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# Impaired Extinction of Learned Contextual Fear Memory in Early Growth Response 1 Knockout Mice

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**Inductive expression of early growth response 1 (Egr-1) in neurons is associated with many forms of neuronal activity. However, only a few Egr-1 target genes are known in the brain. The results of this study demonstrate that Egr-1 knockout (KO) mice display impaired contextual extinction learning and normal fear acquisition relative to wild-type (WT) control animals. Genome-wide microarray experiments revealed 368 differentially expressed genes in the hippocampus of Egr-1 WT exposed to different phases of a fear conditioning paradigm compared to gene expression profiles in the hippocampus of KO mice. Some of genes, such as serotonin receptor 2C (Htr2c), neuropeptide B (Npb), neuronal PAS domain protein 4 (Npas4), NPY receptor Y1 (Npy1r), fatty acid binding protein 7 (Fabp7), and neuropeptide Y (Npy) are known to regulate processing of fearful memories, and promoter analyses demonstrated that several of these genes contained Egr-1 binding sites. This study provides a useful list of potential Egr-1 target genes which may be regulated during fear memory processing.**

## INTRODUCTION

*De novo* mRNA and protein syntheses are required for both consolidation and reconsolidation of long-term fear memories, which is achieved through alterations of synaptic plasticity and neural circuit (Martin et al., 2000; Nguyen et al., 1994). Previous studies demonstrated that the transcription factors cAMP-response element (CRE) binding protein (CREB), CCAAT enhancer binding protein (C/EBP), activating protein 1 (AP-1), and Rel/nuclear factor κB (Rel/NFκB) are important for regulating expression of genes that modulate the synaptic strength and underlie formation of long-term memories in the brain (Albensi and Mattson, 2000; Alberini, 2009; Alberini et al., 1994; Meberg et al., 1996; Silva et al., 1998).

The transcription factor Egr-1, also known as Krox24, NGFIA, zif268, Zenk, and TZs8, has three zinc finger structures that mediate DNA binding to the element 5′-GCGC/GGGGCG-3′. Egr-1 is an inducible transcription factor that mediates rapid gene expression in response to various cellular stimuli. In rat brains, Egr-1 mRNA is found in the neocortex, primary olfactory bulb, entorhinal cortices, amygdaloid nuclei, nucleus accumbens, striatum, cerebellar cortex, and the hippocampus (Davis et al., 2003). Neuronal long-term potentiation (LTP) in the hippocampus significantly increases expression of Egr-1 in NMDA receptor-dependent manner (Cole et al., 1989; Wisden et al., 1990). Egr-1 knockout (KO) mice show impaired long-term memory formation in both spatial and nonspatial learning tasks and deficiencies in the late-phase of LTP but not in the early-phase of LTP or long-term depression (LTD) (Jones et al., 2001; Wei et al., 2000). A rapid and transient increase in hippocampal Egr-1 mRNA is induced by exposure to a water maze (Guzowski et al., 2001) or to contextual fear retrieval tasks (Hall et al., 2001). In addition, antisense knockdown of Egr-1 in the lateral amygdala (LA) impairs long-term memory formation but not affect acquisition or short-term memory formation (Maddox et al., 2011). However, the Egr-1 target genes that mediate these behaviors and cellular functions are unknown.

In this study, the hippocampal target genes of Egr-1 that mediate contextual fear conditioning, a hippocampus-dependent form of learning and memory, were assessed through genome wide microarray analyses of WT and Egr-1 KO mice. These analyses searched for candidate genes with expression levels that were regulated by either contextual fear conditioning or by retrieval of a contextual fear memory in WT but not in Egr-1 KO mice. Several genes met these criteria and contained promoters with Egr-1 binding sequence motifs. These genes included serotonin receptor 2C (Htr2c), neuropeptide B (Npb), neuronal PAS domain protein 4 (Npas4), NPY receptor Y1 (Npy1r), fatty acid binding protein 7 (Fabp7), and neuropeptide Y (Npy).

