Research Report

**Egr-1 mRNA expression patterns in the prefrontal cortex, hippocampus, and amygdala during variants of contextual fear conditioning in adolescent rats**

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**ABSTRACT**

We report activation of the immediate-early gene Egr-1 in the lateral amygdala (LA), hippocampus (CA1), and medial prefrontal cortex (mPFC) 30-min following the training phase in the context pre-exposure facilitation effect (CPFE) and standard context fear conditioning (180 s context exposure→shock). On day one of the CPFE paradigm, postnatal day (PD) 31 rats (±1) were pre-exposed to Context A (Pre) or Context B (Alt-Pre) for 5 min followed by five additional 1-min exposures. A day later, Pre and Alt-Pre rats received a 2-s, 1.5 mA footshock immediately upon placement in Context A. Animals included in in situ hybridization were then sacrificed 30 (±3) min later. On day three, the behaviorally-tested Pre rats showed significantly more fear-conditioned freezing in Context A than Alt-Pre rats. Standard context fear conditioning groups showed much greater freezing than the Pre group, as well as no shock and immediate-shock controls. Thirty minutes after immediate shock training, Pre rats showed increased Egr-1 mRNA in the prelimbic mPFC relative to Alt-Pre rats. Standard context conditioning selectively increased Egr-1 in CA1. In the LA and mPFC, Egr-1 increased to a similar extent in no shock, immediate shock, and standard context conditioning relative to homecage controls. The present study demonstrates that Egr-1 mRNA expression has a complex relationship to fear learning in different brain regions and variants of context conditioning.

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1. Introduction

In Pavlovian fear conditioning, a conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US) such that future presentations of the CS alone elicit species-typical defensive responses (e.g., freezing). In standard context fear conditioning (sCFC), the training context, rather than a discrete CS, is paired with an aversive stimulus (Maren et al., 1997; O’Reilly and Rudy, 2001). sCFC requires a context exposure period in which a representation of the context is formed prior to learning the context-US association; if animals are shocked immediately upon being placed in a context for the first time, they fail to form the context-US association, a phenomenon termed the “immediate-shock deficit” (Fanselow, 1990). This
deficit is used as a control procedure in a variant of sCFC, the context pre-exposure facilitation effect (CPFE), which permits the dissociation of context learning from context-shock learning by separating them into different procedural phases. This is important because it is not possible to distinguish between these different forms of learning in a standard context fear experiment. In the CPFE paradigm, animals pre-exposed to Context A (Pre) and given an immediate shock in Context A 24-h later exhibit a freezing response when returned to Context A the following day, whereas animals pre-exposed to an alternative Context B on the first day (Alt-Pre) do not display freezing when tested in Context A. Our research group has recently employed this paradigm to study correlates of immediate-early gene expression associated with the context acquisition and the context-shock association phases of the CPFE (Asok et al., 2013b).

Our previous investigations have focused on the expression of the immediate-early gene early growth response gene 1 (Egr-1, a.k.a. Krox 24, NGFI-1, Zif268, TIS8), an inducible transcription factor associated with neuronal plasticity and implicated in various forms of learning, such as fear conditioning and spatial learning (for reviews, see Alberini, 2009; Davis et al., 2003; Knapska and Kaczmarek, 2004; Rosen, 2004; Rosen and Donley, 2006; Veyrac et al., 2014). Egr-1 expression is increased in the lateral nucleus of the amygdala (LA) of adult animals that learn context fear in sCFC relative to those that display the immediate-shock deficit (Malkani and Rosen, 2000) and context fear conditioning is abolished following antisense knockdown of EGR-1 protein in the LA (Malkani et al., 2004). Recently, we have extended these findings to the CPFE in adolescent rats (Asok et al., 2013b) by examining patterns of activity in the dorsal hippocampus (dHPC) and LA, as well as in the prelimbic (PL) and infralimbic (IL) divisions of the medial prefrontal cortex (mPFC) following the preexposure and training phases of the CPFE procedure. Taken together, these studies suggest a unique role for Egr-1 during consolidation of context fear memories throughout different brain regions.

