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Research report

Expression of *egr-1* (*zif268*) mRNA in select fear-related brain regions following exposure to a predator

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Abstract

Research has demonstrated that immediate-early genes/inducible transcriptional factors (e.g., *c-fos*, *egr-1*) are increased in amygdala nuclei (lateral, basal and central nuclei) known to be involved in fear conditioning, footshock stress and novelty. Although these data suggest that expression of inducible transcriptional factors are involved in fear, other non-shock ethologically based paradigms (predator or predator odor exposure) do not appear to increase *c-fos* in the lateral and basal nuclei. While the lack of *c-fos* expression may indicate that predator stress does not engage the lateral and basal amygdala nuclei, it may be that *c-fos* in the amygdala is not responsive to predator exposure. Therefore, *egr-1*, which increases in the lateral nucleus following fear conditioning, footshock and novelty, was assessed to determine if its expression is induced in rats exposed to a cat. Five minutes of cat exposure did not increase expression of *egr-1* mRNA in the lateral nucleus of the amygdala. *egr-1* was increased in the paraventricular nucleus of the hypothalamus, indicating cat-induced stress, and visual cortex compared to rats that were either confined for 5 min or handled. In the lateral periaqueductal gray, handled rats displayed a left hemisphere dominance, which disappeared in both the cat-exposed and confined group, suggesting that immobility, induced by either cat-induced stress or unstressed confinement, increased right hemisphere *egr-1* expression. The results are discussed in a context of differences and similarities in neural circuitry for conditioned and unconditioned fear.

Keywords: *c-fos*; *egr-1*; Amygdala; Fear; Paraventricular nucleus; PVN; Periaqueductal gray; PAG; Predator; Stress; Sensory cortex

1. Introduction

The study of the neurobiology of fear has primarily relied on conditioned fear paradigms (e.g., conditioned emotional responses, Pavlovian fear conditioning) and behavior in unfamiliar situations that are fear- or anxiety-provoking (e.g., elevated-plus maze, open field) in rodents. These paradigms exploit the rodent's normal behavior to threat or danger and have produced our best understanding of the neurobiology of fear. In the last several years, other paradigms have been designed that are arguably more ethological relevant (e.g., social defeat, predator or predator odor exposure) because they emphasize interaction with other conspecifics or predators and predator odors that are part of normal evolutionarily derived ecological niches (for example, [13]). In the laboratory, some of the paradigms are quite ecologically natural, such as cat or cat odor exposure in a rodent visual burrow environment, while others take the naturally fearful stimuli or derivatives of these stimuli (e.g., cat odors or synthetic predator odors) into more traditional experimental chambers. One rationale for the development of these paradigms is that these unconditioned stimuli are more "natural" than those typically employed in fear-conditioning paradigms (i.e., electrical shock) but are still aversive or threatening without necessarily being painful. Additionally, these ethologically based paradigms rely on the unconditioned or unlearned nature of the fear stimuli, whereas fear-conditioning paradigms

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explicitly study learning and memory of fear. (The term ethologically based does not suggest that learned fear is not ethologically important nor evolutionarily relevant, but simply that some ethologically based stimuli have a specific unconditioned quality derived from survival pressures in the ecological niches animal species evolved.) Both conditioned and unconditioned paradigms measure similar defensive behaviors, so differences are in the nature of the eliciting stimuli and not the behavioral responses. Therefore, unconditioned, ethologically based paradigms may contribute to our understanding of the neurobiology of fear in unique ways that differ from conditioning paradigms.

One of the most widely studied of these paradigms is exposure to a predator or predator odor. Large lesions of the amygdala in rats block defensive behavior in response to a cat [12,30]. Smaller lesions or chemical inactivation of specific amygdaloid nuclei have shown that the medial nucleus and the associated bed nucleus of the stria terminalis reduce defensive freezing to cat or fox odor [28,39], but lesions or inactivation of the basal, lateral or central nuclei of the amygdala have little effect on freezing to these predator odors [28,39,56,63]. Interestingly, while not producing major effects on unconditioned freezing, the lesions of the basal, lateral or central nuclei of the amygdala severely disrupt fear conditioned responses [39,61,63]. These results are corroborated by a lack of an effect of MK-801, an NMDA antagonist, in the amygdala on unconditioned passive and escape responses to a live cat [8], but a lasting reduction of fearful sensitization induced by cat exposure [1,9,21]. Both the lesion and pharmacological studies suggest that conditioned and unconditioned fear responses may rely on different amygdala circuitry.

Another way of addressing the neurobiology and neurocircuitry of fear is to map activation patterns during or just following exposure to a fearful stimulus. Expression of genes that are rapidly transcribed and translated, particularly inducible transcription factors and immediate-early genes, has been used as neuronal markers of activity (e.g., [34]). One of these, *c-fos* and its protein product Fos, is activated in a number of amygdala nuclei and periaqueductal gray following fear conditioning and retention tests of fear learning [15,19,35,49,52,54,57–59]. However, *c-fos* expression is not increased in the lateral and basal nuclei of the amygdala with exposure to a live cat or predator odors [24,27,29,47], whereas it is in the periaqueductal gray [18,24,27,47]. While these data suggest that the lateral and basal nuclei of the amygdala are involved in fear conditioning but not unconditioned fear to a predator or predator odor, *c-fos* expression may not be an appropriate activity marker for the lateral and basal amygdala nuclei during fear. While some have found *c-fos* mRNA and Fos protein increased in the lateral and basal amygdala nuclei following fear conditioning or presentation of a conditioned fear stimulus [11,49,58], others have not [52,57,59].