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## MATERIALS AND METHODS

### Animal model

The Korea University Institutional Animal Care and Use Committee approved all of experimental procedures performed with animals. In addition, all experiments were performed in accordance with the guidelines of Korea University. All mice used in this study were on the C57BL/6 genetic background. Adult male mice (WT and Egr-1 KO mice) were obtained by crossing heterozygous parent mice bearing targeted mutation of the *Egr-1* gene (Lee et al., 1995). Mice were housed in a temperature (22°C), humidity (50%), and light-controlled vivarium (12 h/12 h light/dark cycle) with *ad libitum* access to food and water.

### Contextual fear conditioning (CFC) and extinction

The fear conditioning paradigm procedures were performed in the black Plexiglas chamber (14 × 15 × 26 $cm^3$) that was in a sound-attenuating cubicle (58 × 58 × 68 $cm^3$) with a ventilation fan. Mice were gently handled for 3 days before being subjected to experiment. The fear conditioning procedures involved a 2 min habituation to the context, and then three electrical foot shock (0.5 mA foot shock, 2 s duration, and 1 min inter-trial interval). Electrical foot shocks were automatically delivered through a grid floor composed of 16 stainless steel rods (0.2 cm diameter, 0.5 cm apart) using a customized program designed with LabVIEW (National Instruments). The behaviors of the animals through the procedure were videotaped for behavioral analyses. The mice were returned to their home cage after completing the conditioning procedure. CFC was then assessed 24 h after the conditioning session by placing mice in the conditioning chamber for 5 min. Total amounts of freezing time were measured and converted to freezing percentage (total amount freezing time/5 min) to assess fear activity for each mouse. Freezing percentages were calculated in a similar fashion for the 2 min habituation and 3 min training phases. Extinction training sessions lasted for 10 min and were performed for 4 consecutive days with 24 h intersession intervals. The extinction trials consisted of additional exposures to the conditioning context. Spontaneous recovery was measured by placing the mice in the conditioning context 7 days after the last extinction trial.

### Microarray analysis

Hippocampi were dissected from mouse brains immediately after decapitation and were stored at -80°C in RNA*later* RNA Stabilization Solution (AM7020, Ambion, USA). Total RNA was extracted with a RNeasy Mini Kit (74104, Quiagen, USA) and the quantity and quality were assessed by RNA gel electrophoresis and UV spectrometric analysis. Double-stranded cDNA was generated, prepared, and labeled with an Ambion WT expression kit (4411973, Ambion) and a GeneChip WT terminal Labeling and Controls Kit (901525, Affymetrix, USA). The cDNA was hybridized to GeneChip Mouse Gene 1.0 ST Array (901171, Affymetrix), washed, and scanned according to standard Affymetrix protocols. GeneChip arrays were scanned with a GeneChip Scanner 3000 7G. Raw scanned image DAT files and processed CEL files were acquired with Affymetrix GeneChip Command Console software (AGCC).

### Microarray data processing

Raw data CEL files were imported into GeneSpring Analysis software, version GX12.1 (Agilent Technologies). These files were preprocessed with Probe Logarithmic Intensity Error (PLIER), and replicas from the conditioning group arrays (Fig. 2) and the control (naïve) arrays were then grouped using the grouping feature of the software program. Fold changes (FCs) in genes were assessed by comparing signal intensities between control naïve samples and conditioning samples. Genes with an FC of at least 1.3 were designated as DEGs.

### *In silico* inspection of promoter regions of candidate target genes

Gene2Promoter (http://www.genomatix.de) software was used to extract the promoter regions from the potential Egr-1 target genes. Specially, 2,101 bp regions [-2,000 to +100; +1 indicates the transcriptional start site (TSS)] were extracted. MatInspector (http://www.genomatix.de) software, which utilized the TRANSFAC database containing eukaryotic transcription factors (TFs) and their genomic binding sites to identify matches, was used to locate putative Egr-1 sites in these extracted DNA sequences. The MatInspector output consisted of a table indicating all putative Egr-1 binding sites. The core similarity was set at the default level of 0.85 in the MatInspector software.

