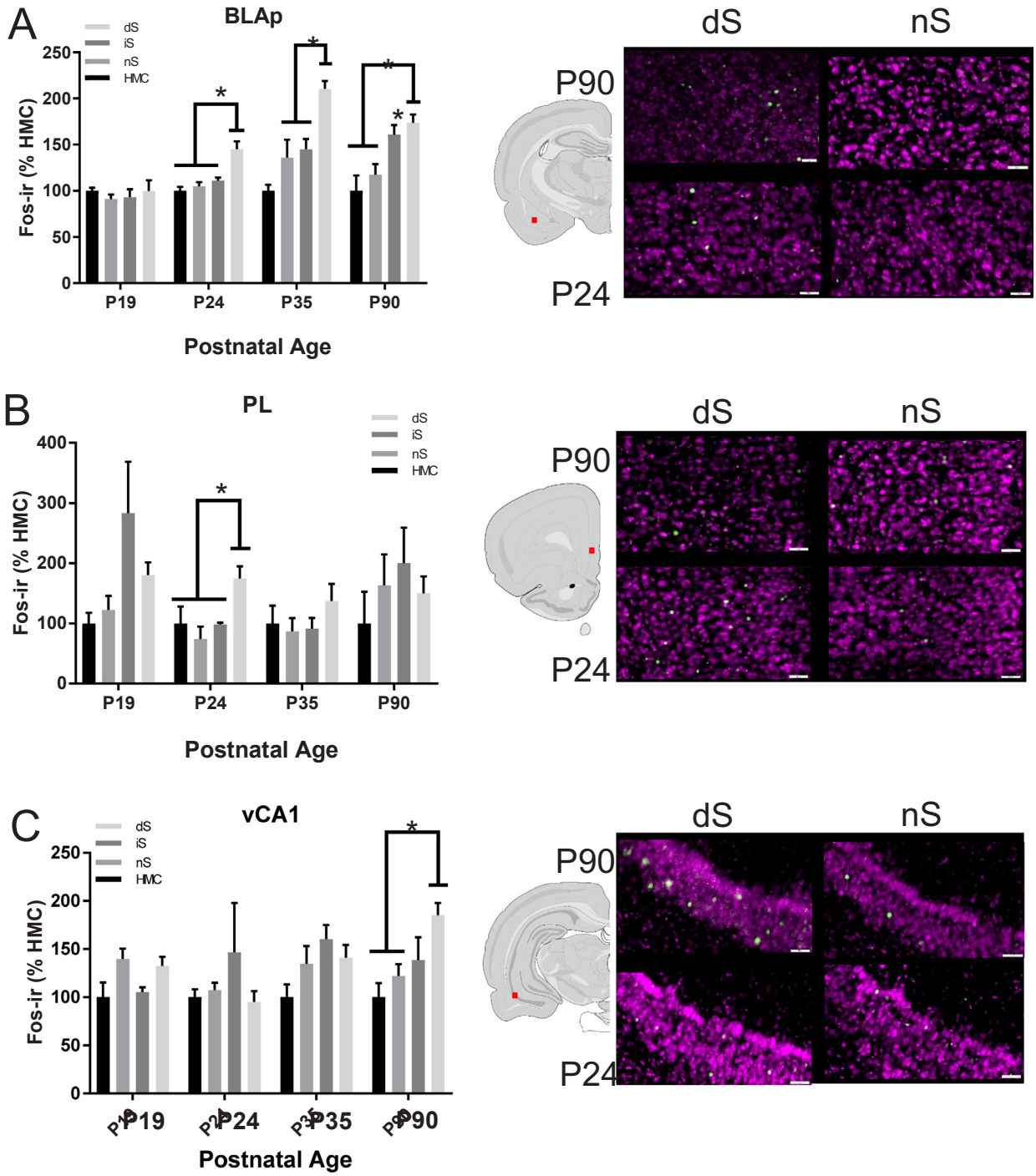

Fig. 2. The average numbers of context fear retrieval induced Fos-ir neurons + SEM as a percent of average numbers of Fos-ir neurons in the home cage condition (Fos-ir, %HMC) quantified at 1 d following initial conditioning by a 1.5 mA footshock. A–C: Fos-ir %HMC quantified in sampled subregions within the BLAp (A), PL (B) and CA1v (C). D–F: Representative photomicrographs of coronal sections for Fos-ir (green) and the background Nissl stain, Neuro-Trace (magenta), depicting BLAp (D), PL (E) and CA1v (F) among dS and nS conditions. Asterisks (*) denote significance at the $p < 0.05$ threshold for *a priori* tests of the hypothesis. The number sign (#) denotes a trend toward significance at the $p < 0.1$ threshold for *a priori* tests of the hypothesis. Scale bars in D–F 50 μM.

($r_{(16)} = 0.528$, $p < 0.05$)).

Our hypothesis of context fear retrieval-dependent activation within the BLAa and LA was not supported, as delayed shock did not differ from nS or iS for any of the age groups tested. ANOVAs performed to examine the number of Fos-ir neurons within the BLAa and LA identified a main effect of condition that was limited to P35 rats [BLAa ($F_{(3,19)} = 3.929$, $p < 0.05$); LA ($F_{(3,19)} = 4.564$, $p < 0.05$)]. Post-hoc comparisons revealed that the dS, but not iS nor nS conditions, exhibited a significant increase in the numbers of Fos-ir neurons compared to the HMC condition [BLAa ($t_{(10)} = 3.958$, $p < 0.05$); LA ($t_{(10)} = 5.347$, $p < 0.05$)]. No significant correlations were found between freezing and the numbers of Fos-ir neurons in the LA or BLAa. Importantly, the