Another inducible transcription factor/immediate-early gene, early-growth response 1 gene (*egr-1*, also called *zif268*, *ngfi-a*, *krox 24*, *tis-8*), does increase in the lateral nucleus of the amygdala with fear [32,33,40–42,57]. Some research suggests that *egr-1* increases in the lateral nucleus of the amygdala shortly following fear conditioning in a fear-conditioning specific manner [41,57], whereas others suggest that its increase is involved in the stress of unconditional fear or novelty and not specifically to its learning ([32]; for discussion of this issue, see [37]). In any case, *egr-1* expression may indicate that the lateral nucleus of the amygdala is activated during unconditioned fear to a predator.

Expression of *egr-1*, as opposed to *c-fos*, has not been examined with exposure to a predator or predator odor. While expression of *egr-1* in the lateral nucleus of the amygdala is of particular interest, whether increased *egr-1* is also found in other regions that display *c-fos* expression during stress and predator exposure is also not known. Thus, we have investigated the expression of *egr-1* mRNA by in situ hybridization in the lateral nucleus of the amygdala, the paraventricular nucleus of the hypothalamus, periaqueductal gray, and sensory cortex.

2. Methods

2.1. Animals

Thirty naïve male Long-Evans rats, about 60 days old, were purchased from Harlan. The rats were housed individually with a 12 h light:12 h dark (Memorial University Newfoundland) cycle and ad libitum access to food and water. All behavioral experiments were conducted at Memorial University Newfoundland. The Animal Care and Use Committee of Memorial University Newfoundland approved experimental protocols. Brains were shipped to the University of Delaware for in situ hybridization.

2.2. Apparatus

2.2.1. Cat exposure chamber

The chamber was a 1.52 m × 1.83 m room without separate cat and rat compartments and not cleaned of cat odors from previous experiments. The floor of the testing environment was divided into 0.1 m^2 with masking tape.

2.2.2. Confinement chamber

The chamber used to confine rats was Plexiglas cylinder (8.6 cm diameter, 20 cm long) from a commercial startle chamber (SR-Lab animal enclosure, San Diego Instruments, San Diego, CA).

3. Procedure

3.1. Cat exposure

For 3 days prior to treatments, all rats were handled for 1 min each day. Rats were randomly assigned to three groups of 10 rats each: (1) handled rats were handled for 1 min on the day of cat exposure; (2) confined rats were first acclimated to

the Plexiglas confinement cylinder over the 3 days of initial handling (day 1—2 min, day 2—3 min, and day 3—4 min) and on test day were placed in the confinement cylinder for 5 min; (3) cat-exposed rats were placed in a room with a cat for 5 min. Rats were returned to their home cages following manipulation.

Behavior of the cat and rats in the test situations was videotaped for later analysis. The percent time rats spent immobile (no movement except for breathing) during exposure to the cat and during confinement were calculated. Responses of the rats to cat approach were also monitored. Frequencies of active, passive and escape defensive responses were measured as described elsewhere [9].

Behavior of the cat in the test situation was also analyzed to provide a quantitative measure of the cat exposure experience. The cat behaviors scored from videotape were: latency to approach, and time spent near the rat; latency to sniff and time spent sniffing the rat. Time spent near the rat was scored when the cat was within 0.3 m of the rat.

3.2. In situ hybridization of egr-1

Thirty minutes after treatments, all rats were sacrificed by decapitation. The brains were removed quickly and frozen in −45 °C isopentane. They were stored at −70 °C until sectioned. Sixteen micrometer coronal brain sections corresponding to the amygdala, bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, and periaqueductal gray were sectioned on a cryostat (Leica Inc., Deerfield, IL) using the atlas of Paxinos and Watson [51] as a guide and thawed onto superfrost plus microscope slides (VWR, West Chester, PA). Two adjacent brain sections were placed on each slide. These slides were stored at −70 °C until processed for in situ hybridization.

An antisense RNA probe (riboprobe) was transcribed from a plasmid containing an antisense cDNA coding for a 230 bp sequence of *egr-1* (gift from Jeffrey Milbrandt, Washington University, St. Louis). The riboprobe was labeled by in vitro transcription with ^{35}S-UTP (approximately 10^6 dpm μg^{-1}) using a T7 RNA polymerase Maxiscript kit according to the manufacture's instructions (Ambion Inc., Austin, TX).