### Statistical analysis

Statistical significance was evaluated using one-way ANOVA followed by Fisher's post hoc least significant difference (LSD) *t*-tests. $P < 0.05$ was considered significant. Values are expressed as mean ± SEM.

## RESULTS

### Fear extinction is delayed in Egr-1 KO mice

We utilized a classical fear conditioning paradigm to assess the behavioral responses of Egr-1 KO mice to contextual cues (Figs. 1A and 1B). In this paradigm, an association between a foot shock (unconditioned stimulus; US) and a context (conditioned context; CS) results in learned fear. Freezing responses are measured at later time points in the same apparatus that was used for the conditioning. We could not observe any significant differences in freezing levels between WT and Egr-1 KO mice 30 min or 24 h after contextual fear conditioning (CFC and Test, respectively; Fig. 1C). These data indicated that an Egr-1 deficiency did not affect hippocampus-dependent formation of fear memories. Interestingly, Egr-1 KO mice displayed delayed fear extinction relative to WT mice (Fig. 1D). We also assessed spontaneous recovery, which tests the preservation of the CS-US association after several extinction trials. Interestingly, Egr-1 KO mice showed enhanced freezing responses relative to WT mice in the spontaneous recovery test after 1 week of contextual fear extinction (Fig. 1D). These findings suggest that Egr-1 is important for ameliorating relapses to contextual fear cues.

### Microarray analysis

To identify Egr-1 target genes that are involved in hippocampus-dependent contextual fear memory formation and retrieval, we dissected hippocampi from the mice exposed to the CFC paradigm and subjected to these tissues to microarray analyses. The mice used for the microarray analyses were trained in parallel to the mice that were used for the behavior testing. Previous studies reported that Egr-1 mRNA expression is induced in various brain regions during acquisition of fear memories (Hall et al., 2001; Han et al., 2012; Maddox et al., 2011; Rosen et al., 1998). The hippocampal gene expression profiles were examined in mice taken from five different phases of a fear conditioning paradigm. These phases included naïve control (no treatment), 30 min after CFC, 1 day after CFC, 30 min after the retrieval test, and 2 h after retrieval test (Fig. 2A). Indi-