injection of Fluorogold tracer (see Section 2) into the right BLA (results for which are being reported elsewhere: Santarelli et al., 2016), did not produce any obvious impairments in learning, nor was the ability of the ipsilateral BLA complex to mount Fos responses

dependent on condition (see Supplemental Fig. S1).

3.3. mPFC activation

For the portion of the mPFC we sampled, we counted context fear-retrieval induced Fos-ir neurons in the PL (Fig. 2B), ILA (Supplemental Fig. S2), and ACA (Table 1). In support of the hypothesis that early juvenile (P24) but not adult rats would show enhanced numbers of PL and ILA Fos-ir neurons that correspond with freezing behavior, we found a main effect of condition within the PL ($F_{(3,20)} = 4.305$, $p < 0.05$) and a trend toward significance for the ILA ($F_{(3,20)} = 3.075$, $p < 0.1$) for P24, but not P90 rats. However, the effects of condition upon the levels of ACA Fos-ir neurons were not significant for any age groups. Planned comparisons for Fos-ir neurons in the PL revealed that P24 rats showed a significant elevation in the numbers of Fos-ir neurons in dS-trained compared to nS-trained rats within both the PL ($t_{(9)} = 3.447$, $p < 0.01$) and IL ($t_{(9)} = 2.417$, $p < 0.05$). Post-hoc comparisons revealed no other significant differences among trained rats within either regions. In P19 rats, analysis for the numbers of Fos-ir neurons within the PL demonstrated a main effect of condition ($F_{(3,212)} = 3.205$, $p < 0.05$); however, post-hoc tests failed to reveal distinct group differences. P35 rats showed no effects of training condition in either the ILA or PL.