In situ hybridization was performed on two slides (four brain sections) per brain area per animal. Sections were fixed in 4% formaldehyde in 1× PBS and then rinsed in PBS. The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature. This was followed by dehydration in which the sections were treated with increasing concentrations of ethanol, defatted in chloroform, and then followed by another ethanol rinse. The sections were air-dried. ^{35}S-labeled riboprobe (1×10^6 cpm) was added to 100 μL of hybridization buffer and applied to each slide. The slides were covered with a glass coverslip and incubated in a humidified box overnight at 55 °C. The hybridization buffer contained 20 mM Tris–HCl (pH 7.4), 50% formamide, 300 mM NaCl, 1 mM EDTA (pH 8), 1× Denhardt's, 250 μg mL^{-1} yeast total RNA, 100 μg mL^{-1} salmon sperm DNA, 10% dextran sulfate, 100 mM dithiothreitol, 0.1% SDS, and 0.1% sodium thiosulfate. The following day, the sections were rinsed four times for 5 min each in 4× SSC. They were then treated with 20 mg L^{-1} RNAse A (Boerhinger Mannheim, Indianapolis, IN) in an RNAse Buffer solution containing 0.5 M NaCl, 1 mM EDTA, 10 mM Tris (pH 8) for 30 min at room temperature. The slides were then washed in decreasing concentrations of 1×, 0.5×, and 0.1× SSC for 5 min each. This was followed by two 30 min washes in 0.1× SSC at 65 °C. Finally, the slides were washed in increasing concentrations of ethanol containing 300 mM ammonium acetate, and allowed to air-dry. The slides were exposed to Kodak Biomax MR film for 2 days. ^{14}C standards (Amersham, Arlington Heights, IL) were also placed on the film.

egr-1 autoradiograms were digitized and converted to gray values using a Dage CCD video camera with Image 1.63 program (Wayne Rasband, NIMH) on an Apple G4 and then analyzed with the same program. The Image program was used to subtract the background (2D-rolling ball option) and measure the mean density (mean gray value) within the area of interest. Because the response of the film to the radioisotope is not linear, the gray values are not an accurate representation of the radiolabeled signal of the hybridized probe. Therefore, the gray values were converted to standardized units with a third degree polynomial equation from a standard curve constructed from the ^{14}C standards that were exposed to each film. The standardized units are not meant to be the precise amount of mRNA in a measured area, but are an accurate relative measure of *egr-1* mRNA density in the area of interest that can be used for statistical analysis. The density of *egr-1* labeling was statistically analyzed in the dorsolateral portion of the lateral nucleus of the amygdala (Plate 33 of Paxinos and Watson [51], paraventricular nucleus of the hypothalamus (Plate 27 of Paxinos and Watson [51], and periaqueductal gray (PAG, Plate 49 of Paxinos and Watson [51], and visual (Plate 49 of Paxinos and Watson [51] and somatosensory (Plate 27 of Paxinos and Watson [51] cortices. The densities of the right and left side of the brain for the four brain sections per animal were averaged into a single score in each brain area for each rat. The dorsal and lateral aspects of the PAG were analyzed separately. The area of the dorsal PAG was defined by drawing a horizontal line intersecting the most dorsal point of the aqueduct and consisted mostly of the dorsomedial PAG and some of the dorsolateral PAG. The lateral PAG consisted of the area between two horizontal lines, one intersecting the most dorsal point of the aqueduct and the other intersecting the most ventral point. The area consisted of the dorsolateral and lateral PAG. In addition, the right and left sides of the lateral PAG were analyzed separately. These demarcations of the dorsal and lateral PAG were used because previous research demonstrated hemispheric differences in phosphorylated CREB in the lateral PAG following cat exposure [4]. To test for statistically significant group differences, ANOVAs were performed for *egr-1* mRNA of each brain region separately followed by post-hoc analyses de-

scribed in the results section. Statistical significance was set at $p < 0.05$.

4. Results

4.1. Behavior

Cat-exposed rats spent 114 ± 15 s (Mean ± S.E.M.) of the 5 min exposure in a defensive immobile posture, whereas the confined rats spent 189 ± 16 s of their 5 min confinement in an immobile position. As shown in Fig. 1, confined rats spent significantly more time immobile than the cat-exposed rats ($t_{18} = 3.46$, $p < 0.003$).

Other behaviors of both the rats and the cat are shown in Table 1. These behaviors included rat approaches to the cat, rats' active and passive defense and escape responses (as defined in [9]), the latency of the cat's approach to the rats, and the time near (within 0.3 m) and latency for the cat to sniff the rats. The cat did not touch or bite any of the rats.

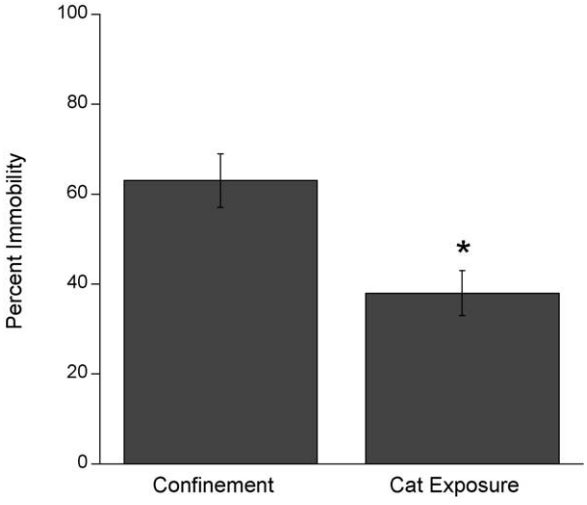

Fig. 1. Mean ± S.E.M. immobility expressed in percent time spent immobile. Both cat-exposed and retrained rat were immobile for a significant amount of time, however, immobility in the confined condition was statistically greater than immobility in the cat-exposed group ($^{*}p < 0.003$).