Interestingly, Asok et al. (2013b) obtained findings that indicate a novel role of Egr-1 in the mPFC during the context-shock association in the CPFE. This adds to a recent growing literature on the role of prefrontal cortex in contextual fear conditioning: this region seems to be particularly involved in learning of standard contextual fear (No Shock/Imm Shock vs. sCFC), and (3) whether a multiple-exposure CPFE paradigm might reduce uncertainty-based Egr-1 responses in LA and CA1 that might “unmask” fear-based responses that were not evident in our previous study (Asok et al., 2013b).

2. Results

2.1. Behavior

After excluding outliers (N=1 female, Standard Context Fear; N=2 males, Alt-Pre; N=1 female, 1 male, Pre; N=1 female, 1 male, Handled Immediate Shock; N=2 males, Handled No Shock), freezing behavior was scored for the remaining 102 animals across the five training conditions.

A 5 (Condition) × 2 (Sex) factorial ANOVA revealed a main effect of condition only [F(4, 92) = 41.77, p<0.01] (see Fig. 1). There was no main effect of sex, or sex x condition interaction (p’s=0.06), indicating no sex differences in freezing behavior. Newman–Keuls analysis conducted on the main effect of condition showed that freezing in the Standard Context Fear condition (40.66±3.87%) was significantly higher than all other conditions. Animals in the Pre-condition (21.10±2.00%) froze significantly more than those in all remaining control conditions, which did not differ among themselves: Alt-Pre (8.54±1.84%), Handled Immediate Shock (5.22±1.60%), or Handled No Shock (0.53±0.23%) conditions. Importantly, animals in the Pre condition froze significantly more than animals in the Alt-Pre condition (p<0.001), confirming the CPFE in this study.

In summary, our behavioral data replicate and extend our previous findings with the multiple-exposure CPFE paradigm (Dokovna et al., 2013). Standard Context Fear conditioning produced higher levels of freezing than any group, including...
the Pre group, indicating that this is a stronger form of fear conditioning than the CPFE. Lastly, animals in the Alt-Pre condition do not show significantly different amounts of freezing from animals that do not learn either because of the immediate shock deficit (Handled Immediate Shock) or because of the lack of an aversive stimulus (Handled No Shock).

2.2. Gene expression

An omnibus ANOVA simultaneously comparing all of the experimental groups in this study is underpowered by the large number of groups relative to effect sizes and also includes many irrelevant contrasts (e.g. Alt-Pre vs. sCFC). We therefore analyzed CPFE and sCFC data separately, in a comparable fashion to previous studies (Asok et al., 2013b; Malkani and Rosen, 2000).

2.2.1. CPFE analysis

To examine our data under similar conditions as Asok et al. (2013b), we analyzed all brain regions across three groups that define the CPFE: Baseline, Alt-Pre, and Pre. Initially, analyses were conducted in a factorial (condition x sex) ANOVA. Because there were no significant main effects or interactions involving sex (p’s > 0.22), we collapsed across this variable and all further analyses were conducted with a one-way ANOVA. The data from this analysis are depicted in Fig. 2.

In CA1, there was no significant main effect of condition (p>0.3). Thus, the multiple-exposure procedure used in this study may have reduced the “novelty response” in CA1 that appeared on the training day with a single exposure procedure in our previous study (Asok et al., 2013b).

In the LA, there was a significant main effect of condition [F(2, 26) = 12.61, p < 0.001]. Dunnett’s test showed that both Pre and Alt-Pre groups differed significantly from Baseline (p’s < 0.001). Newman–Keuls analysis showed that gene expression in Pre (1.69 ± 0.12) and Alt-Pre (1.69 ± 0.14) groups did not differ from one another (p > 0.98). Thus, greater fear conditioning in Group Pre relative to Alt-Pre (Fig. 1) did not differentially elevate Egr-1 in the LA.

