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Variants of contextual fear conditioning induce differential patterns of *Egr-1* activity within the young adult prefrontal cortex

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Abstract

Contextual fear conditioning is a form of associative learning where animals must experience a context before they can associate it with an aversive stimulus. Single-trial contextual fear conditioning (sCFC) and the context preexposure facilitation effect (CPFE) are two variants of CFC where learning about the context is temporally contiguous (sCFC) with or separated (CPFE) from receiving a footshock in that context. Neural activity within CA1 of the dorsal hippocampus (CA1), amygdala (LA), and prefrontal cortex (PFC) may play a critical role when animals learn to associate a context with a footshock (i.e., training). Previous studies from our lab have found that early-growth-response gene 1 (*Egr-1*), an immediate early gene, exhibits unique patterns of activity within regions of the PFC following training in sCFC and the CPFE of juvenile rats. In the present study, we extended our studies by examining *Egr-1* expression in young adult rats to determine (1) if our previous work reflected changes unique to development or extend into adulthood and (2) to contrast expression profiles between sCFC and the CPFE. Rats that learned context fear with sCFC showed increased *Egr-1* in the anterior cingulate, orbitofrontal and infralimbic cortices relative to non-associative controls following training, but expression in prelimbic cortex did not differ between fear conditioned and non-associative controls. In contrast, rats trained in the CPFE also showed increased *Egr-1* in all the prefrontal cortex regions, including prelimbic cortex. These findings replicate our previous findings in juveniles and suggest that *Egr-1* in specific PFC subregions may be uniquely involved in learning context-fear in the CPFE compared to sCFC.

Keywords

prefrontal cortex; *Egr-1*; *zif268*; context preexposure facilitation effect; spatial learning; context fear

1. Introduction

A major strength of single-trial contextual fear conditioning (sCFC) paradigms is that animals rapidly acquire and express long-term fear to the training context [1–4]. Animals are

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typically given a few minutes to experience the context and then one or more footshocks occur. During the test for long-term memory of the learned fear, the animals are brought back to the context later, usually 24 hours, and the amount of time spent freezing is measured as the strength of conditioned fear [5]. However, given that all learning about the context and context-shock association occurs within the same trial in sCFC, it is difficult to distinguish which brain regions are uniquely recruited during different phases of learning, and uniquely contribute to learning about the context vs. the context-shock association. To overcome this limitation, the context preexposure facilitation effect (CPFE) paradigm has been used to temporally dissociate learning about the context from learning the context-shock association [6].

The CPFE paradigm relies on the immediate shock deficit – a phenomenon in which animals fail to show fear conditioned freezing to a context during a retention test if they do not have enough time to form a representation of the training context before receiving a foot-shock [2, 7]. However, a preexposure trial in the training context on the day prior to receiving an immediate shock overcomes this deficit, allowing animals to exhibit fear-conditioned freezing [2]. By temporally separating context learning from context-shock learning, the unique contributions of different brain regions during different phases of acquisition may be dissociated [8–10].

Although the behavioral characteristics of fear learning are well understood, our understanding of the neural activity within specific brain regions during discrete phases of learning is continually evolving [11–13]. Recent work has used immediate early gene (IEG) activity to measure the changes occurring within specific nuclei during different phases of contextual fear learning and expression [14–20]. Of particular interest, early-growth-response gene 1 (*Egr-1*, also known as *zif268*, *krox24*, and *NGFI-A*), a transcription factor associated with synaptic plasticity and learning and memory, is increased in structures necessary for contextual fear learning, such as the hippocampus and amygdala [1, 21, 22]. Using *in situ* hybridization and antisense knockdown strategies, work from our lab and others has shown that *Egr-1* mRNA in the dorsolateral division of the lateral nucleus of the amygdala (LA) and the dorsal hippocampus (dHC) is necessary for acquiring the context-shock association in sCFC and the CPFE paradigm [1, 14, 23, 24]. However, *Egr-1* mRNA is also increased following exposure to unpredictable situations and exposure to novel contexts – suggesting its role in learning contextual information may be quite complex [15, 16, 25–27]. While a number of studies have examined the functional role and molecular changes occurring within the hippocampus and amygdala during different phases of learning in sCFC and the CPFE, recent evidence has begun to point to a role for the prefrontal cortex (PFC) in contextual fear learning [10, 19, 20, 28, 29]. Although the neuroanatomical pathways between the PFC, hippocampus, and amygdala are fairly well characterized, the function of the PFC in contextual fear conditioning is just starting to be explored [30–33].

Recently, we have begun examining activation of the PFC during contextual fear learning using the CPFE paradigm in developing rats [29, 34]. We have found rats that learn the CPFE via preexposure to the training context (relative to rats preexposed to an alternate context) show increased *Egr-1* mRNA levels in the prelimbic and infralimbic regions of the PFC following the context-shock learning phase. However, there is no difference in PFC

Egr-1 expression between rats that learn the CPFE and those that do not during the context preexposure phase. This suggests that neural activation, as measured by *Egr-1* mRNA expression, in the PFC is related to the novelty of experiencing a new environment, but also in learning to associate an immediate foot shock with a previously acquired representation of the training context. However, given the dynamic nature of the PFC during development, it is unclear if these findings in developing rats would also be present in adulthood when the PFC is more mature [35, 36]. It is also unclear how *Egr-1* activity in the PFC differs between learning sCFC and the CPFE in adult rats.

