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Altered expression of synaptotagmin 13 mRNA in adult mouse brain after contextual fear conditioning

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ABSTRACT

Contextual fear memory processing requires coordinated changes in neuronal activity and molecular networks within brain. A large number of fear memory-related genes, however, still remain to be identified. Synaptotagmin 13 (Syt13), an atypical member of synaptotagmin family, is highly expressed in brain, but its functional roles within brain have not yet been clarified. Here, we report that the expression of Syt13 mRNA in adult mouse brain was altered following contextual fear conditioning. C57BL/6 mice were exposed to a novel context and stimulated by strong electrical footshock according to a contextual fear conditioning protocol. After 24 h, the mice were re-exposed to the context without electrical footshock for the retrieval of contextual fear memory. To investigate the relationship between Syt13 and contextual fear memory, we carried out *in situ* hybridization and analyzed gene expression patterns for Syt13 at four groups representing temporal changes in brain activity during contextual fear memory formation. Contextual fear conditioning test induced significant changes in mRNA levels for Syt13 within various brain regions, including lateral amygdala, somatosensory cortex, piriform cortex, habenula, thalamus, and hypothalamus, during both acquisition and retrieval sessions. Our data suggest that Syt13 may be involved in the process of contextual fear memory.

1. Introduction

Contextual fear conditioning is a typical example of classical associative learning which capitalizes on the capacity of animals to associate environmental context with an aversive stimulus. A lot of neural substrates underlying contextual fear memory have so far been identified in hippocampus and amygdala as well as other brain regions [1]. Several lines of evidence indicate that the contextual fear memory processing requires cooperative changes in brain activity and molecular networks, and that a large number of genes are dynamically regulated during contextual fear conditioning [2]. Nevertheless, numerous fear memory-related genes still remain to be identified. It is, therefore, important to uncover genes involved in the process of contextual fear memory and to elucidate their functional roles and contributions to complex behavioral changes.

Synaptotagmins are a large protein family known as membrane trafficking proteins with a short extracellular N-terminal region, a one transmembrane domain, two tandem C2 domains and a short C-terminal region. They can be subclassified based on biochemical and phylogenetic analyses. Unlike other isoforms, Syt13 lacks an N-terminal extracellular domain. Moreover, although the C-terminus of Syt13 is highly conserved as other synaptotagmin family members, its C2 domains lack essential amino acid residues responsible for calcium binding and consequent calcium-dependent regulation of phospholipid-binding activity which are key features of several other isoforms [3,4]. Because of these atypical characteristics as well as high basal level of gene expression in brain, Syt13 has been proposed to be involved in constitutive vesicular transport. Nevertheless, its exact roles within brain have not yet been elucidated. Furthermore, to our best knowledge, there have been no reports describing altered changes of Syt13 gene expression within brain in an activity-dependent manner.

In the present study, we examined the relationship between contextual fear memory and Syt13 using *in situ* hybridization and found that Syt13 mRNA expression in various brain regions was enhanced after contextual fear conditioning and retrieval. Our findings suggest that Syt13 may be associated with contextual fear memory.

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2. Materials and methods

2.1. Experimental animals

All experimental procedures with animals were approved by Korea University Institutional Animal Care and Use Committee and performed in accordance with its guidelines of Korea University. C57BL/6 mice (Orient Bio, Gyunggi-do, Korea) were 10 weeks old (20–25 g) for contextual fear conditioning. They were housed in cages with a 12 h/12 h light/dark cycle at 21–24 °C and given ad libitum access to food and water.

2.2. Behavioral apparatus

All behavioral procedures were performed in the black Plexiglas chamber (14 cm × 15 cm × 26 cm) placed in a sound-attenuating cubicle (58 cm × 58 cm × 68 cm) with a ventilation fan. Each chamber was equipped with a video camera. The chamber as a context for fear conditioning was illuminated by four red bulbs in a cubicle and floored with a grid composed of 16 stainless steel rods (0.2 cm diameter, 0.5 cm apart) connected to a shock generator (Coulbourn Instruments, Whitehall, PA) for the delivery of electrical footshock. At the termination of each behavioral session, the chamber and its grid were cleaned up with 70% ethanol.