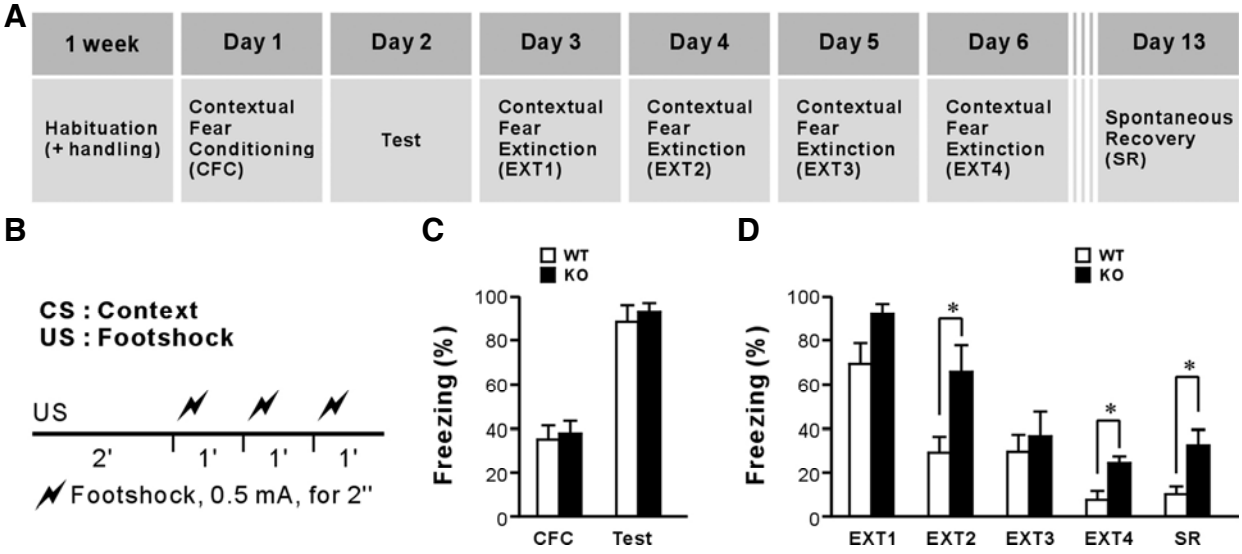


**Fig. 1.** Contextual fear conditioning (CFC) of Egr-1 KO mice. (A) Time line of CFC procedure. (B) Association between conditioned stimulus (CS, context) and unconditioned stimulus (US, foot shocks). (C) Freezing behaviors of indicated genotypes during conditioning and retrieval sessions. All mice subjected to CFC displayed robust freezing behavior. (D) Freezing behaviors of mice during extinction trials (4 day) and during spontaneous recovery of CFC (7 days after the last extinction trial). Bars ± error bars in the histogram represent mean ± SEM (n = 5 for wild type; n = 3 for KO mice). Asterisks represent level of significance (*$P < 0.05$; **$P < 0.01$) indicated by post hoc ANOVA Fisher's LSD test. CFC, contextual fear conditioning; EXT1-4, contextual fear extinction day1-4; and SR, spontaneous recovery.