In the present study we investigated changes in *Egr-1* expression during discrete phases of contextual fear learning in sCFC and the CPFE of young adult rats. Our primary aim was to determine whether *Egr-1* mRNA expression patterns in the PFC, dorsal hippocampus, and lateral amygdala correspond to contextual fear learning in young adult rats in a manner similar to our previously reported findings in developing rats [29, 34].

2. Methods

2.2 Subjects

Procedures for animal husbandry were identical to our previous reports (e.g., [37]). Rats were approximately 60 days of age at the start of the behavioral experiments. Subjects were 34 male Long Evans rats derived from 8 time-bred dams in the University of Delaware breeding colony and 181 male Long Evans rats purchased from Harlan Breeders (Indianapolis, IN). In-house bred and vendor bought rats did not differ on their fear conditioning behavior or gene expression, so their data were pooled for all analyses (data not shown). Out of a total of 215 animals, 113 were assigned to sCFC and 102 were assigned to the CPFE. For the sCFC study, 50 animals were assigned to the gene expression assays and 63 were assigned to the behavior assays. For the CPFE study, 68 animals were assigned to the gene expression assays and 34 were assigned to the behavioral assays.

Animals were pair-housed in opaque polypropylene cages (45x24x21cm) five days prior to the start of the experiment. Animals were given *ad libitum* access to food and water throughout the experiment. All procedures were approved and subjects were treated in accordance with the Institutional Animal Care and Use Committee at the University of Delaware.

2.3 Apparatus and Stimuli

Animals were fear conditioned in two distinct chambers distinguished as “Context A” and “Context B.” Context A consisted of a Plexiglas and metal chamber measuring 25 × 31 × 32cm. The chamber floor was made of 19 stainless steel bars (0.5 cm diameter placed 1.25 cm apart) connected to a shock scrambler that delivered a 1s 1.5 mA alternating current footshock (Med Associates, Georgia, VT ENV-414S). Two walls were made of clear Plexiglas, allowing the animal to view a unique pattern of black and white stripes and circles specific to Context A. The other two walls consisted of opaque Plexiglas and metal panels to prevent animals from seeing other subjects in adjacent chambers. Context B was similar to Context A in overall chamber size but differed in its lack of the visual pattern of stripes and circles found in Context A. In addition, Context B contained a mesh insert which altered the

spatial configurations and tactile cues of one wall. Both contexts were placed in a room on a metal rack in which background noise and light were controlled. All chambers were cleaned with a 5% ammonium hydroxide solution prior to the start of each trial. Cameras affixed to the roof of each chamber recorded animal activity and transmitted data to a nearby computer running FreezeFrame software (Coulbourn Instruments, Whitehall, PA).

FreezeFrame software was used to analyze all animal behavior. Activity thresholds were adjusted on an individual basis to exclude small movements from being calculated as part of an animal's total amount of freezing. Bouts of freezing were defined as 1.00s without changes in pixel luminance. Animals were transported to and from each training session in their homecage. Animals in the sCFC condition were only trained in Context A. Animals in the CPFE experiments were preexposed in either Context A or Context B and then trained and tested in Context A.

2.4 Design and Procedures

2.4.1 Handling—Prior to experimentation, all animals were transported to a holding room in the behavioral lab and handled for five minutes a day for five consecutive days by the same experimenter, similar to Malkani, et al. (2004) and Hamilton et al. (2011) [15, 38].

2.4.2 Single-Trial Contextual Fear Conditioning (sCFC)—The general training procedure used was identical to Malkani and Rosen (2000) [1]. Four experimental conditions (abbreviated sCFC, ImmShck, NoShck, and HC) were run over a period of two days. The sCFC condition, consisted of animals that were exposed to the context for 3 minutes, given a 1s, 1.5mA footshock, and left in the context for another 4 minutes to examine post-shock freezing. The ImmShck, immediate shock deficit, group consisted of animals shocked immediately upon placement in Context A, followed by a 4 minute postshock freezing test. The NoShck, no-shock context only group, consisted of animals that were placed in Context A for seven minutes without receiving any shock. The HC, homecage control, group was only exposed to the five days of handling but not trained and tested. The HC group animals remained in their homecage for the duration of the training, but were sacrificed at the same time as the other groups. A subset of trained animals were sacrificed along with homecage controls thirty minutes after training. Remaining animals from all behavioral groups (sCFC, ImmShck, and NoShck) were returned to Context A 24h after training and tested for fear conditioned freezing during a five minute retention test.

2.4.3 Context Preexposure Facilitation Effect (CPFE) Conditioning—The general training procedure used was similar to Murawski & Stanton (2010) and Hamilton et al. (2011) [38, 39]. Two experimental conditions were run over three consecutive days in the CPFE paradigm). Rats were assigned to one of three groups: PRE (preexposed to Context A), ALT-PRE (preexposed to Context B), or HC (homecage controls). Animals in the PRE and ALT-PRE groups were preexposed to either Context A or B (see Apparatus and Stimuli section above) on Day 1, given an immediate footshock in Context A during training on Day 2 and tested for freezing to Context A on Day 3. Pair-housed cage-mates were assigned to the same condition and trained together on each day. All groups were transported in their

homecage from the animal colony to a holding room on each day and left undisturbed prior to the start of experimentation.