In the PL, there was a significant main effect of condition [F(2, 30) = 17.43, p < 0.001]. Dunnett’s test showed that both Pre and Alt-Pre groups differed significantly from Baseline (p’s < 0.01). Newman–Keuls analysis showed that gene expression in the Pre group was significantly higher than in the Alt-Pre group (p < 0.04). This replicates and extends our previous finding with the single-exposure CPFE procedure (Asok et al., 2013b).

In the IL, there was a significant main effect of condition [F(2, 29) = 17.45, p < 0.001]. Dunnett’s test showed that both Pre and Alt-Pre groups differed significantly from Baseline (p’s < 0.001). Newman–Keuls analysis showed that gene expression in the Pre group was significantly higher than in the Alt-Pre group (p < 0.04). This replicates and extends our previous finding with the single-exposure CPFE procedure (Asok et al., 2013b).
expression in Pre (2.68 ± 0.26) and Alt-Pre (2.36 ± 0.20) groups did not differ from one another (p > 0.27). This outcome contrasts with our previous study (see Section 4).

In summary, following context-shock training in the CPFE paradigm there was a significant fear-conditioning related difference between Pre and Alt-Pre groups in the PL wherein animals in the Pre-condition showed elevated levels of Egr-1 above animals in the Alt-Pre condition. This differential effect was not present in any of the other brain regions, although there were increases over baseline in the LA, PL, and IL in Pre and Alt-Pre conditions (see Fig. 4 for representative digitized enhanced contrast images).

2.2.2. Standard contextual fear conditioning (Baseline, No Shock, Imm Shock, sCFC)
To examine our data under similar conditions to Malkani and Rosen (2000), we analyzed all brain regions while contrasting four conditions: Baseline, No Shock, Imm Shock, and sCFC (Fig. 3). There were no significant main effects or interactions of sex (p > 0.20) in an initial 2 × 4 ANOVA, so we collapsed across this variable and all further analyses were conducted with a one-way ANOVA.

In CA1, there was a significant main effect of condition [F(3, 36) = 3.10, p < 0.04]. Dunnett’s test showed that gene expression in the sCFC group was significantly higher than Baseline (p < 0.02). Newman–Keuls analysis also showed that Egr-1 expression did not differ among the No Shock, Imm Shock, or sCFC groups (p > 0.05).

In the LA, there was a significant main effect of condition [F(3, 38) = 3.18, p < 0.04]. Dunnett’s test showed that gene expression in the Imm Shock and sCFC groups were elevated above baseline (p < 0.05), but not the No Shock group (p > 0.15). Newman–Keuls showed that Egr-1 expression did not differ among the No Shock, Imm Shock, or sCFC groups (p > 0.49).

In the PL, there was a significant main effect of condition [F(3, 34) = 16.33, p < 0.001]. Dunnett’s test showed that gene expression in the No Shock, Imm Shock, and sCFC groups were elevated above baseline (p < 0.001). Newman–Keuls analysis showed Egr-1 expression in these groups was not significantly different from one another (p > 0.16).

In the IL, there was a significant main effect of condition [F(3, 35) = 7.36, p < 0.001]. Dunnett’s test showed that gene expression in the No Shock, Imm Shock, and sCFC groups were elevated above baseline (p < 0.01). Newman–Keuls analysis showed Egr-1 expression in these groups did not significantly differ from one another (p > 0.48).

In summary, in the sCFC paradigm there was a significant elevation of Egr-1 expression above baseline in the PL and IL in all groups, but these elevations did not differ between the No Shock, Imm Shock, and sCFC groups. The sCFC group showed an increase in Egr-1 expression in both CA1 and the LA, and the Imm Shock group showed an increase over Baseline in the LA (see Fig. 4). The lack of a significant difference between the sCFC group and either No Shock or Imm Shock groups suggests that the LA effect reported by Malkani and Rosen (2000) might not extend to the conditions of the present study (see Section 4).