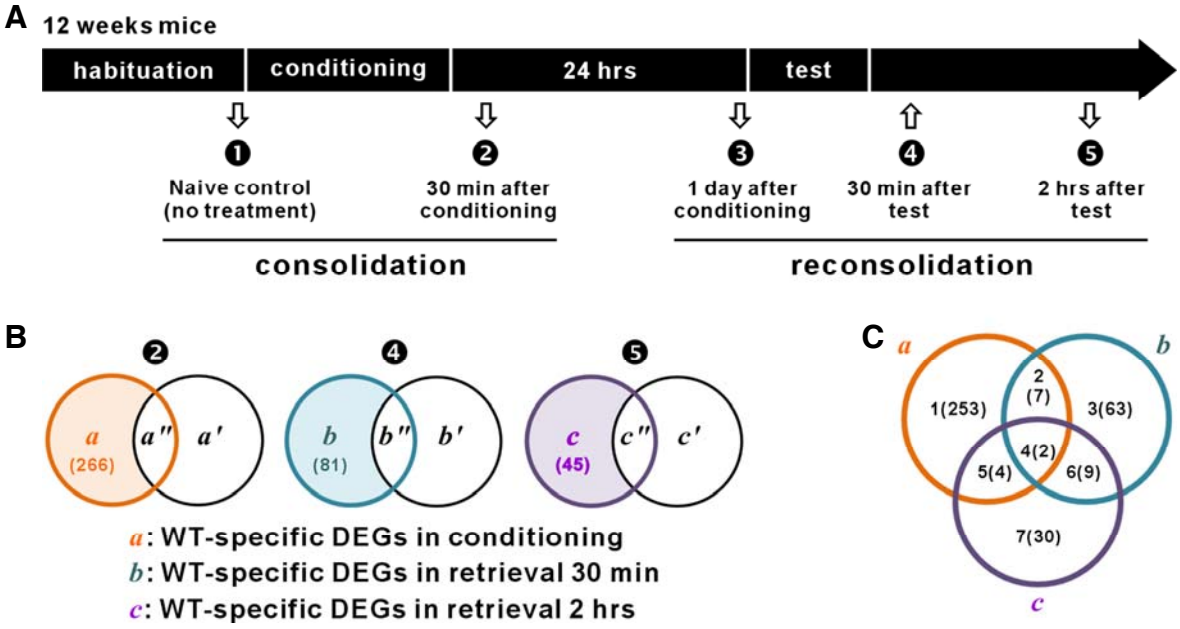


**Fig. 2.** Microarray experiments and analyses. (A) Schematic diagram illustrating the CFC procedures. ❶, ❷, ❸, ❹, and ❺ indicate five different groups: ❶, naïve control (no treatment); ❷, 30 min after CFC; ❸, 1 day after conditioning; ❹, 30 min after the retrieval test; and ❺, 2 h after the retrieval test. All mice (two WT and two KO mice per group) were sacrificed at the indicated time points. (B) Gene expression microarrays were performed on hippocampi dissected from Egr-1 KO and WT mice. Venn diagrams show the differentially expressed genes (DEGs) with a fold change of 1.3 greater based on pairwise comparisons of each experimental group relative to naïve groups in Egr-1WT ($a + a''$, $b + b''$, and $c + c''$) and KO ($a' + a''$, $b' + b''$, and $c' + c''$) mice. The genes labeled *a*, *b*, and *c* were DEGs specific to WT mice. (C) Summary of the microarray results. Circles represent the WT-specific DEGs in each group. These groups were: 30 min after fear conditioning (orange circle), 30 min after the retrieval test (emerald circle), and 2 h after the retrieval test (purple circle). The numbers in parentheses inside each compartment indicate the number of DEGs. The intersections of the sets indicate DEGs that were found in multiple groups.

**Table 1.** Promoter analysis for Egr-1 binding site

| Group | DEG | Full name | # of Egr-1 binding sites |
|---|---|---|---|
| 1 | *Arhgef5* | Rho guanine nucleotide exchange factor 5 | 1 |
| | *Ccl3* | Chemokine (C-C motif) ligand 3 | |
| | *Esr1* | Estrogen receptor 1 (alpha) | 1 |
| | *Gabrb2* | Gamma-aminobutyric acid (GABA) A receptor, subunit beta 2 | |
| | *Htr2c* | 5-Hydroxytryptamine (serotonin) receptor 2C | 1 |
| | *Il2* | Interleukin 2 | |
| | *Il5* | Interleukin 5 | |
| | *Il15* | Interleukin 15 | |
| | *Il16* | Interleukin 16 | |
| | *Il25* | Interleukin 25 | 2 |
| | *Ins2* | Insulin II | |
| | *Mc3r* | Melanocortin 3 receptor | |
| | *Npb* | Neuropeptide B | 1 |
| | *Pias1* | Protein inhibitor of activated STAT1 | 4 |
| | *Rasgrf2* | RAS protein-specific guanine nucleotide-releasing factor 2 | 1 |
| | *Shank2* | SH3/ankyrin domain gene 2 | 3 |
| 2 | *Arhgap6* | Rho GTPase activaing protein 6 | |
| | *Npas4* | Neuronal PAS domain protein 4 | 10 |
| | *Npy1r* | Neuropeptide Y receptor Y1 | 1 |
| | *Scgn* | Secretagogin, EF-hand calcium binding protein | |
| 3 | *Erbb3* | v-Erb-b2 erythroblastic leukemia viral oncogene homolog 3 | 3 |
| | *Fabp7* | Fatty acid binding protein 7, brain | 2 |
| 5 | *Unc13c* | Unc-13 homolog C (C. elegans) | |
| 6 | *Il2rg* | Interleukin 2 receptor, gamma chain | 2 |
| 7 | *Cbln1* | Cerebellin 1 precursor protein | 9 |
| | *Gh* | Growth hormone | 7 |
| | *Npy* | Neuropeptide Y | 2 |
| | *Pde11a* | Phosphodiesterase 11A | 3 |
| | *Prkcd* | Protein kinase C, delta | 2 |