Table 1
Behavior of cat and rats during cat exposure

Frequency of rat behavior to cat (mean ± S.E.M.)	
Approach to cat	0.6 ± 0.3
Active defense	0.6 ± 0.3
Passive defense	6.7 ± 1.4
Escape	3.0 ± 0.9
Latency or amount of time cat spent engaged in behavior toward rats (s) (mean ± S.E.M.)	
Latency to move toward rats	139.2 ± 27.4
Time spent near rats	21.2 ± 4.6
Latency to sniff rats	159.4 ± 25.4
Time spent sniffing rats	6.9 ± 1.8

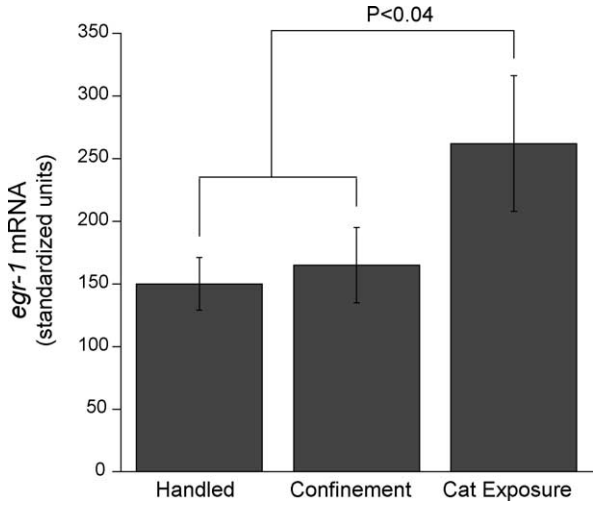

Fig. 2. Mean ± S.E.M. *egr-1* mRNA levels in the paraventricular nucleus of the hypothalamus. Expression levels in the cat-exposed group differed from a combined handled and confined group ($p < 0.04$).

4.2. Expression of egr-1 following cat exposure and confinement

4.2.1. Paraventricular nucleus of the hypothalamus (PVN)

egr-1 in the PVN was analyzed as a measure of stress. With visual inspection of both a graph of *egr-1* levels and the brain images, it appeared that there was increased *egr-1* expression in the PVN of rats exposed to the cat compared to the handled and confined rats, but there was no difference between the handled and confined rats (Figs. 2 and 3). Therefore, the *egr-1* expression in the handled and confined groups formed a combined control group for comparison to the cat-exposed group. Some of the brain sections containing the PVN were damaged (one handled and three confined) and not included in the analysis. A combined control vs. cat exposure *t*-test demonstrated a significant difference ($t_{24} = 2.21$, $p < 0.04$),

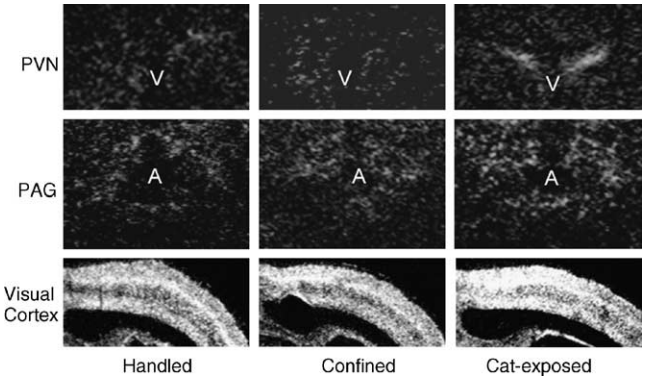

Fig. 3. Representative images of *egr-1* mRNA in the paraventricular nucleus of the hypothalamus (PVN), periaqueductal gray (PAG), and visual cortex of handled, confined and cat-exposed rats. V, ventricle; A, aqueduct. The digitized images are reversed to dark field and the contrast increased for demonstration purposes.

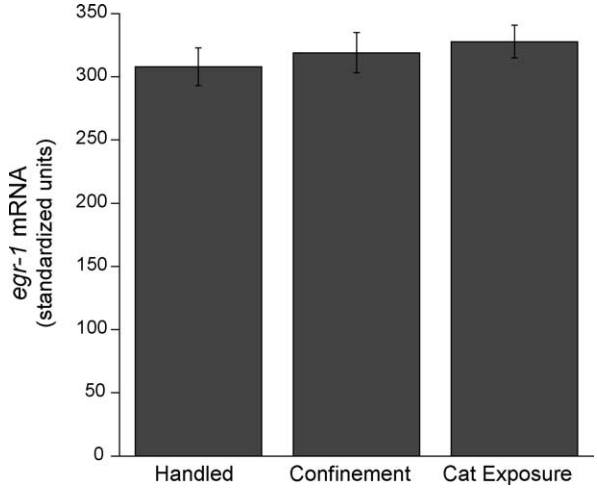

Fig. 4. Mean ± S.E.M. *egr-1* mRNA levels in the dorsolateral division of the lateral nucleus of the amygdala. There were no statistical differences between any of the groups.

indicating that cat exposure induced significantly more *egr-1* in the PVN compared to handling and confinement.