Preexposure: On Day 1, animals in the PRE and ALT-PRE groups were placed, respectively, in either Context A or Context B. After a five-minute period (during which time their behavior was recorded), animals were placed back in their homecages and either returned to the holding room or sacrificed for *in situ* hybridization assays.

Training: For the training phase of the CPFE, animals in the PRE and ALT-PRE groups were transported to the conditioning chambers (i.e., Context A) in their homecage and given an immediate 1s 1.5 mA footshock. Animals were removed from the context within 10s of being shocked either returned to the holding room or sacrificed for *in situ* hybridization assays.

Retrieval: For the retrieval (testing) phase of the CPFE, animals in the PRE and ALT-PRE groups were transported in their homecage from the holding room to the conditioning chamber (i.e., Context A). Animals were placed in Context A for 5 minutes to assess freezing. During this time, behavior was recorded and later analyzed. At the end of the testing period animals were returned to the holding room.

2.5 Brain Collection

Thirty (± 2) min after the end of each session (i.e., sCFC training, CPFE preexposure, and CPFE training), animals used for *in situ* hybridization were sacrificed. Homecage controls were sacrificed along with conditioned animals to ensure that controls received a similar experimental history as their counterparts. Rats were sacrificed by rapid decapitation. Brains were removed within two minutes, immediately frozen in isopentane on dry ice, and stored at -80°C until they were sectioned.

2.6 Brain Sectioning

Sixteen micrometer coronal brain sections were sliced on a Jung CM 3000 cryostat (Leica, Germany), thawed onto glass slides (VWR Superfrost Plus Micro Slides, Radnor, PA) and frozen at -80°C . The sections corresponded to the prelimbic cortex (PL), infralimbic cortex (IL), ventral and lateral orbitofrontal cortex (OFC), anterior cingulate (AC, cingulate cortex area 1 of Paxinos and Watson, 2007 [40]), dorsolateral division of the lateral nucleus of the amygdala (LA), and CA1 subfield of the dorsal hippocampus (CA1) as defined by Paxinos and Watson, 2007 [40]. Each slide held two sections from the same region (either PFC or dHc/LA) from one animal.

2.7 *In situ* hybridization

In situ hybridization was conducted identically to that described in Asok, et al. (2013) and Thompson and Rosen (2006) [29, 41]. A 218 bp *Egr-1* antisense RNA probe was transcribed from a sense cDNA plasmid and radioactively labeled with a ^{35}S UTP (approximately $1-10^6$ dpm) using a T3 RNA polymerase Maxiscript kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY). The *Egr-1* sense cDNA sequence was PCR amplified from an original *Egr-1* cDNA plasmid (gift from J. Milbrandt, Washington

University, St. Louis, MO) and re-cloned into a TOPO-TA 4.0 vector (Life Technologies, Grand Island, NY) flanked by a T3 promoter sequence. Slides were washed, dried, and exposed to Kodak Biomax MR Film for 2 days along with ^{14}C standards (nCi/g).

2.8 *In situ* hybridization and image analyses

Autoradiograms were captured from film exposed to the radioactively labeled sections. They were digitized to 8-bit gray values on an Apple computer using a Dage CCD video camera with ImageJ 1.45m (Wayne Rasband, NIMH). ImageJ was used to subtract the background (2D-rolling ball radius of 50.0 pixels) and measure the mean density (mean gray value) of mRNA within the regions of interest. The AC, PL, IL, and OFC were measured using Plate 11 of Paxinos and Watson (2007) [40] as a guide. The LA and CA1 were measured using Plate 57 of Paxinos and Watson (2007) [40].

Two sections per slide per region for each animal were used for *in situ* hybridization. For each brain region the mean gray value of the left and right side of each section was averaged within slices and then within slides. Mean gray values were then converted into a known amount of radioactivity using ^{14}C standards exposed along with the experimental slides. The standards were used to generate a 3rd degree polynomial equation and convert the mean gray values from the slides to a known amount of radioactivity, nCi/g (See Table 1 for nCi/g for each of the regions and experimental groups analyzed). The nCi/g value was then normalized against the average nCi/g of all HC animals in each region to obtain a proportionate gene expression score. To collapse across films, the proportionate scores were averaged together and multiplied by 100 so that the average of all home-cage animals would equal 100%.

2.9 Statistical Analyses

SPSS 23.0 (IBM, Chicago, IL) was used for all statistical analyses. Any animal which fell ± 2.0 S.D. outside the group mean for behavior or *in situ* hybridization assays was excluded from further statistical analyses. The final number of animals used for each contrast are reported in the results section. For behavior statistical analyses, a mixed model, repeated measures ANOVA was used to compare freezing levels between sCFC postshock and testing (comparing sCFC, ImmShck, and NoShck groups), and CPFE preexposure and testing (comparing PRE and ALT-PRE groups). For *in situ* hybridization statistical analyses, each region was analyzed separately by one-way ANOVA for sCFC training (sCFC, ImmShck, NoShck, and HC) and CPFE preexposure and training (PRE, ALT-PRE, and HC). Levene's Test for homogeneity of variance was used to evaluate differences in variance across behavior and gene expression groups prior to ANOVA. Given that no groups violated this assumption, a one-way ANOVA was used to test for main effects at $p < .05$. Tukey's HSD post hoc multiple means comparison test was used at $p < .05$ to determine group differences following a main effect. Tukey-Kramer post hoc analyses were used to control for unequal group sizes when necessary.