Group, indicate each compartment in Fig. 2C; DEG, differentially expressed gene

vidual Affymetrix microarrays were used for bilateral hippocampi from each mouse (WT: n = 2 arrays from two mice for each group; KO: n = 2 arrays from two mice for each group). Differentially expressed genes (DEGs) were defined as genes that showed an arbitrary fold change (FC) of 1.3 or greater (30% increase or decrease in expression) in mice exposed to the fear conditioning paradigm relative to naïve control mice. The reliability of the microarray data was validated by quantitative PCR (qPCR) on the total RNA samples used in the microarray experiment during contextual fear conditioning of WT and KO mice (Supplementary Fig. 1).

To epitomize the Egr-1-dependent target genes, we subtracted DEGs of KO from those of WT for all phases. These criteria resulted in 266, 81, and 45 genes WT-specific DEGs in the conditioning (group *a*), 30 min retrieval (group *b*), and 2h retrieval (group *c*), respectively (Fig. 2B). We next compared these potential Egr-1 target DEGs among three different groups (30 min after conditioning, 30 min after retrieval, and 2 h after retrieval) with Venn diagram (Fig. 2C). These analyses revealed substantial overlap in the DEGs. Two DEGs are found in all three groups. In addition, 9 DEGs were found in group *a* and *b*, 11 were found in group *b* and *c*, and 6 were found in group *a* and *c* (Fig. 2C and Supplementary Table 1).

## Promoter analysis of candidate Egr-1 target genes

We next analyzed the potential Egr-1 target DEGs to assess whether any of these genes were known to be associated with fear memory formation and retrieval and for the presence of putative Egr-1 binding sites. We used the MatInspector Web-based search algorithm available from Genomatix Software to search for the Egr-1 binding sites in the DEG promoter sequences that spanned -2000 to -1 (Quandt et al., 1995) (Table 1). These analyses revealed that several fear memory associated genes such as Htr2c, Npb, Npas4, Npy1r, Fabp7, Il2rg, and Npy, were differentially expressed and contained putative Egr-1 binding motifs.

## Functional classification of DEGs

To further classify the potential Egr-1 target genes into biological and molecular categories, we analyzed the Gene Ontology