4.2.2. Amygdala

As previous studies have demonstrated [32,33,41,57], basal expression of *egr-1* mRNA is consistently found in the dorsolateral division of the lateral nucleus of the amygdala (LaDL), and following contextual fear conditioning and footshock stress *egr-1* expression increases in the LaDL [32,41,57]. Similar to what we have reported previously [41,57], expression of *egr-1* was very light in other nuclei of the amygdala. Therefore, *egr-1* was only analyzed in the LaDL. One of the brains of the cat-exposed group was damaged at the amygdala and not included in the analysis. Expression in the LaDL was found in all groups (Fig. 4), however none of the groups differed with an ANOVA ($F_{2,26} < 1$, ns) or a *t*-test after combining the handled and confined data into a single control group ($t_{27} < 1$, ns). Thus, it appears that exposure to a cat did not induce *egr-1* mRNA expression in the LaDL compared to the handled and confined groups.

4.2.3. Periaqueductal gray

Although the *egr-1* mRNA signal in the PAG was fairly light, there appeared to be higher levels in the cat-exposed rats. Therefore, expression of *egr-1* was analyzed in the dorsal and lateral aspects of the PAG because Fos expression was previously shown to be highest in these regions of the PAG following cat exposure [18] or predator odor exposure [24,27,47]. In addition, differences in expression in the left and right sides of the lateral PAG were analyzed because previous work has shown that the right lateral PAG is more critical for potentiation of defensive behavior than is the left PAG [2,3]. Representative images of *egr-1* mRNA labeling in the PAG are shown in Fig. 3.

One of the brains of the handled group was damaged in the PAG and not included in dorsal or lateral PAG analyses.

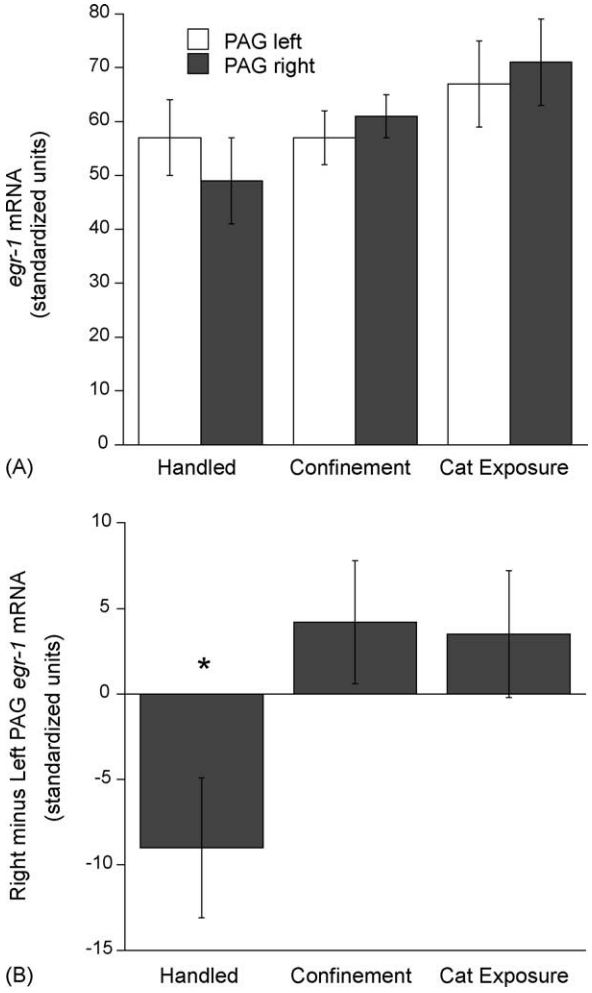

Fig. 5. Mean ± S.E.M. *egr-1* mRNA levels in the lateral periaqueductal gray. (A) Expression in the left and right hemispheres are shown. There was no main groups or main side (left vs. right) differences. However, there was a significant interaction ($p < 0.04$). (B) The interaction effect is displayed by the graph of the change in hemispheric expression of *egr-1*. Plotted over groups is mean ± S.E.M. of the difference in expression in the lateral PAG of the right and left hemispheres (*egr-1* levels in the right minus left hemispheres). The handled group displayed a left hemiphere dominance, whereas the confined and unprotected exposure groups had dominance of the right hemisphere. The difference scores of the confined and unprotected exposure groups were statistically different from the handled group ($p < 0.03$).

Analysis of *egr-1* expression in the dorsal aspect of the PAG did not reveal any differences (group effects: $F_{2,26} = 1.44$, ns; side effects: $F_{1,26} < 1$, ns; interaction: $F_{2,26} = 1.79$, ns).

Analysis of the lateral aspect of the PAG also did not find group and side effects ($F_{2,26} < 1$ and $F_{1,26} < 1$, respectively). However, there was a significant interaction of group by side of the PAG ($F_{2,26} = 3.73$, $p < 0.04$). The data are shown in graphic form in Fig. 5A.

To analyze this interaction further, a left–right difference score in *egr-1* expression in the lateral PAG was calculated for each subject (right side minus the left side). These difference scores were subjected to individual comparison tests (Fig. 5B). The unprotected cat-exposed and confined groups

significantly differed from the handled group ($p < 0.03$ for each comparison). Moreover, the difference scores of the cat-exposed and confined groups did not differ from each other. In addition, the handled difference score was biased to the left hemisphere, being less than zero ($t_8 = 2.19$, $p < .04$). In contrast, the difference scores of the unprotected cat-exposed and confined groups did not differ from zero. The right–left side PAG analysis suggests that basal (handled group) asymmetry in the lateral PAG is weighted toward more activation in the left side. However, following exposure to a cat or confinement the asymmetry was eliminated with equal activation of both hemispheres due to increased activation of the right hemisphere (Fig. 5A).