3. General discussion
The present study extends previous research from our laboratory on amygdala Egr-1 expression in context fear conditioning (Malkani and Rosen, 2000; Malkani et al., 2004), as well as amygdala, hippocampal, and prefrontal Egr-1 expression in the CPFE (Asok et al., 2013b). We examined if the post-training pattern of Egr-1 expression observed by Asok et al. (2013b) in the medial prefrontal cortex extended to context-shock learning in the multiple pre-exposure CPFE paradigm and to standard contextual fear conditioning. Additionally, we used a multiple pre-exposure procedure in an attempt to reduce the additive effect of environmental novelty on fear-learning-related increases of Egr-1 mRNA in the amygdala and hippocampus (Hall et al., 2000; Malkani and Rosen, 2000).

The present study extends previous reports by comparing how sCFC and CPFE training procedures activate Egr-1 in prefrontal cortical regions. The CPFE differs from sCFC in some important respects. First, the CPFE depends on an association between a retrieved context representation and shock (Rudy et al., 2004). The retrieved representation is arguably not as salient as the context itself, which can be directly associated with shock in sCFC. Second, the CPFE is a weaker form of conditioning, as reflected in levels of freezing that are typically half of what is observed in sCFC. The levels of fear conditioning in our study (Fig. 1) show strong agreement both with our previous studies of the CPFE (Asok et al., 2013b).
Fig. 4 – Digitized enhanced contrast images of animals across all experimental conditions in the Prelimbic (PL) and infralimbic (IL) of the prefrontal cortex (PFC), CA1 region of the dorsal hippocampus (dHPC), and lateral nucleus of the amygdala (LA).
The functional significance of *Egr-1* expression patterns in CA1, the LA, the PL, and the IL is discussed in Asok et al. (2013b). It is possible that during CPFE training, animals that were exposed to an alternative context (Context B; Alt-Pre) form a context-shock association with the previously acquired context (Context A; Rudy and O’Reilly, 2001; Rudy et al., 2004). Our data do not support this theory, specifically because of the lack of significant difference in freezing between animals exposed to the alternate context and animals shocked without preexposure to either context (Alt-Pre vs. Imm Shock). It is also worth noting that the learning-dependent expression of *Egr-1* mRNA in the prelimbic mPFC reported by Asok et al. (2013b) has since been replicated and reported twice outside of previous reports (e.g., Asok et al., 2013b; Schifino et al., 2011) and with Malkani and Rosen’s (2000) study of sCFC. Malkani and Rosen (2000) found an increase in LA *Egr-1* in a standard context fear conditioning group over an immediate-shock control group (Malkani and Rosen, 2000; Rosen et al., 1998). The present experiment did not obtain evidence of this effect in the LA and failed to replicate or extend it to any of the brain regions analyzed. Various methodological differences could contribute to these findings, such as handling procedures, post-shock behavior testing, or foot shock salience (length and intensity; Rosen and Donley, 2006). Additionally, Malkani and Rosen (2000) used adult male Sprague-Dawley rats, whereas the present study used adolescent Long-Evans rats of both sexes (although there were no significant sex differences in any of our reports). Fear learning during development differs from adulthood, especially with respect to the anatomical and psychological changes that characterize the adolescent developmental period (Kim et al., 2011; Markham et al., 2007; Pattwell et al., 2011; Richardson and Hunt, 2010; Rubinow and Juraska, 2009; Spear, 2000; Stanton, 2000). However, recent findings from our research group suggest that adult rats show similar patterns in *Egr-1* expression as adolescents when trained with the same general procedures used here (Chakaborty et al., 2013), suggesting that ontogenetic differences between adolescents and adults cannot explain the different outcomes of the present study versus Malkani and Rosen (2000).