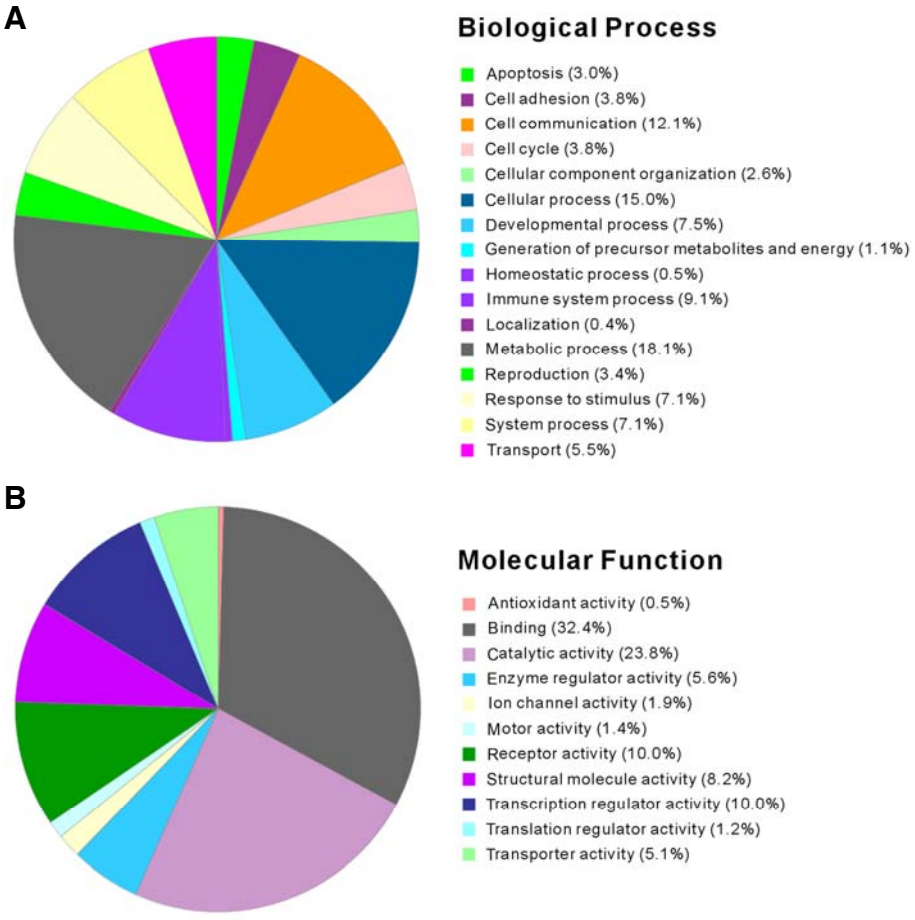


**Fig. 3.** Gene ontology (GO) annotation of WT-specific DEGs. Each pie chart represents the distribution of the GO annotations into functional categories for biological process (A) and molecular functions (B). The values in parentheses indicate the percentage of functional annotations.

annotations of the 368 DEGs with the PANTHER protein classification system (http://www.pantherdb.org). As shown in Fig. 3, the 368 DEGs were classified by the PANTHER system into 16 biological processes (genes involved in the same biological process are likely regulated in a coordinated manner) and 11 molecular functions (the biological functions of gene products). The biological process annotations that contained the greatest number of DEGs were metabolic process (18.1%), cellular processes (15.0%), and cell communication (12.1%). The molecular function annotations that contained the greatest number of DEGs were binding (32.4%) and catalytic activity (23.8%). These function annotations together comprised greater than 50% of the total number of molecular functions identified by gene ontology (GO) analyses (Fig. 3B).

## DISCUSSION

This study presented behavioral and brain transcriptome analyses of Egr-1 KO mice exposed to a contextual fear memory paradigm. The results provide further evidence that Egr-1 and its target genes are important for extinction, but not formation, of fearful memories. Ko et al. (2005) did not find any differences in formation or in extinction of contextual fear memories in Egr-1 KO mice relative to WT mice. In this study, however, we found that Egr-1 KO mice showed delayed fear extinction relative to WT mice (Figs. 1C and 1D). We did not see any effects on the formation of fearful memories. Ko et al. (2005) used a continuous scrambled foot shock at 0.75 mA for 2 s with a tone (80 dB) as a US during the fear conditioning procedure. By contrast, we presented three foot shocks without a tone (Fig. 1B). Thus, the different behavior results likely resulted from different conditioning protocols. In addition, the extinguished response (spontaneous recovery) was enhanced in Egr-1 KO mice relative to WT mice 7 days after the last extinction trial (Fig. 1D). This enhanced spontaneous recovery in Egr-1 KO mice might be due to a failure of these mice to achieve extinction learning, which is a form of long-term memory formation, suggesting that Egr-1 is required for long-term extinction memory formation.

Our microarray and promoter analyses indicate that several genes known to be associated with processing of fearful memories were potential Egr-1 target genes. More specifically, Htr2c, Npas4, Il2rg, Npy, and Npy1r are important for fear memory formation and retrieval (Burghardt et al., 2007; Fendt et al., 2009; Ramamoorthi et al., 2011; Verma et al., 2012; Wu et al., 2010). In addition, we found putative Egr-1 binding sites in the promoters of these genes (Table 1). Npas4 and Htr2c were downregulated in Egr-1 KO hippocampi relative to WT hippocampi. Npas4 is a neuronal activity-dependent immediate early gene that regulates inhibitory synapse development (Lin et al., 2008). Npas4 knockdown in the lateral amygdala (LA) impairs formation of fear memories and retention of reactivated fear memory (Ploski et al., 2011). In addition, mice that are Npas4-deficient and mice that lack Npas4 specifically in the CA3 sub-