3 Results

3.1 sCFC

3.1.1 sCFC Behavior (Figure 1A)—Of the original 63 animals, 5 subjects were excluded as outliers from the baseline freezing, postshock, and retention analyses (1 sCFC, and 2 NoShck and 2 ImmShck). Pre-shock freezing scores were not included in the analysis because they were very low in NoSchk and sCFC groups, and there was no pre-shock phase for the ImmShck group. A 2x3 repeated measures ANOVA demonstrated that the sCFC, ImmShck, and NoShck groups significantly differed from one another in the level of freezing during postshock and retention tests ($F(1, 57) = 8.560, p < .01$). A significant interaction effect between conditioning groups and testing phase was also found ($F(2, 56) = 14.920, p < .001$). Posthoc analyses of postshock freezing using Tukey's HSD test showed that animals in the sCFC condition froze significantly more than animals in the ImmShck ($p < .001$) and NoShck ($p < .001$) groups, but the ImmShck and NoShck groups did not differ from each other ($p > .05$; see Figure 1A). In the sCFC group, freezing decreased in the retention test compared to the post-shock freezing. However, the sCFC group still froze more than both the ImmShck ($p < .01$) and NoShck ($p < .01$) groups during the retention test, whereas ImmShck and NoShck groups did not differ from one another ($p > .05$).

In summary, only the sCFC group displayed contextual fear conditioning. Animals exposed to the conditioning context 3 min prior to receiving a shock in the same training session (group sCFC) exhibited significantly more freezing to the context during the postshock and retention tests than animals receiving an immediate shock (group ImmShck) or no shock (group NoShck).

3.1.2 sCFC *Egr-1* Gene expression (Figure 1B and 1C)—Fourteen animals in the sCFC and twelve animals each in the HC, ImmShck, and NoShck conditions were assayed. Analysis of *Egr-1* expression after training was based on the number of animals per condition calculated after outlier exclusion or had damage the region. The final number of animals in each group were: AC ($n_{\text{sCFC}} = 13, n_{\text{HC}} = 12, n_{\text{ImmShck}} = 11, n_{\text{NoShck}} = 11$), PL ($n_{\text{sCFC}} = 13, n_{\text{HC}} = 12, n_{\text{ImmShck}} = 12, n_{\text{NoShck}} = 11$), IL ($n_{\text{sCFC}} = 13, n_{\text{HC}} = 12, n_{\text{ImmShck}} = 11, n_{\text{NoShck}} = 10$), OFC ($n_{\text{sCFC}} = 13, n_{\text{HC}} = 12, n_{\text{ImmShck}} = 11, n_{\text{NoShck}} = 10$), CA1 ($n_{\text{sCFC}} = 13, n_{\text{HC}} = 12, n_{\text{ImmShck}} = 11, n_{\text{NoShck}} = 11$), and LA ($n_{\text{sCFC}} = 11, n_{\text{HC}} = 12, n_{\text{ImmShck}} = 10, n_{\text{NoShck}} = 10$).

In general, *Egr-1* expression was increased in all of the prefrontal cortex regions of the sCFC, NoShck and ImmShck groups compared to the HC group, with increases in the sCFC group mostly being greater than the NoShck and ImmShck groups. The ImmShck group also had increased expression in CA1 compared to HC, but there were no differences between NoShck, ImmShck and sCFC groups. In the LA, ImmShck and sCFC groups had increased expression compared to HC.

One-way ANOVAs demonstrated a significant main effect of condition in all PFC regions—AC, $F(3, 43) = 13.252, p < .001$; PL, $F(3, 44) = 11.530, p < .001$; IL, $F(3, 42) = 18.849, p < .001$; and OFC, $F(3, 42) = 14.065, p < .001$. Significant main effects were also found in CA1 of the hippocampus ($F(3, 43) = 3.323, p < .05$) and in the lateral amygdala ($F(3, 39) =$

3.7883, $p < .02$). Post hoc analyses found selective group differences in the various regions analyzed.

In the AC, the sCFC group significantly differed from all other control groups (sCFC vs. ImmShck, $p < .05$; sCFC vs. NoShck, $p < .05$; sCFC vs. HC: $p < .001$). In addition, the ImmShck and NoShck groups expressed higher levels of *Egr-1* expression compared to HC controls (ImmShck vs. HC $p < .05$, NoShck vs. HC $p < .01$).

In the PL, sCFC, ImmShck, and NoShck groups expressed significantly higher levels of *Egr-1* compared to HC controls (ImmShck vs. HC: $p < .05$; NoShck vs. HC: $p < .05$; sCFC vs. HC: $p < .001$), but the sCFC group did not differ significantly from the ImmShck and NoShck groups ($p = .248$ and $.242$, respectively).

In the IL, sCFC, ImmShck, and NoShck groups expressed significantly higher levels of *Egr-1* compared to HC controls (sCFC vs HC: $p < .001$; ImmShck vs HC, $p < .05$; NoShck vs HC, $p < .05$). The sCFC group also exhibited significantly more *Egr-1* expression compared to the ImmShck and NoShck groups (sCFC vs. ImmShck, $p < .05$; sCFC vs. NoShck $p < .05$).