2.3. Contextual fear conditioning

Mice were gently handled for 3 days prior to the experiment. After habituation in context for 2 min, all mice received three presentations of electrical footshock for 3 min (0.5 mA footshock, 2 s duration, 1 min inter-trial interval). Electrical footshock was delivered automatically using a customized program built on LabVIEW (National Instruments) and the animals' behaviors were videotaped for behavioral analyses. After conditioning, mice were returned to their home cage. To test the contextual fear conditioning, 24 h after the conditioning session, mice were placed for 5 min in the same chamber where they had received contextual fear conditioning training. Freezing time was measured and converted to freezing percentage to assess emotional reactivity during habituation (2 min), conditioning (total three trials 3 min, each trial 1 min), and test 24 h after conditioning (5 min). Freezing behavior was defined as a complete lack of movement excluding respiration.

2.4. In situ hybridization

In situ hybridization of mouse Syt13 was performed as previously described [5]. Briefly, ribo-probes used in this study were directed against 353–749 nt of Syt13 (Genbank access # NM_030725.4). Ribo-probes against c-*fos* and Egr1 were directed against 257–679 nt (Genbank access # NM_010234.2) and 712–1171 (Genbank access # NM_007913.5), respectively. Antisense and sense probes were produced by *in vitro* transcription in the presence of ^{35}S-UTP (Amersham). Sections were hybridized overnight at 52 °C with labeled probe (1.2×10^6 cpm/slide). On the next day, sections were treated with RNase A (Boehringer–Mannheim) for 30 min at 37 °C, washed, dehydrated in a graded series of ethanol and air-dried. Hybridized radioactivity for Syt13 was visualized by exposure to Biomax film (Kodak). To reduce variations, all the tissues were processed at the same time.

2.5. Quantification and statistical analysis

For quantification of autoradiographic films, images were captured by high-resolution. Gray values between 0 (brightest) and 255 (darkness) were assigned to the grayness of certain selections of autoradiographic images, and the film background was subtracted. Statistical significance was evaluated using one-way ANOVA followed by Tukey's *post hoc* test. $P < 0.05$ was considered significant. Values are expressed as mean ± SD.

3. Results

3.1. Contextual fear memory

To understand the relationship between contextual fear memory and Syt13, we designed the experiment to observe temporal changes in the Syt13 mRNA expression during contextual fear memory formation. For this purpose, C57BL/6 mice were trained using a contextual fear conditioning procedure in which animals were conditioned to associate an environmental context with an electrical footshock. Mice were then divided into four groups, representing temporal changes in brain activity during contextual fear memory formation, and they were decapitated at each indicated time points for gene expression analysis using *in situ* hybridization autoradiography. A schematic diagram of the contextual fear conditioning procedures is presented in Fig. 1A. The association of a