field of the hippocampus display learning and memory deficits during CFC (Ramamoorthi et al., 2011). These results suggest that Npas4 is required for consolidation and reconsolidation of fearful memories. In addition, acute treatment of selective serotonin reuptake inhibitors (SSRIs), such as citalopram and fluoxetine, enhance acquisition of auditory fear conditioning. These SSRI effects are blocked by pretreatment with specific Htr2c antagonist (Burghardt et al., 2007), indicating that Htr2c mediates the acute anxiogenic effects of SSRIs in humans. Thus, down regulations of Naps4 and Htr2c mRNAs in Egr-1 KO during fear conditioning and retrieval likely affects the activity of neuronal circuits that underlie contextual fear processing. In contrast to Naps4 and Htr2c, Npy and Npy1r were upregulated in Egr-1 KO hippocampi. Npy and its receptors (Y1, Y2, Y3, and Y4) are concentrated in the limbic system of the brain, a circuit that includes the hippocampus and the amygdala. Interestingly, Y1 and Y2 receptor functions in the limbic system are important for fear conditioning and extinction (Verma et al., 2012).

The results in this study indicate that Egr-1 deficiency inhibits hippocampus-dependent extinction learning, but does not affect retrieval (Figs. 1C and 1D). Because Egr-1 transcriptionally regulates the distinct genes by fearful stimuli such as electrical foot shock during fear conditioning and context alone during retrieval, however, there are two possibilities to explain the behavioral differences of the Egr-1 KO mice compared to WT. One possibility is that the process of extinction is not separate from the initial acquisition process. One of the early studies of extinction in which a critical structure, the medial prefrontal cortex (mPFC) has been damaged, showed that normal acquisition can precede impaired extinction of conditioned fear response in subsequent sessions, indicating that the initial formation of fear memory could affect later extinction process (Morgan et al., 1993). Similarly, we suspect that fear memory formation in Egr-1 KO mice might have underwent different encoding process despite their seemingly normal level of fear response during acquisition phase. To support, we also found activation of Egr-1 target genes in the hippocampus, another structure that has been strongly implicated in acquisition and extinction of fear response (Ji and Maren, 2007). Therefore, it is reasonable to assume that the disruption of the Egr-1-mediated processes in the KO mice during acquisition might have facilitated abnormal encoding of fear memory, which eventually led to delayed extinction in later sessions. Another is that Egr-1 is required for reconsolidation of contextual fear memory, e.g. fear extinction. Retrieval of contextual fear memories is tested by presenting only the CS. However, retrieval test also initiates extinction of the conditioned response, since the CS no longer predicts the US. Thus, retrieval and extinction occur simultaneously in the brain since presentation of only the CS results in new learning (extinction learning) (Garelick and Storm, 2005). Egr-1 is required for the late-phase of long-term memory formation and Egr-1 expression is regulated by extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling that is activated during LTP in the dentate gyrus subfield of the hippocampus (Davis et al., 2000). Interestingly, disruption of ERK/MAPK signaling impairs both retrieval and extinction learning of contextual fear memories (Szapiro et al., 2000; 2003). Following this logic, Egr-1 and its target genes specifically enhance new fear extinction memory. This way, we could emphasize the functional link between Egr-1 target genes and the acquisition and/or extinction of fear response, which in turn would improve the cohesiveness of the data interpretation.

The microarray experiments in this study assessed hippocampal tissues during different phases of a fear conditioning paradigm. Future studies should assess whether the potential Egr-1 target genes found in this study are also altered in the amygdala of Egr-1 KO mice. Furthermore, it will be interesting to test whether altered expression of the potential Egr-1 target genes results in abnormal fear memory processing.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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