4.2.4. BNST

The BNST has been shown to be important for unconditioned fear responses in rats to a predator odor and a brightly lit environment [28,61]. The BNST was visually examined, but no expression of *egr-1* was apparent. Therefore, *egr-1* expression in the BNST was not subjected to image analysis.

4.2.5. Visual cortex and somatosensory cortex

With visual inspection of the brain images it appeared that there was increased *egr-1* expression in the visual cortex of rats exposed to a cat (Fig. 3). Image analysis was conducted on an area that included primary and secondary visual cortices of the coronal sections sliced for analysis of expression in the PAG (Plate 49 of Paxinos and Watson [51]). Because expression in the handled and confined groups did not appear to differ, a combined control group was formed for comparison to the cat-exposed group. One cat-exposed and one handled brain had damage to the visual cortex and were used not in the analysis. A combined control vs. cat exposure *t*-test including all of the animals did not find a significant difference ($t_{26} = 1.53$, $p < 0.14$). However, there were two subjects, one each in the handled and confined groups that had scores about 2 standard deviations from the group means. Analysis with these two animals removed revealed a significant difference between the combined control and cat-exposed groups ($t_{24} = 2.37$, $p < 0.03$). The analysis suggests that cat exposure induced an increase in *egr-1* expression in primary and secondary visual cortices (Fig. 6A).

Because the cat exposure group had increased *egr-1* expression in the visual cortex, image analysis was performed on the somatosensory cortex to determine if the increases also occurred in another sensory cortex. The brains from one handled, two cat-exposed, and three confined rats were damaged and not used in the analysis. In contrast to the visual cortex, there were no differences in *egr-1* expression in the somatosensory cortex with an ANOVA ($F_{2,21} < 1$, ns) or a *t*-test after combining the handled and confined data into a single control group ($t_{22} < 1$, ns). Thus, cat exposure or confinement did not induce *egr-1* in the somatosensory cortex (Fig. 6B).

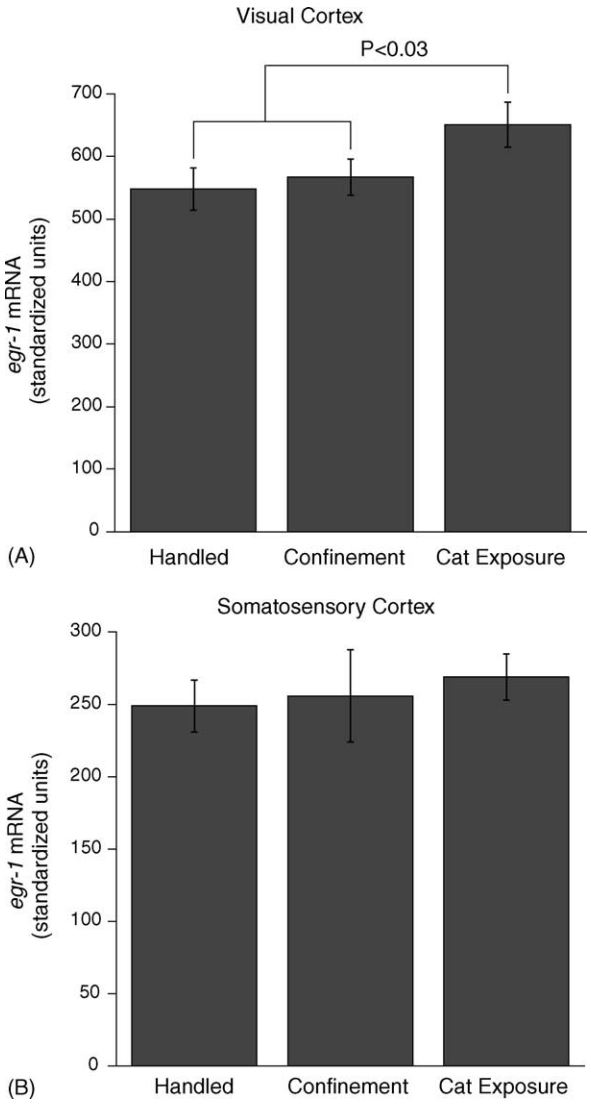

Fig. 6. Mean ± S.E.M. *egr-1* mRNA expression in the visual cortex (A) and somatosensory cortex (B). In the visual cortex, expression levels in the cat-exposed group differed from a combined handled and confined group, $p < 0.03$. *egr-1* mRNA expression in the somatosensory cortex was not different between any of the groups.

5. Discussion

The results of the present study demonstrate that only some of the selected brain regions known to be involved in fear are activated in rats exposed to a cat, as measured by *egr-1* mRNA expression. Increased expression was found in the PVN and PAG in exposed rats, whereas expression in the amygdala and the BNST was not augmented. In the cortex, cat exposure increased *egr-1* expression in the visual cortex, but not in the somatosensory cortex. These data generally agree with studies using *c-fos* in situ hybridization and Fos protein immunohistochemical analyses following cat exposure or predator odor exposure, but not in all regions [16,18,24,27,29,47]. Our behavioral and *egr-1* expression re-

sults will be discussed and compared to the Fos studies and lesion or inactivation studies.