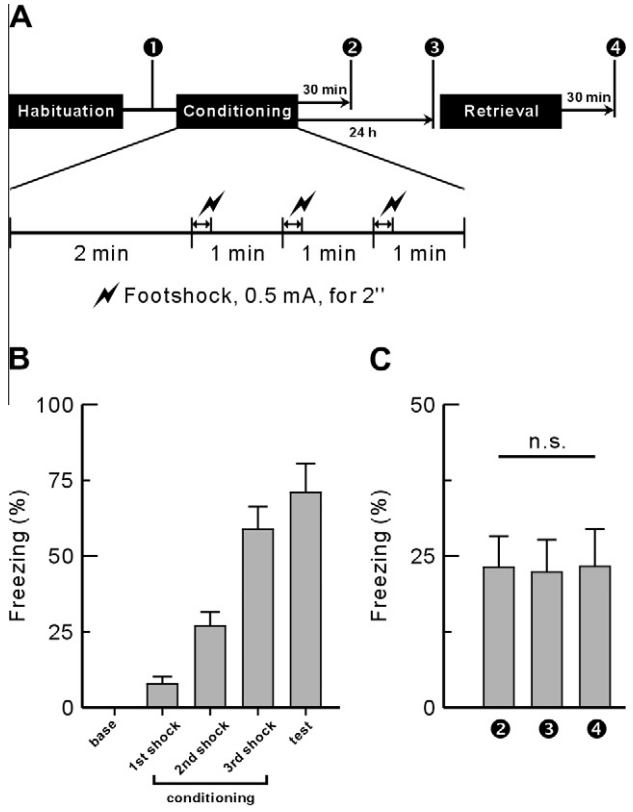

Fig. 1. Experimental design and behavioral validation of contextual fear conditioning. (A) Schematic diagram illustrating the contextual fear conditioning procedure. ❶–❹ indicate experimental groups in four different phases, i.e., naïve control (no treatment), 30 min after contextual fear conditioning with unconditioned stimulus (US), 1 day after conditioning, and 30 min after retrieval test, respectively. All the mice in the groups were decapitated at each indicated time points and sampled for gene expression analysis. (B) Freezing rate for mice during habituation, training and retrieval sessions. Freezing levels during total three trials of conditioning were separately presented and it showed increasing pattern according to trials. All the mice that had received contextual fear conditioning showed significantly robust freezing behavior against the same context even in the absence of aversive stimuli. Histogram represents mean ± SEM ($n = 12$, animals for each group). (C) Freezing levels for experimental groups during training session (3 min). Each experimental group showed statistically non-significant differences in freezing levels during conditioning. ($n = 4$, animals for each group; n.s., not significant; ANOVA Tukey's test).

footshock (unconditioned stimulus; US) with a context (conditioned stimulus; CS) results in learned fear, and freezing responses were measured for behavioral validation of the experimental system in the same apparatus that was used for conditioning. Re-exposure to the identical context elicited robust freezing behaviors even in the absence of aversive US (Fig. 1B). This result indicated that mice have successfully learned predictive value of the context for aversive stimuli and the behavioral procedures performed herein are appropriate for our experimental purpose. There were no significant differences in freezing levels during fear conditioning session among experimental groups (Fig. 1C). This result excludes the possibility of variable intensity in fear memory acquisition, which can be reflected in gene expression levels of experimental animals decapitated at different time points.

3.2. Immediate early genes during contextual fear conditioning

Previous studies showed that c-*fos* and Egr1 mRNAs, so-called immediate early genes (IEGs), are up-regulated in various brain regions during the process of fear memory acquisition [6–8]. We, therefore, used *in situ* hybridization to verify whether mRNA expressions of IEGs were enhanced in various brain regions. Analysis of c-*fos* and Egr1 mRNA expression showed significant increases in limbic regions (hippocampal CA1 and lateral amygdala; Fig. 2D–G), cortex (somatosensory and piriform cortices; Fig. 3A–C), and hypothalamus (Fig. 4A and B) at 30 min after fear conditioning. Of note, the mRNA expressions for IEGs were rapidly induced and reached the peak immediately after contextual fear conditioning process. Re-exposure to the same context without aversive stimuli generally showed similar increasing tendency in the mRNA expressions of these IEGs, but its impact were relatively low compared with the conditioning process itself. These results are consistent with previous studies and indicated that our experimental procedure was appropriate for the formation of contextual fear memory.

3.3. Syt13 mRNA expression is increased in amygdala and cortex during contextual fear conditioning

While synaptotagmins (Syts) have extensively been studied in synaptic plasticity and memory formation in a variety of memory systems, the roles of Syts in fear memory-dependent process have not yet been clearly understood. Ferguson et al. [9,10] reported earlier that Syt4 mutation disrupts contextual fear conditioning and affects hippocampus-dependent learning and memory. Therefore, to explore whether other synaptotagmin isoforms are