It remains to be determined whether *Egr-1* expression serves exclusively as a molecular marker of learning in the CPFE. In Lee (2010), an analog to our Pre group (including multiple pre-exposure) showed an increase in *EGR-1* protein in the dHPC following training over all their experimental groups (including one similar to our Imm Shock group). The CPFE was prevented when *Egr-1* antisense was infused into the dHPC 90-min before training, leading Lee (2010) to conclude that *EGR-1* protein in the hippocampus was specifically involved in updating contextual memories to include foot-shock. In our experiment, the only significant increase in hippocampal *Egr-1* expression above baseline was observed in the sCFC group (Figs. 2 and 3), although this could be owing to a difference between the use of mRNA vs. protein expression in measuring *Egr-1* responses (Maier et al., 2009). It is also known that antisense knockdown of *Egr-1* in the LA (Maddox et al., 2011; Malkani et al., 2004) causes deficits in fear learning, suggesting that *Egr-1* is causally and specifically related to fear conditioning. However, the broader literature on *Egr-1* expands its purview beyond “fear learning” (e.g. Pre vs. Alt-Pre) to include “novelty/uncertainty responses” (e.g. Imm Shock vs. sCFC; see Davis et al., 2003; Hall et al., 2000; Knapska and Kaczmarek, 2004; Rosen and Donley, 2006; Veyrac et al., 2014). Our data suggest that elevated *Egr-1* mRNA expression in various regions during fear conditioning may be driven by multiple processes, including novelty and threat detection appraisals of uncertain and novel situations.

In summary, the present study expands upon previous research indicating that the CPFE causes *Egr-1* expression in the PL in which *Egr-1* mRNA is higher in group Pre than in group Alt-Pre, corresponding to behavioral expression of acquired fear. This increase was not seen during standard contextual fear conditioning, suggesting that the mPFC becomes more critical for contextual fear conditioning when context retrieval is important or when context cues are weak. Our findings also suggest that regional *Egr-1* expression may not be a “marker” of fear learning per se, but rather likely has a broader role as a regulator of neuronal plasticity and as a molecular priming agent that responds to novelty, uncertainty, and other factors which are necessary for learning to occur.

4. Experimental procedures

4.1. Subjects

Subjects and animal husbandry were as described in our previous reports (e.g., Asok et al., 2013b; Schifino et al., 2011). Subjects were 185 (101 males and 84 females) Long Evans rats derived from 36 time-bred dams in the University of...
Table 1 – Subject assignment and experimental design.

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Delaware breeding colony. Of these, 74 animals (40 males and 34 females) were assigned to the 6 experimental groups in the in situ hybridization assay, and 114 (63 males and 51 females) were assigned to the 5 groups that underwent behavioral testing (see Table 1, design and procedure below). The date of birth (PD 0) was determined by checking for births during the light cycle on GD 21 and 22. On PD 3, litters were culled to 8 pups (usually 4 males and 4 females) and paw-marked by subcutaneous injections of non-toxic black ink for identification purposes. Pups were kept with the dam in a clear polypropylene cage (45 × 24 × 21 cm$^3$) until PD 21, after which they were weaned and housed with same-sex littermates in 45 × 24 × 17 cm$^3$ cages. Two days prior to the start of the experiment (PD 29 ± 1), rats were individually-housed in opaque white cages (24 × 18 × 13 cm$^3$), where they lived for the remainder of the study. Same-sex littermates were assigned to different behavioral or assay conditions so that no more than one same-sex littermate was represented in a particular experimental condition. In three cases in which same-sex littermates were inadvertently assigned to the same experimental group in the behavioral experiment (2 males in group Pre, 1 female in group No Shock), the animals’ data were averaged together to yield a single data point. The final number of animals in the behavioral assay was 111 (61 males and 50 females). Animals had ad libidum access to food and water throughout the experiment. All subjects were treated in accordance with the Institutional Animal Care and Use Committee at the University of Delaware.