5.1. Behavior and egr-1 expression in the PVN

Cat-exposed and confined rats spent a significant amount of their time (about 40% and 60%, respectively) immobile, and the confinement procedure actually induced significantly more immobility than cat exposure. While immobility in situations of threat or danger is generally considered a defensive behavior and indicative of a state of fear or stress, *egr-1* levels in the PVN of the confined rats were no greater than those of the handled rats. This is likely due to habituation to the confinement procedure and chamber as expression of both *c-fos* and *egr-1* decreases in the PVN with repeated restraint [20,64]. In comparison, *egr-1* expression in the PVN was significantly greater in the cat-exposed rats compared to the confined and handled rats. Because expression of immediate-early genes in the PVN is reliably induced by acute stress procedures [22], including cat odor exposure ([18,27]; however, see [29]), it is typically used as a measure of stress. Although *c-fos* expression in the PVN can be dissociated from CRH and ACTH release, *c-fos* response in the PVN has stronger links to stress than hypothalamus–pituitary–adrenal axis activity [14]. Thus, according to *egr-1* expression in the PVN, exposure to a cat was stressful, but confinement was less stressful. Furthermore, because confinement induced high levels of immobility without increased fear, a confinement procedure can be used independently as a control for the effects of immobility when measuring fear-induced freezing and *egr-1* induction in brain regions other than the PVN (see PAG below).

5.2. egr-1 expression in the visual and somatosensory cortices

As just discussed, *egr-1* in the PVN suggests that cat exposure induced fear or stress compared to confinement and handling. *egr-1* in cat-exposed rats also increased in the visual cortex compared to a combined handled-confined group. In contrast, *egr-1* expression in the somatosensory cortex did not differ in any of the groups. Taken together, the data indicate that the visual cortex, but not the somatosensory cortex, is activated when encountering a predator. The lack of increased *egr-1* expression in the somatosensory cortex differs from that found by Figueiredo et al. [29], where both test chamber and cat exposure increased *c-fos* in the somatosensory cortex. While differences in experimental procedures may contribute to the discrepant outcomes of the studies, in our experiment, it would have been interesting to see if the somatosensory cortex would be engaged in other circumstances, like a direct tactile encounter with the cat. Unfortunately, in this experiment, at no time did the cat touch any of the rats.

Other primary sensory areas were not analyzed. However, exposure to cat odor has been shown to increase the number of Fos labeled cells in both the main and accessory olfactory bulbs [47]. Exposure to a cat, which has an olfactory component, should also induce *egr-1* expression in the olfactory bulb, but this awaits demonstration.

5.3. egr-1 expression in the amygdala

In previous studies with fear conditioning, *egr-1* was induced in the dorsolateral division of the lateral nucleus of the amygdala [32,41,55,57] suggesting that *egr-1* in the lateral nucleus of the amygdala is important for learning and memory of fear [41,56,57], footshock stress or novelty [32]. In the present experiment, cat exposure did not induce *egr-1* in the lateral nucleus of the amygdala compared to both the handled and confined groups. Taken together, the results intimate that induction of *egr-1* in the amygdala is involved in transcriptional processes during fear conditioning or novelty, but not unconditioned fear of a predator. Further support for this notion is found in experiments where knocking down the levels of *egr-1* in the amygdala with an *egr-1* antisense oligodeoxynucleotide blocks long-term memory of conditioned fear but not unconditioned freezing to a predator odor [43].

The lack of change in *egr-1* expression in lateral nucleus of amygdala is of interest because cat exposure induces NMDA-dependent neuroplasticity (i.e., LTP), fear sensitization, as measured by elevated plus maze behavior and startle [7,8], and phosphorylation of CREB in the lateral and basal nuclei of the amygdala (Adamec et al., [5]). Whereas these changes appear to be independent of *egr-1* induction, an NMDA antagonist blocks both fear conditioning and increases of *egr-1* in the lateral nucleus induced by fear conditioning [42]. Together, these studies suggest different molecular processes in the lateral and basal amygdala during fear conditioning as opposed to predator stress induced fear sensitization. They further suggest that non-associative learning associated with exposure to a predator does not involve induced transcription of *egr-1* in the lateral nucleus of the amygdala, but likely activation of other transcriptional pathways (e.g., phosphorylated CREB).

In contrast to the lack of an increase in *egr-1* in the amygdala with cat exposure, small increases in Fos protein have been found in the lateral and basal nuclei with exposure to fox urine [31]. However, the importance of these increases is not known, particularly because they have not been replicated with exposure to a live cat, cat fur odor or a synthetic fox feces odor [24,27,29,47]. Furthermore, lesions or inactivation of the basolateral complex or central nucleus do not block freezing to predator odors [28,39,56,63]. Significant Fos increases have also been found in the medial nucleus of the amygdala, but not other amygdaloid nuclei, following exposure to a live cat, or cat and fox odor [24,27,29,47]. While Fos increases in the medial nucleus are not specific to unconditioned fear of predator stimuli [25,29,49,57], a recent study found a blockade of defensive freezing to cat odor following lesions of the medial nucleus of the amygdala [39]. These studies, and others [e.g., 61], suggest that the amygdala

circuitry for fear conditioning and unconditioned fear may be different [56], although other interpretations are possible [62]. Further studies on the effects of lesions or inactivation of specific amygdaloid nuclei would help clarify the amygdala circuitry for responses to predators and specific predator associated stimuli.