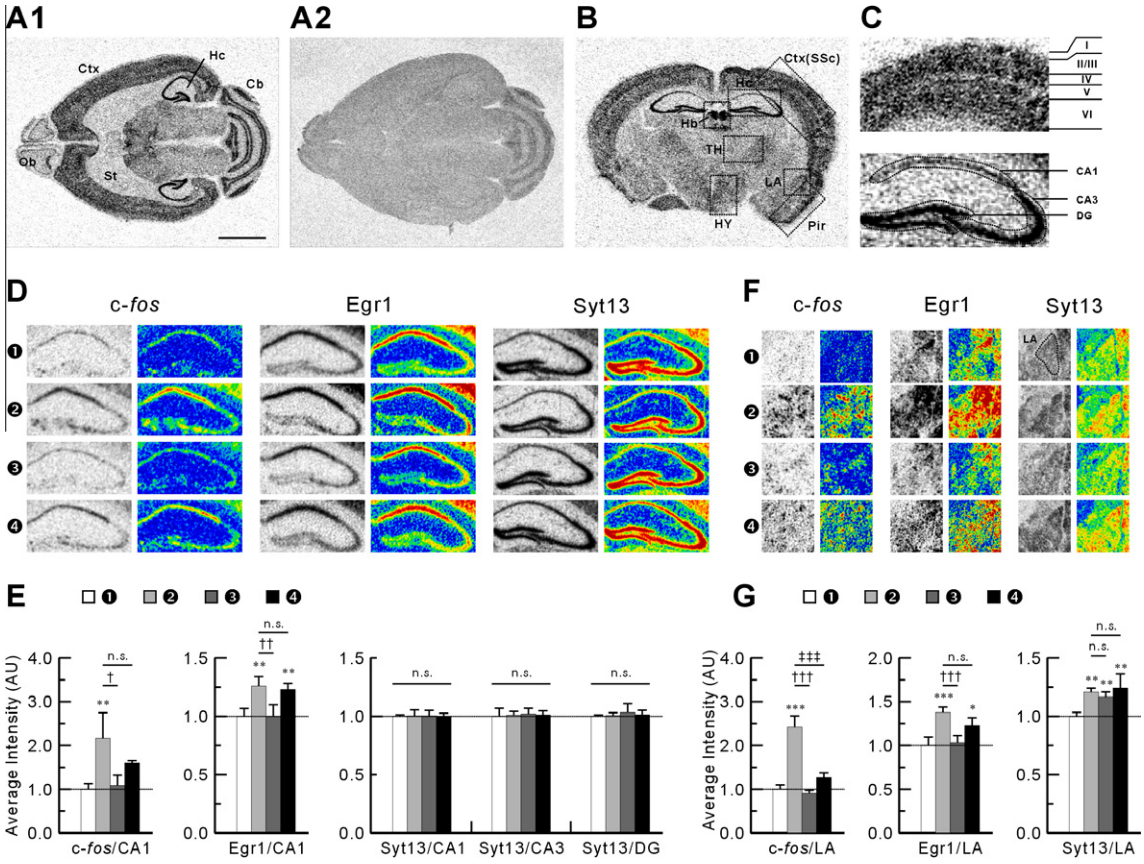

Fig. 2. Contextual fear conditioning elevates c-*fos*, Egr1 and Syt13 mRNA levels in the hippocampus and lateral amygdala of mouse brain. (A and B) Distribution patterns of Syt13 mRNAs in the adult mouse brain. Horizontal (A1) and coronal (B) sections of adult mouse brain (8 weeks) were hybridized with a Syt13 riboprobe. Riboprobe-specificity against Syt13 was verified with sense riboprobe as a negative control (A2). Ob, Olfactory bulb; Ctx, Cortex; St, Striatum; Hc, Hippocampus; Cb, Cerebellum; SSc, Somatosensory cortex; Pir, Piriform cortex; LA, Lateral amygdala; Hb, Habenula; TH, Thalamus; HY, Hypothalamus. (C) Boundary lines of brain subregions for the measurement of gene expression levels were indicated by dotted lines. (D–G) Original grayscale and pseudocolor autoradiograms of *in situ* hybridization with ^{35}S - radiolabeled probes for c-*fos*, Egr1 and Syt13 genes in the hippocampus (D) and lateral amygdala (F). LA, lateral amygdala (indicated by dotted lines). In pseudocolored images, no detectable gene expression level is indicated by *blue* and the highest expression level is indicated by *red*. Relative differences in mRNA expression for c-*fos*, Egr1 and Syt13 genes among four groups (average intensities normalized to those of naïve control) were examined in the hippocampus (E) and lateral amygdala (G). Histogram represents mean SD ($n = 4$, animals for each group; the statistical significance of each group was compared with naïve control (❶) and its value was indicated with asterisks (*) above the bars. Also, the statistical significance between experimental groups was indicated with crosses (†, between ❷ and ❸ groups; ‡, between ❷ and ❹ groups); $^{*}p \leqslant 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; n.s., not significant; ANOVA Tukey's test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