In the OFC, sCFC animals showed significantly higher levels of *Egr-1* compared to the ImmShck and HC groups (sCFC vs. ImmShck, $p < .01$; sCFC vs. HC, $p < .001$), but did not differ from the NoShck group. NoShck animals expressed more *Egr-1* compared to HC controls (NoShck vs. HC $p < .01$). The ImmShck group expressed trending increases in *Egr-1* mRNA compared to the HC group (ImmShck vs. HC, $p < .10$). The ImmShck and NoShck animals did not differ from each other (ImmShck vs. NoShck, $p > .05$).

In CA1 only the ImmShck group differed significantly from HC controls ($p < .05$). Post hoc tests did not reveal significant differences between the sCFC, NoShck and ImmShck groups.

Lastly, in the LA, the sCFC group and the Immshck group expressed significantly higher *Egr-1* expression compared to HC controls (sCFC vs HC, $p < .05$; ImmShck vs HC, $p < .05$ (see Figures 1B and 1C).

3.2 CPFE

3.2.1 CPFE Behavior (Figure 2A)—Thirty-four subjects were assayed for behavior, of which 5 subjects (3 PRE and 2 ALT-PRE) were excluded from behavioral analyses because their mean percent freezing scores exceeded criterion for an outlier (2.0 *S.D.* from the mean). A 2x2 repeated measures ANOVA comparing freezing levels between the PRE and ALT-PRE groups between preexposure to a context and retention testing found significant differences in freezing levels between the two groups ($F(1, 27) = 22.959$, $p < .001$). A significant interaction effect was also found ($F(1, 27) = 12.554$, $p < .001$), such that animals in the PRE group froze most during testing compared to ALT-PRE controls (see Fig 2A).

In summary, animals preexposed to the conditioning context (group PRE) showed significantly more freezing during a retention test in the same context, whereas animals preexposed to an alternate context (group ALT-PRE) did not.

3.2.2 CPFE *Egr-1* Gene Expression

3.2.2.1 CPFE Preexposure (Figure 2B and 2D): Six animals in each of the HC, PRE, and ALT-PRE conditions were assayed. One subject in the ALT-PRE group was excluded from the AC analyses due to tissue damage, leaving an $n=5$ in the ALT-PRE group for the AC. One-way ANOVAs demonstrated a significant main effect of condition in the AC, $F(2,13) = 14.616, p < .001$, PL, $F(2,14) = 35.604, p < .001$, IL, $F(2,14) = 23.764, p < .001$, OFC, $F(2,44) = 56.498, p < .001$, and CA1, $F(2,14) = 5.009, p < .05$, but not the LA, $F(2,14) = 1.554, p > .05$. Post-hoc analyses revealed the PRE and ALT PRE groups had greater expression than the HC group in all regions except the LA, while the PRE and ALT-PRE groups did not differ from each other in any brain region (p 's $< .05$).

In summary, following exposure to a novel chamber on the preexposure day, both PRE and ALT-PRE groups expressed significantly higher *Egr-1* levels in the AC, PL, IL, OFC, and CA1 compared to HC controls, but did not differ from one another (see Figures 2B and 2D).

3.2.2.2 CPFE Training (Figure 2C and 2E): In the PFC, eight animals in the HC, 20 animals in the PRE and 22 animals in the ALT-PRE conditions were assayed. Analysis of *Egr-1* expression after training was based on the number of animals per condition after exclusion because of tissue damage to the region. The final number of animals in each group were: AC ($n_{HC} = 8, n_{PRE} = 20, n_{ALT-PRE} = 22$), PL ($n_{HC} = 8, n_{PRE} = 20, n_{ALT-PRE} = 22$), IL ($n_{HC} = 8, n_{PRE} = 20, n_{ALT-PRE} = 22$), OFC ($n_{HC} = 8, n_{PRE} = 19, n_{ALT-PRE} = 22$), CA1 ($n_{HC} = 8, n_{PRE} = 20, n_{ALT-PRE} = 22$), and LA ($n_{HC} = 8, n_{PRE} = 19, n_{ALT-PRE} = 20$).

Statistical analyses using a one-way ANOVA of each regions demonstrated a significant main effect of condition in all PFC regions - AC $F(2,47) = 9.921, p < .001$, PL, $F(2,47) = 9.267, p < .001$, IL $F(2,47) = 9.921, p < .001$, OFC, $F(2,46) = 5.731, p < .01$ - as well as the LA, $F(2,44) = 3.825, p < .05$. There were no differences between groups in the CA1 of the hippocampus, $F(2, 47) = 0.137, ns$. In all PFC regions, post hoc analyses revealed significant differences between PRE and ALT-PRE groups relative to HC controls (p 's $< .001$). Furthermore, significant differences between PRE and ALT-PRE groups in all of the PFC regions were detected (p 's $< .05$). In the LA, post hoc analyses revealed significant differences between PRE and ALT-PRE groups relative to HC controls ($p < .05$), but no differences between the PRE and ALT-PRE groups were detected.

In summary, on the training day following immediate-shock, *Egr-1* was significantly elevated in the PRE and ALT-PRE groups compared to HC controls in the PFC and LA, but not the CA1 of the hippocampus. Importantly, compared to the ALT-PRE group, the PRE group expressed greater levels of *Egr-1* in all PFC regions, but not CA1 or LA (see Figures 2C and 2E).