4.2. Apparatus and stimuli

The apparatus is the same one described previously (Asok et al., 2013b; Murawski and Stanton, 2010, 2011). There were two pre-exposure contexts, the training context (Context A) and an alternate context (Context B). Context A was a clear Plexiglas chamber measuring 16.5 × 21.1 × 21.6 cm$^3$ with a floor consisting of 9 stainless steel bars (0.5 cm diameter placed 1.25 cm apart) connected to a shock scrambler that delivered a 2 s 1.5 mA footshock (Med Associates, Georgia, VT). Four chambers (Context A) were placed on a Plexiglas stand (2 chambers per row and column) within a fume hood, which provided background light and ambient noise. The sides of each chamber were made opaque to prevent animals from viewing one another. Activity was recorded with a camera connected to a computer running FreezeFrame software (Actimetrics, Wilmette, IL). Freezing was defined as a bout of 0.75 s or longer without changes in pixel luminance. Context B was a modification of Context A to include a wire mesh insert covering the floor and protruding into the chamber to alter the spatial configuration of the context. Opaque paper was draped across three of the four outside walls of Context B; only the wall facing the camera remained visible. Chambers were cleaned with a 5% ammonium hydroxide solution immediately prior to use. Transport cages (11 × 11 × 18 cm$^3$) made of Lexan and surrounded with opaque paper on all four outside walls were used to move individual rats to and from their home cages in the colony room.

4.3. Design and procedures

Design. There were eleven experimental conditions in this study with different littersmates being assigned to each condition (Table 1). These eleven conditions were comprised of five core conditions: Handled No Shock (handled and exposed to the context without receiving a footshock; “No Shock”), Handled Immediate Shock (handled and shocked immediately upon placement in the training context; “Imm Shock”), Alt-Pre (pre-exposed to Context B, see Section 2.2), Pre (pre-exposed to Context A, see Section 2.2), and Standard Context Fear (handled on pre-exposure day, received 180 s acclimation period followed by a shock on training day; “sCFC”). These 5 conditions were assigned to behavioral testing (Fig. 1) or were sacrificed on the training day for an in situ hybridization assay that included a sixth home-cage baseline group (Figs. 2 and 3).

Procedure. The general training procedure has been described previously (Dokovna et al., 2013). Contextual fear conditioning occurred over three days (pre-exposure/handling, training, and testing) starting on PD 31(±1). Pre-exposure consisted of a 5-min exposure to one of two distinct contexts (Context A or Context B) followed by an additional five 1-min exposures. Animals that did not receive pre-exposure (Handled Immediate Shock, Handled No Shock, and Standard Context Conditioning) received equivalent
4.3.1. Pre-exposure

Animals were weighed and transported to a place outside the conditioning room. Animals were loaded into individual contexts (A or B) and allowed to explore the context for a 5-min period, after which they were returned to their transport cages. Approximately one minute later, animals received an additional five sessions of 1-min exposures to the pre-exposure context, separated by 1-min periods in their transport cages. The total procedure consisted of six exposures: one 5-min exposure followed by five 1-min exposures. After the final 1-min pre-exposure, animals were loaded into their transport cages and brought back to their home cages in the colony room.

While animals in the Pre and Alt-Pre conditions were being exposed to Context A or B, animals in the Imm Shock, No Shock, and sCFC groups received similar handling from an experimenter in a hallway outside of the behavior room (i.e., they were briefly picked up, and then replaced in their transport cages), but were not exposed to either Context A or Context B on the pre-exposure day.

4.3.2. Training

Twenty-four hours after pre-exposure, animals in the in situ hybridization condition (Table 1) were again weighed and transported four at a time to the behavior room (see Section 2.3.1). Animals were then individually brought into the conditioning room and received a 2-s 1.5 mA footshock immediately upon placement into Context A (except sCFC animals, which experienced a 180-s acclimation period prior to shock, see Section 2.3). Animals in the No Shock condition were placed in chambers with inactivated shock grids, although all other elements of the training procedure were identical to animals in the Pre, Alt-Pre, and Imm Shock groups. Animals were immediately removed, returned to their transport cages, brought back to their home cages in the colony room, and sacrificed 30 (±3) min later (see below). HC (baseline control) animals remained undisturbed in their home cages and were sacrificed while their littermate counterparts were undergoing training. Care was taken to ensure that HC controls had the same experimental history as their counterparts. For the in situ hybridization condition, the HC control group was comprised approximately equally of rats that had received Pre or Alt-Pre exposures the previous day (Table 1).