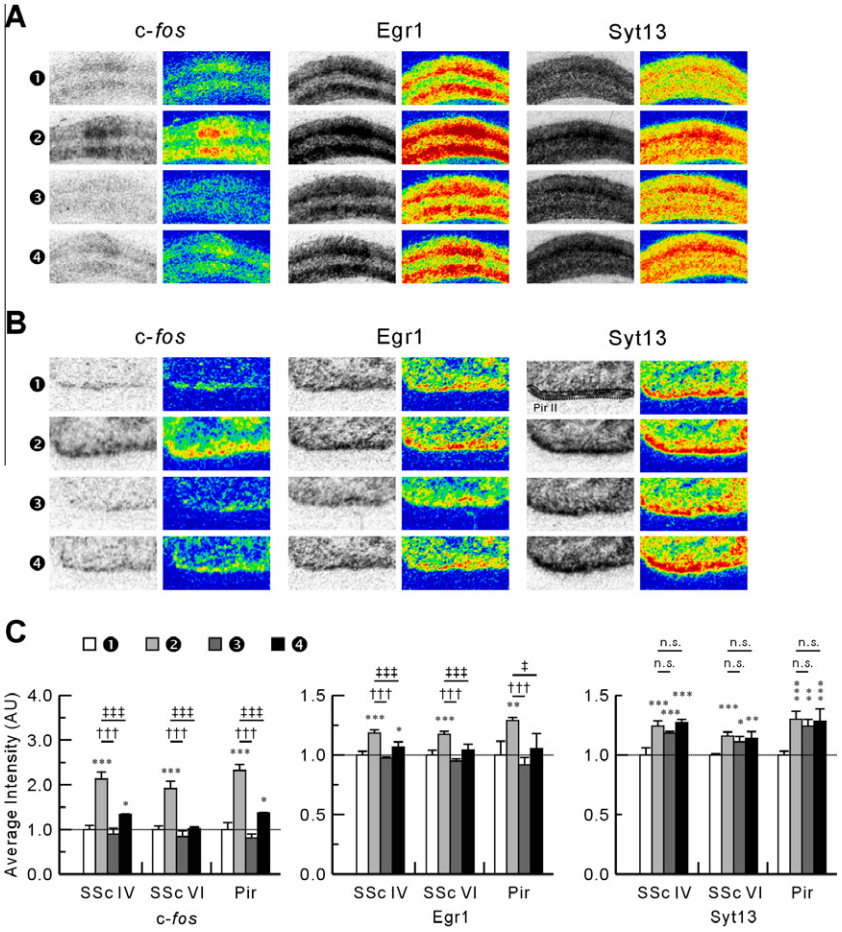

Fig. 3. Contextual fear conditioning elevates c-*fos*, Egr1 and Syt13 mRNA levels in the Somatosensory cortex (SSc) and Piriform cortex (Pir) of mouse brain. (A and B) Original grayscale and pseudocolor autoradiograms of *in situ* hybridization for c-*fos*, Egr1 and Syt13 genes in somatosensory cortical layer IV and VI and piriform cortical layer II as outlined in Fig. 2B and C. The color palette is same as in Fig. 2D and F, with *blue* showing no signal and *red* showing the highest in mRNA expression. Pir II, Piriform cortical layer II (indicated by dotted lines). (C) Relative differences in mRNA expression for c-*fos*, Egr1 and Syt13 genes among four groups (average intensities normalized to those of naïve control) were examined according to detailed anatomical regions. Histogram represents mean SD ($n = 4$, animals for each group; the statistical significance of each group was compared with naïve control (❶) and its value was indicated with asterisks (*) above the bars. Also, the statistical significance between experimental groups was indicated with crosses (†, between ❷ and ❸ groups; ‡, between ❷ and ❹ groups); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; n.s., not significant; ANOVA Tukey's test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