3.4. Hippocampal and extrahippocampal cortical activation

The numbers of Fos-ir neurons that we observed within the dorsal hippocampus (dH) failed to support our hypothesis that context fear retrieval would result in an overall increased neuronal activation in fields CA1. No significant differences were detected across age groups within the dH or its constituent subregions with respect to numbers of dS-specific Fos-ir neurons (Fig. 2C and D). However, within the vH (Level 37: Fig. 2CE), a main effect of condition (elevated numbers of Fos-ir neurons) was observed within field CA1 in P90 ($F_{(3,23)} = 3.487$, $p < 0.05$), but not P24 or P35 groups. Planned comparisons between dS and nS conditions indicated that the numbers of Fos-ir neurons was elevated in dS animals ($t_{(9)} = 3.2646$, $p < 0.05$). Unplanned comparisons revealed that dS levels for these neurons were also elevated from HMC ($t_{(10)} = 3.935$, $p < 0.01$). Interestingly, a main effect of group was also detected for P19 rats ($F_{(3,22)} = 3.382$, $p < 0.05$). However, post hoc comparisons did not achieve statistical significance.

Within the extra-hippocampal cortical regions we sampled, dS training failed to increase the numbers of Fos-ir neurons relative to those observed in nS and iS groups. P90 rats showed a significant main effect of condition within the PERI ($F_{(3,22)} = 5.377$, $p < 0.01$) and the rostral portion of the ENTl ($F_{(3,22)} = 4.967$, $p < 0.05$) (Level 28; Supplemental Fig. S2). The numbers of ENTl Fos-ir neurons were elevated in the nS group ($p < 0.05$) relative to the HMC group. No other post hoc comparisons of ENTI or PERI achieved statistical significance. A one-way ANOVA analysis for Fos-ir neuron numbers in a more caudal portion of the ENTI did not reveal any main effects at any age.

4. Discussion

The ontogeny of fear retention was examined in this study using a standard contextual fear-conditioning paradigm and was accompanied by a description of induced Fos-ir in brain regions defined within the nervous system nomenclature ontology of Swanson (1992, 1999, 2004, 2018). In agreement with the literature, this study showed that juvenile (P24, P35) and adult rats (P90) are able to express context fear memory following a one-day retention period. Moreover, fear expression involved elevations in the numbers of Fos-ir neurons relative to controls throughout the subregions of the BLA complex that correlated with the observed freezing behavior. In forebrain regions known for their functional links to nuclei of the BLA complex, age-dependent differences were observed in response to conditioning. Specifically, juvenile rats (P24) exposed to delayed-shock displayed an enhancement in the numbers of Fos-ir cells throughout the portions of the PL and ILA that we sampled. In contrast, adults exhibited such enhancement only within the sampled regions of the ventral CA1 field. Interestingly, P35 rats exhibited a pattern distinct from P24 or P90 suggesting that this age may represent a transition period between the early juvenile and adult rats. Although further analysis of the tissue series used in this study is underway – in which Fos expression is analyzed in neuroanatomically traced circuits among these regions (Santarelli et al., 2016) – these results suggest that the emergence of context fear retrieval in young rats may depend on a functionally activated circuit that is distinct from that activated in the adult.

Many studies have demonstrated a critical role of the hippocampus in context mediated fear behaviors (Fanselow, 2000; Anagnostaras, Gale, & Fanselow, 2001; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Rudy & Matus-Amat, 2005; Hobin, Ji, & Maren, 2006; Raineki et al., 2010; Jin & Maren, 2015; Xu et al., 2016). In rats, the maturation of the hippocampus continues throughout postnatal life (Bayer, 1980; Gould & Cameron, 1996; Markham, McKian, Stroup, & Juraska, 2005). Only recently has evidence appeared for the involvement of the mPFC during the emergence of context fear (Chakraborty et al., 2016; Robinson-Drummer et al., 2017). Indeed, the expression of fear is dependent upon the PL during juvenile development and emerges around P25 (Stella et al., 2012).

While little is known how this circuit changes over development, prefrontal inputs to the BLA subregions are pruned during adolescence (Cressman et al., 2010). However, in adult animals, mPFC involvement in context-fear processing depends on the functional engagement of the dorsal hippocampus: rats with an intact hippocampus do not require mPFC function, while animals with damage to the hippocampus require mPFC–BLA function (Zelikowsky et al., 2013). Adding to this body of work, the present study suggests further that the mPFC involvement is later disengaged in adulthood or may act as a moderator of hippocampal regulation of context fear, as is observed in extinction and renewal (Giustino & Maren, 2015).