4.3.3. Retention testing

Twenty-four hours after training, the remaining littermates in the behavior condition (Table 1) were weighed and transported identically as described in the pre-exposure and training phases. Animals were loaded into the same chambers where they received training (Context A), and were monitored for freezing behavior over a 5-min testing period.

4.4. Brain collection

The procedure was the same as previously described (Asok et al., 2013b). Rats were sacrificed by rapid decapitation, and brains were removed and frozen in −45°C isopentane and stored at −80°C until sectioned. Sixteen micrometer coronal brain sections corresponding to the medial prefrontal cortex, lateral nucleus of the amygdala, and CA1 subfield of the dorsal hippocampus were sectioned on a cryostat (Leica Inc., Deerfield, IL) using the Paxinos and Watson stereotaxic brain atlas as a guide (Paxinos and Watson, 2007). Two brain sections were placed on each slide, and slides were stored at −80°C until they were processed for in situ hybridization.

4.5. In situ hybridization

In situ hybridization was conducted as described previously (Asok et al., 2013a, 2013b). An antisense RNA probe (riboprobe) was transcribed from a plasmid containing a sense cDNA sequence coding for a 230 bp sequence of Egr-1 (gift from J. Milbrandt, Washington University, St. Louis, MO). The transcribed riboprobe incorporated a radioactively labeled 35S UTP (approximately 1 × 106 dpm) using a T7 RNA polymerase Maxiscript kit according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). After hybridization and washing, the dry slides were exposed to Kodak Biomax MR Film for 2 days.

4.6. In situ hybridization image and statistical analysis

As previously described (Asok et al., 2013b), autoradiograms were captured and digitized to 8-bit gray values via a Dage CCD video camera with ImageJ 1.45m program (Wayne Rasband, NIH). ImageJ was used to subtract the background (2D-rolling ball radius of 50.0 pixels) and measure the mean density (mean gray value) within a specific area. The mean density of all mRNA labeling was analyzed for the prelimbic and infralimbic divisions of the medial prefrontal cortex (using Plate 11 of Paxinos and Watson as a guide; Paxinos and Watson, 2007), as well as the lateral nucleus of the amygdala and CA1 of the hippocampus (using Plate 57 of Paxinos and Watson as a guide; Paxinos and Watson, 2007).

The mean gray value of the left and right side of the brain was averaged within slices and then within slides. A 14C standard with known amounts of radioactivity was exposed and captured with the slides. The standard was used to generate a 3rd degree polynomial equation and calibrate the mean gray values from the slides against the radioactive standard (in nCi/g). The nCi/g value was then normalized against the average nCi/g of all home-cage animals in that region to obtain a proportionate score (the average proportionate score of the home cage group equaled 1). For each assay, when nCi/g scores differed by ±1.96 standard deviations from the nCi/g group mean for a particular region, that score was defined as an outlier and was excluded from the calculation of proportionate scores and further analysis.

In situ hybridization data (proportionate scores) were analyzed by separate one-way ANOVAs on each brain region and type of experiment (CPFE, Fig. 2; sCFC, Fig. 3; see below). Post-hoc analyses included Dunnett’s tests to contrast each
group with home-cage baseline and Newman–Keuls tests to contrast the remaining groups with each other (statistically significant differences, \( p < 0.05 \)).

4.7. Behavioral statistical analysis

Freezing behavior was scored using FreezeFrame software by an observer blind to the experimental condition of the animals as described previously (Schiffino et al., 2011). Activity thresholds were adjusted on an individual basis to exclude small movements from being calculated as part of an animal’s total freezing. Data were analyzed via one-way ANOVA, as well as Newman–Keuls and Dunnett’s post-hoc tests (Fig. 1).

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