involved in hippocampus-dependent contextual fear memory, we performed the microarray analysis of mouse hippocampus which was taken from contextual fear conditioning models. However, we failed to find any altered expressions of 17 isoforms in hippocampus (data not shown). Even though Syt13 is enriched and shows widespread distribution in brain (Fig. 2A and B) consistent with previous report [5], its functional role is not known. Using *in situ* hybridization, therefore, we investigated Syt13 mRNA expression in the various brain regions during the contextual fear memory formation. As shown in Figs. 2 and 3, there was a significant enhancement of the gene expression in lateral amygdala (Fig. 2F and G), somatosensory cortical layer IV and VI (Fig. 3A and C) and piriform cortex layer II (Fig. 3B and C), but not in hippocampal CA1, CA3, and DG regions (Fig. 2D and E), consistent with our microarray results. It should be noted that analysis of Syt13 mRNA expression in the mouse brain revealed patterns distinct from those of IEGs: While gene expression levels of IEGs, c-*fos* and Egr1, reached the peaks during acquisition session of fear memory, decreased back to the basal levels 24 h after conditioning and increased to the high, but relatively lower than peaks, levels during retrieval session, Syt13 mRNA expression showed sustained enhancement after fear memory acquisition, suggesting the roles of Syt13 distinct from those of IEGs.

3.4. Syt13 mRNA expression is increased in epithalamus, thalamus and hypothalamus during contextual fear conditioning

Habenula, a part of epithalamus, plays a prominent part in such behavioral choice by outputting into many midbrain areas involved in releasing neuromodulators, such as dopamine and serotonin [11]. Recent studies on zebrafish suggested that habenula is important for controlling experience-dependent modification of fear responses [12]. In addition, cytoskeletal actin-binding protein, α-actinin and orphan nuclear receptor, nurr1/nr4a2 showed significant changes of mRNA expression in the habenular region after fear conditioning, but not in hippocampus [2]. These studies suggest that habenula is associated with fear memory formation through mRNA synthesis-dependent manner.

Thalamus is located between the cerebral cortex and midbrain and relays sensory and motor signals to the cerebral cortex [13,14]. However, several studies showed that thalamus is not only a relay for sensory stimuli but also a critical node in the neural circuit that supports fear memory formation in mRNA and/or protein synthesis-dependent manner [15]. In hypothalamus, innate or conditioned fear stimuli activate modulating systems of the hypothalamic–pituitary–adrenal axis (HPA axis) to control stress reactions and to express the conditioned freezing behavior [16].