5.4. *egr-1* expression in the bed nucleus of the stria terminalis

The BNST did not display *egr-1* and could not be analyzed. It was anticipated that *egr-1* would increase in the BNST because the BNST is critical for freezing to a predator odor [28] and *c-fos* and Fos were found to increase in the BNST in rats exposed to a live cat, and predator odors [24,27,29,47]. These results demonstrate differential expression of *egr-1*and *c-fos* in the BNST.

5.5. *egr-1* expression in the periaqueductal gray

The PAG is known to be important for both active escape and passive immobile responses to fear and pain [26,36]. In general, the dorsolateral PAG is responsible for active behavioral and autonomic responses, while the ventrolateral PAG is responsible for passive responses [10], however, another functional parcellation is that the dorsolateral PAG is involved in unconditioned fear responses, whereas the ventrolateral PAG is part of a conditioned fear circuit [60]. Fos expression in the caudal parts of the ventrolateral PAG increases with conditioned fear [19]. Cat and cat odors exposure increases Fos expression in all aspects of the PAG [18,27,47], while *c-fos* mRNA in both the ventro- and dorso-lateral PAG was not increased by synthetic fox odor above a no odor condition [24]. Moreover, predator stress lastingly potentiates central amygdala efferent transmission to right but not left lateral PAG [4,6], and phosphorylated CREB increases in the right lateral PAG with cat exposure [4]. We therefore examined *egr-1* expression in dorsal and lateral aspects of the PAG, and hemispheric differences in the lateral aspect.

Contrary to Fos [18,27,47], there was no change in expression of *egr-1* in the dorsal PAG. This suggests that *egr-1* is not the appropriate activity marker for the dorsal PAG. As we have seen, *egr-1* is not expressed in the BNST or medial nucleus of the amygdala—both regions that Fos has been found to increase following cat and fox odor exposure [24,27,47].

In contrast to the lack of an effect in the dorsal PAG, different patterns of *egr-1* expression were found in the lateral PAG between the groups. While there were no main group or side (left or right PAG) effects, there was a significant group by side interaction. Previous studies demonstrated a right hemispheric dominance for long-lasting fear in cats and rats in the amygdala–PAG circuit [2,3]. In the present study, a similar hemispheric effect emerged in the lateral PAG of the cat-exposed and confined groups, although a distinct right hemispheric dominance did not occur. Whereas the handled group displayed a left dominance in *egr-1* expression, this shifted to symmetric hemispheric expression in the cat-exposed and confined groups due to increasing expression in the right hemisphere. Because cat-exposed rats appeared to be more fearful than confined rats (as indicated by *egr-1* expression in the PVN), the changes in hemispheric expression in the lateral PAG are likely not associated specifically with fear, but more generally with immobility. This is not universal for all transcription factors. Phosphorylation of CREB was shown to increase in the lateral PAG following cat exposure, but not confinement [4]. Whether Fos displays a right PAG dominance has not been examined.

5.6. Conclusions: functional neurocircuitry for conditioned and unconditioned fear

Expression of *egr-1* and *c-fos* following exposure to a predator or predator odors suggest a circuit for unconditioned fear that has considerable overlap with circuitry proposed for Pavlovian conditioned fear, but with some striking differences. Canteras [16] proposed an unconditioned fear circuit where a medial hypothalamic defensive system is a major interface between sensory input and motor output. Both lesion and Fos expression studies support this notion that the anterior and ventromedial hypothalamic nuclei and the dorsal premammillary nucleus are involved in fear responses to predator and predator odors [17,18,24,27,29,46,47]. Whether this system is important for fear conditioning is unknown; results from one Fos study suggest the possibility [11]. However, what appear to be missing from the unconditioned fear circuit are amygdala nuclei that are central to conditioned fear (i.e., lateral, basal, and central nuclei of the amygdala) [23,38,45,56]. *egr-1*, which is increased in the lateral nucleus of the amygdala following contextual fear conditioning, footshock stress, or novelty [32,41,57], and Fos, which has been found increased in the lateral, basal and central nuclei following fear conditioning in some studies [11,35,49,58], but not all [52,57,59], are not induced in the lateral, basal or central nuclei in response to a cat or predator odors [24,27,29,47], except in one study testing fox urine exposure [31]. Furthermore, whereas large lesions of the amygdala reduce fear responses to cat exposure [12,30], small lesions or inactivation confined to the basolateral complex or central nucleus of the amygdala do not disrupt fear responses to predator odors [28,39,56,63]. Deficits found following large amygdala lesions may be due to destruction of the medial nucleus. Indeed, a lesion confined to the medial nucleus was shown to interfere with unconditioned fear to predator odor [39]. Interestingly, the medial nucleus does not appear to be involved in fear conditioning [39,50].

In conclusion, answering questions of whether the neural circuitry instantiating conditioned and unconditioned fear are different and whether plasticity occurs in unconditioned fear circuits as it appears to occur in fear conditioned circuits [44,48,53] will benefit from application of both conditioning and ethoexperimental approaches [13] to the study of the neurobiology of fear.

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