4. Discussion

The present study evaluated *Egr-1* mRNA expression patterns in the brain across two variants of contextual fear conditioning (CPFE and sCFC paradigms) in young adult rats. This allowed us to examine (1) unique activity driven by incidental contextual learning (PRE vs. ALT-PRE groups during CPFE preexposure), (2) unique activity driven by

learning the context-shock association in young adult rats preexposed to the training context (PRE vs. ALT-PRE during CPFE training), and (3) neural activity when context-shock learning occurred in the same, and on a different, occasion as incidental contextual learning (sCFC vs. CPFE paradigms during training). Our results show that incidental context learning drove *Egr-1* expression in all of the PFC regions analyzed and in the CA1 of the hippocampus. Furthermore, context-shock training in the CPFE and in sCFC uniquely drove *Egr-1* expression in animals that learned context fear beyond expression induced by shock without fear learning (PRE vs. ALT-PRE and sCFC vs. ImmShck) across the PFC, except in the PL during sCFC training. Thus, the major finding of the study shows that fear learning in both of these variants of contextual fear conditioning induced increased *Egr-1* expression across the PFC, but with some regional differences suggesting functional roles of the PFC areas in the acquisition of incidental learning of context and context-shock associations. These and other findings are discussed below.

4.1 CPFE vs sCFC Behavior

As expected, rats in all groups exhibited low-levels of baseline freezing to the context (“preexposure” in the CPFE and “baseline” in sCFC) prior to footshock, but following training only rats preexposed to the training context (sCFC and PRE groups) exhibited fear conditioned freezing to the context during a retention test the following day. Interestingly, rats that learned context-fear in the CPFE froze more than rats that learned context-fear in sCFC during the retention test (32.8% vs. 19.2%), although post-shock freezing in sCFC was 35% - the same freezing level of the PRE group in the CPFE retention test. Previous work from our lab, and other labs, have observed that training in the CPFE (with a single preexposure session and a single shock during training) typically produces levels of freezing that are significantly less than those observed with sCFC – suggesting that the CPFE paradigm produces weaker contextual conditioning (see [3, 34]). Reduced freezing during the sCFC retention test may reflect the inclusion of a post-shock freezing measure in the sCFC study. The extra 4 minutes of (nonreinforced) context exposure occasioned by this measure may have reduced freezing during the retention test relative to the PRE group in the CPFE study, which did not undergo a post-shock freezing test. Inclusion of a post-shock freezing measure in the CPFE tends to depress retention performance [42]. It is also possible that other procedural differences explain the difference between the present finding and other studies (i.e., more handling by the experimenter, the use of adult rats, larger training chamber size, shock intensity and shorter duration of foot-shock) [1, 29, 34]. Despite these procedural differences, only rats that learned context-fear (PRE and sCFC groups) showed significantly elevated freezing relative to their baseline levels and relative to other behavioral control groups during the retention test.

4.2 *Egr-1* mRNA Expression in the Prefrontal Cortex

In the PFC, all animals across the sCFC experiment (except group ImmShck in the OFC) and the CPFE experiment expressed greater levels of *Egr-1* relative to HC controls – a group which controlled for housing, transport, and handling across experiments. Of greater importance, after CPFE training, the PRE group exhibited significantly higher levels of *Egr-1* in all four PFC regions relative to the key shock control group ALT-PRE, whereas following sCFC training there was differential *Egr-1* expression between the sCFC group

and the NoShck and ImmShck groups in the AC, IL and OFC, but not in the PL. These data suggest the AC, IL and OFC are involved in context-shock association processing, regardless of whether the shock is associated with a context that is present (sCFC) or retrieved from memory (CPFE). For the PL, the results could possibly reflect greater involvement in context novelty in the PL, than specifically in fear conditioning. Recent work showing context exposure activates *Arc* in the PL supports the idea of a role for rapid gene expression in the PL during context acquisition [32, 33]. The PL has also been suggested to be involved in learned fear expression and/or predictive fear conditioning [43–46]. Our *Egr-1* expression in the CPFE experiment supports these views. However, data from the sCFC do not support the fear expression view because expression in the NoShck, Immshck and sCFC groups did not differ although fear behavior did. The data might support a role of the PL in processing unexpected stimuli because rats in these three groups experienced something novel and *Egr-1* expression was greater than the HC group. Studies specifically addressing the functional role of the PL in the CPFE and sCFC are warranted.

Increases in *Egr-1* expression in IL of the sCFC group might be due to the nonreinforced 4min postshock period. Speculatively, during the sCFC experiment, the fear memory may have been partially extinguished during the postshock period, resulting in reduced freezing during the retention test and increased *Egr-1* expression in the IL, which is known to be involved in fear extinction [47, 48]. If so, omitting post-shock context exposure would reduce *Egr-1* expression in IL (as we've reported in juvenile rats) [34]. Furthermore, in the sCFC experiment the NoShck and ImmShck groups did not differ from each another in any prefrontal regions assessed – suggesting that exposure to the context alone without a shock (NoShck) and exposure to a shock without prior context exposure (ImmShck) produces similar levels of *Egr-1* expression. Taken together, the results from both the sCFC and CPFE experiments indicate increased *Egr-1* expression in the AC, IL and OFC in the fear conditioning groups (sCFC and PRE) above all of the other groups is due to contextual fear learning.