A few considerations must be borne in mind with the analysis presented in this study. First, any analysis of Fos-ir *post mortem* is at best a limited proxy of electrical activity of neurons networked *in vivo* (Hoffman & Lyo, 2002; Watts et al., 2006). Notwithstanding this limitation, the data in this study suggest that as in adult animals, the context fear neural system across development is dynamic and can engage anatomically distinct neural pathways (Ponnusamy, Poulos, & Fanselow, 2007; Poulos et al., 2009, 2010; Zelikowsky et al., 2013). Second, we report Fos-ir patterns using the nomenclature of the Swanson adult rat brain atlas (2004), in part, because extant atlases of postnatal rat brain (Sherwood & Timiras, 1970; Ramachandra & Subramanian, 2011; Calabrese, Badea, Watson, & Johnson, 2013; Khazipov et al., 2015) are restricted primarily to ages earlier than the earliest age we examined here (P19), do not delimit subregional boundaries for certain regions we analyzed, and/or lack the cellular-level spatial resolution required for our analysis. Moreover, a uniform, internally consistent ontology of defined neuroanatomical terms for the rat brain is provided in the nomenclature tables in the Swanson atlases (1992, 1999, 2004, 2018), all of which are based on the same, single rat brain. Using this ontology allows us to contextualize our data in relation to other molecular expression patterns that have been mapped in this spatial model across a variety of behavioral conditions (e.g., Scicli, Petrovich, Swanson, & Thompson, 2004; Yao, Gouraud, Paton, & Murphy, 2005; Zséli et al., 2016). This approach also ensures that our data can migrate to future atlases of the developing rat that may utilize the same ontology of defined anatomical terms (see Khan, Perez, Wells, & Fuentes, 2018, for a discussion of data migration).

Collectively, the results of the present study along with prior work suggest that the application of a standard adult neural circuit model of context fear conditioning to juvenile (P24 and P35) animals may be an overgeneralization. Our results permit us to propose three key

hypotheses: (1) The relative engagement of the vH and mPFC with respect to the BLA is normally regulated by developmental age; (2) in juvenile animals, context-fear processing is biased toward mPFC–BLA pathways and less dependent on vH–BLA pathways; and (3) conversely, in adult animals, context-fear processing is biased toward vH–BLA pathways and less dependent on mPFC–BLA connections.

Support for the first hypothesis is demonstrated by the context fear retrieval dependent activation of the vH in adult, but not early juvenile rats (Fig. 2C), while the ILA is activated in juvenile, but not adult rats (Supplemental Fig. S2). The second hypothesis is supported by the correlated activation between the PL and ILA with the BLAp in juvenile (P24, P35) rats, but not in adult rats. The third hypothesis is supported by research indicating that lesion or inactivation of the adult vH (Rudy & Matus-Amat, 2005; Kim & Cho, 2017), but not the mPFC alone, prevents the adult expression of context fear responding (Zelikowsky et al., 2013). Though another interpretation could be that the relative engagement of these structures is based upon the level of fear acquired or expressed, as P90 rats showed greater freezing than P24 rats. This interpretation seems less likely, when one considers CA1v Fos-ir under similar levels of freezing as in P90 and P35 animals, where the former, but not the latter displayed conditioning related effects. The extent to which the vH and its projections to the BLA are critical in juvenile context-fear conditioning remains to be analyzed. Future work, assessing the neuroanatomical maturation and functional development of vH–BLA and mPFC–BLA pathways in juvenile and adult animals, can test further the role of these pathways in the acquisition, storage and retrieval of context-fear memories.

Acknowledgments

We thank Kenichiro Negishi, Anais Martinez, and Ellen M. Walker (at UT El Paso) for technical assistance. This work was supported by NIH grants R03MH93781 (AMP) and SC3GM109817 (AMK); and SUNY start-up funds (AMP).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.nlm.2018.05.015.

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