In the CPFE paradigm it was possible to examine the effect of incidental context learning separately from the context-shock association. Exposure to context A or B induced similar levels of *Egr-1* expression in all PFC regions, suggesting either novelty or incidental context learning drove expression regardless of context configuration [29]. As discussed above, similar to the sCFC experiment, context-shock training induced greater *Egr-1* expression in the PFC in rats that learned the association than those that received shock without learning (ALT-PRE and ImmShck groups). Understanding the cellular role of the differential activation of *Egr-1* during incidental context learning, fear conditioning and shock without learning in these various PFC regions is a direction for future research.

Our findings fit well with recent ideas that while various regions of the PFC may be involved in functions that are dichotomous (e.g., PL involved in regulation of fear expression or processing novelty and IL involved in fear suppression), these regions may co-vary and participate in the acquisition of fear conditioning and other phase of fear expression and extinction [46]. In the AC, NR2B containing NMDA receptors are necessary for the acquisition and consolidation of contextual fear memories ([49, 50]; for a review see [51]). Excitatory activity in the AC is also thought to process pain-related information

during contextual fear learning [52]. Given that the AC sends strong excitatory projections to portions of the BLA [53], *Egr-1* activation in the AC following training in both sCFC and the CPFE may reflect global contextual-fear related processing of aversive stimuli. Furthermore, the PL and IL have received the majority of attention for their role in the expression and extinction of learned-fear [54–56], respectively, but recent evidence has begun to support a role for both the PL and IL in aspects of contextual processing during fear learning [32, 46, 57]. Finally, while the OFC is primarily investigated for its role in decision making and rapid encoding of associative learning [58], recent evidence also points to a role for the OFC in processing contextual information during fear learning [59].

4.3 Hippocampus and Amygdala

The hippocampus and amygdala have received considerable attention for their roles in contextual fear conditioning [19, 60–62]. *Egr-1* mRNA did not increase in the CA1 subfield of the dorsal hippocampus following fear conditioning in sCFC or CPFE relative to the NoShck, ImmShck or ALT-PRE groups. Only animals exposed to an immediate shock expressed higher levels of *Egr-1* expression compared to homecage controls in the sCFC experiment. The lack of a unique increase in *Egr-1* within CA1 in animals that learn context-fear following training in sCFC is similar to previous findings from our lab [1] and consistent with some *in vivo* LTP studies ([63]; for a review see [21]). However, during the preexposure phase of the CPFE, *Egr-1* was increased in both the PRE and ALT-PRE groups relative to HC controls, suggesting the hippocampus is involved in incidental context learning. These results are also supported by studies in which muscimol or an NMDA antagonist injected into dorsal hippocampus before context preexposure impaired acquisition and retrieval of contextual fear memory during the CPFE [9, 10].

Additionally, we did not observe any increase in *Egr-1* within the LA following preexposure in the CPFE. However, following training in sCFC and training in the CPFE, groups that received shock (ImmShck, sCFC, PRE, and ALT-PRE) exhibited increased *Egr-1* compared to homecage controls. These findings are consistent with inactivation studies in adult rats and recently published reports from our lab in juvenile rats [9, 10, 29, 34]. Previous work from our lab has also observed increased *Egr-1* mRNA and a functional role for *Egr-1* in the LA following training in adult rats that learn contextual-fear with sCFC [1, 15].

4.4 Development

A secondary aim of the present study was to assess *Egr-1* mRNA expression patterns in the PFC of young adult rats (i.e., about postnatal day (PD) 60) as a contrast to our previous work using juvenile rats (i.e., PD 31; see [29, 34]). Given the continued growth of the PFC during adolescence, the present study was important for determining if our previous findings in the PFC following training in the CPFE were unique to development [29, 34]. We found a similar increase in *Egr-1* expression in young adult rats within the AC, PL, IL, and OFC between rats that learned context fear in the CPFE (PRE group) and those that do not (ALT-PRE group) following both context exposure and context-shock training [29]. This suggests that our previous findings in juvenile rats in the CPFE may reflect changes in context representation and contextual fear processing that also occur in adulthood. In contrast, we observed increased *Egr-1* expression throughout the PFC following sCFC training compared

to the control groups in young adults, whereas in our previous study of juveniles [34] the ImmShck and NoShck groups had similar levels of *Egr-1* expression in the PL and IL compared to the CFC group (the AC and OFC were not measured in the juveniles). This might be reflective of immaturity of the PFC at PD 31 or of different interactions of PFC with other brain systems during contextual fear conditioning in adolescence vs. adulthood [64, 65]. However, it is also possible that procedural differences arising from this cross-study age comparison (e.g., apparatus, inclusion of post-shock context exposure, etc.) also contributed to the different outcome and so further studies are needed to examine age differences more directly. That *Egr-1* expression was increased in two juvenile studies in fear conditioning in the CPFE [29, 34] in the PL and IL to a similar level that was found in young adults, suggests that fear conditioning in sCFC and the CPFE might rely on different neural circuits during development.

4.5 Conclusion

The present study used *Egr-1* mRNA expression as a marker for neuronal activation in two variants of fear conditioning, demonstrating activation of similar amygdala, hippocampal and prefrontal cortex circuits during fear conditioning in a standard paradigm and during the CPFE in young adult rats, in which the PL and IL activity was different from our previous study of sCFC in juvenile rats [34]. *Egr-1* is an inducible transcription factor thought to have functional roles in fear conditioning, extinction and reconsolidation in the amygdala, hippocampus and PFC (for review see Veyrac et al, 2014). While our *in situ* hybridization studies indicate differential *Egr-1* activation during development and adulthood in variants of fear conditioning paradigms, assessment of the functional roles of *Egr-1* expression will need to be addressed with inactivation or overexpression of *Egr-1* manipulations in these brain regions.

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Highlights

- Preexposure to the immediate shock context facilitates fear conditioned freezing
- sCFC increases *Egr-1* in the AC, IL and OFC, but not in the PL
- CPFE conditioning increases *Egr-1* in the AC, PL and IL and OFC
- sCFC and CPFE conditioning may differ in the recruitment of different PFC regions

Figure 1. sCFC behavior and gene expression. (A) Mean percent freezing between groups during baseline preexposure, post-shock testing, and retention testing. sCFC animals expressed

increased freezing compared to animals in the ImmShck and NoShck conditions. (B) sCFC training *Egr-1* expression depicted as mean percent change of HC controls in the AC, PL, IL, and OFC of the PFC, the CA1, and the LA. *Egr-1* expression in HC animals was less than all other conditions in prefrontal cortex, and several conditions in DHC and LA. sCFC animals expressed increased *Egr-1* expression in the AC, IL and OFC compared to ImmShck and in the AC and IL compared to NoShck. Expression in PL did not differ between sCFC, ImmShck and NoShck conditions. (C) Digitized images of animals in HC, NoShck, ImmShck, and sCFC conditions after sCFC training containing all brain regions analyzed. Error bars are $\pm$ SEM. $*p < .05$ difference between groups, $**p < .01$ difference between groups, $***p < .001$ difference between groups.

Figure 2.

CPFE behavior and gene expression. (A) Mean percent freezing between groups during preexposure on Day 1 and retention testing on Day 3. PRE animals froze significantly more on during testing than preexposure. PRE animals also froze significantly more than ALT-PRE animals during testing. (B) CPFE preexposure *Egr-1* expression depicted as mean percent change of baseline HC controls in the AC, PL, IL, and OFC of the PFC, CA1, and the LA. PRE and ALT-PRE animals expressed increased *Egr-1* levels in the PFC and CA1 compared to HC, but did not differ from one another. (C) CPFE training *Egr-1* expression depicted as mean percent change of baseline homecage controls in the AC, PL, IL, and OFC of the PFC, the CA1, and the LA. In the PFC, PRE and ALT-PRE animals expressed increased *Egr-1* levels compared to HC, and PRE animals expressed more *Egr-1* compared to ALT-PRE animals. PRE and ALT-PRE animals expressed increased *Egr-1* levels in the

CA1 and LA compared to HC, but did not differ from one another. (D) Digitized images of animals in HC, ALT-PRE, and PRE conditions after CPFE preexposure containing all brain regions analyzed. (E) Digitized images of animals in HC, ALT-PRE, and PRE conditions after CPFE training containing all brain regions analyzed. Error bars are $\pm$ SEM. * $p < .05$ difference between groups, ** $p < .01$ difference between groups, *** $p < .001$ difference between groups.

Egr-1 mRNA (nCi/g $\pm$ SEM) expression in the prefrontal cortex regions, CA1 and amygdala in the various groups of the sCFC and CPFE experiments.

TABLE 1

Standard Contextual Fear Conditioning						
	AC	PL	IL	OFC	CA1	LA
HC	114 $\pm$ 11	148 $\pm$ 10	129 $\pm$ 8	184 $\pm$ 14	453 $\pm$ 22	183 $\pm$ 8
NoShck	165 $\pm$ 7	221 $\pm$ 15	201 $\pm$ 13	282 $\pm$ 20	484 $\pm$ 47	217 $\pm$ 19
ImmShck	162 $\pm$ 11	220 $\pm$ 16	199 $\pm$ 12	233 $\pm$ 18	562 $\pm$ 47	213 $\pm$ 18
sCFC	204 $\pm$ 10	266 $\pm$ 14	254 $\pm$ 15	329 $\pm$ 15	557 $\pm$ 27	240 $\pm$ 16

Context Pre-Exposure (CPFE Preexposure)						
	AC	PL	IL	OFC	CA1	LA
HC	104 $\pm$ 9	199 $\pm$ 10	187 $\pm$ 23	250 $\pm$ 4	425 $\pm$ 24	170 $\pm$ 22
ALT-PRE	192 $\pm$ 8	266 $\pm$ 14	237 $\pm$ 10	339 $\pm$ 16	592 $\pm$ 20	213 $\pm$ 16
PRE	185 $\pm$ 18	307 $\pm$ 24	247 $\pm$ 20	387 $\pm$ 27	531 $\pm$ 52	195 $\pm$ 12

Context-Shock (CPFE Training)						
	AC	PL	IL	OFC	CA1	LA
HC	99 $\pm$ 9	123 $\pm$ 8	127 $\pm$ 8	162 $\pm$ 7	609 $\pm$ 58	144 $\pm$ 13
ALT-PRE	167 $\pm$ 10	209 $\pm$ 14	202 $\pm$ 17	274 $\pm$ 23	440 $\pm$ 35	220 $\pm$ 15
PRE	261 $\pm$ 18	248 $\pm$ 21	308 $\pm$ 23	212 $\pm$ 29	453 $\pm$ 37	221 $\pm$ 14