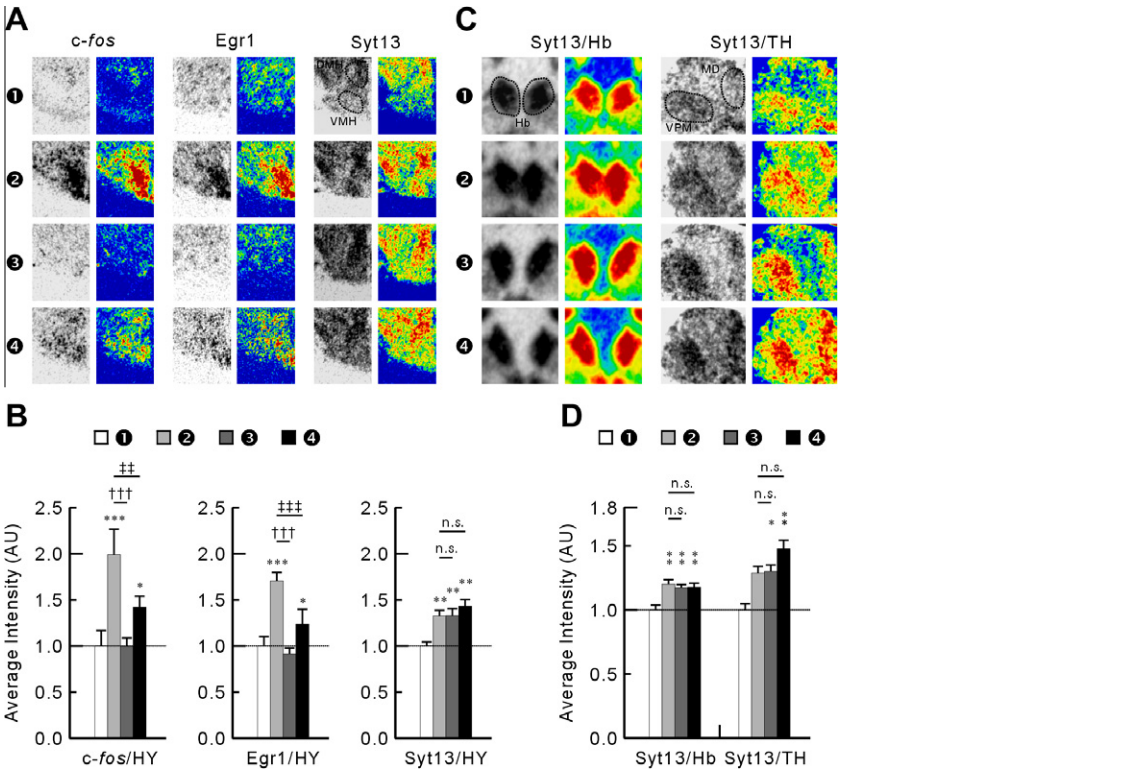

Fig. 4. Gene expression of Syt13 is enhanced in the diencephalon including habenula, thalamus and hypothalamus during both contextual fear memory acquisition and retrieval sessions. (A and B) Contextual fear conditioning significantly increased the mRNA expression of c-*fos*, Egr1 and Syt13 genes in the hypothalamus (HY). DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus (indicated by dotted lines). (C and D) Contextual fear conditioning significantly increased the mRNA expression of Syt13 gene in the habenula (Hb) and thalamus (TH). Hb, habenula; MD, mediodorsal nucleus; VPM, ventral posteromedial nucleus (indicated by dotted lines). (A and C) Original grayscale and pseudocolor autoradiograms of *in situ* hybridization for c-*fos*, Egr1 and Syt13 genes in the hypothalamus (A), habenula (C, left panel) and thalamus (C, right panel). (B and D) Relative differences in mRNA expression for c-*fos*, Egr1 and Syt13 genes among four groups (average intensities normalized to those of naïve control) were examined in the hypothalamus (B), habenula and thalamus (D). Histogram represents mean ± SD ($n = 4$, animals for each group; the statistical significance of each group was compared with naïve control (❶) and its value was indicated with asterisks (*) above the bars. Also, the statistical significance between experimental groups was indicated with crosses (†, between ❷ and ❸ groups; ‡, between ❷ and ❹ groups); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; n.s., not significant; ANOVA Tukey's test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, the ventromedial hypothalamus (VMH) is a part of a large network of hypothalamic nuclei that are involved in defensive or fear-related behavior [17], and numerous neurons within this region show increased c-*fos* expression when rats are exposed to fearful environments [18,19], which are consistent with our present data of enhanced c-*fos* mRNA expression in hypothalamus after contextual fear conditioning (Fig. 4A and B).

We determined changes in Syt13 mRNA expression after fear conditioning in epithalamus, thalamus and hypothalamus. In hypothalamus, Syt13 mRNA expression was induced after fear conditioning, maintained until the next day, and highly enhanced after retrieval process (Fig. 4A and B). This expression pattern of Syt13 in hypothalamus was distinct from those of c-*fos* and Egr1, which is similar to differential expression kinetics observed in hippocampus, lateral amygdala and cortex. Also, in habenula and thalamus, Syt13 mRNA expression significantly increased after fear conditioning and continuously maintained its expression levels (Fig. 4C and D).

4. Discussion

The results presented herein showed that Syt13 mRNA expression was increased in various brain regions after contextual fear conditioning in mice. While neuronal activity-induced IEGs, such as c-*fos* and Egr1, were increased in various brain regions and their expressions usually reached their peaks immediately after fear conditioning with electrical footshock, the highest expression of Syt13 in various brain regions was observed after re-exposure to environmental context. Significant increase in Syt13 mRNA expression during fear conditioning were observed in lateral amygdala, somatosensory cortex, piriform cortical layer II, ventral posteromedial nucleus (VPM) and mediodorsal nucleus (MD) of thalamus, and medial hypothalamus (MH). These brain subregions have been suggested to be related to associative fear memory formation.

Cortical layer IV receives the major inputs from the thalamus and transmits signals to the supragranular (layer II/III) and infragranular layers (layer V and VI). The thalamus receives sensory information from the periphery and all part of the cortex and works together with the cerebral cortex to create feedback circuit by passing information from infragranular cells to the thalamus and then back to the cortical layer VI cells. In fact, cerebral cortex combines sensory processing and memory plasticity to encode emotional behavior of perceiving stimuli [20]. The mediodorsal thalamic nucleus (MD) is an important relay station for the structures of limbic circuit and lesion of the MD significantly attenuates conditioned freezing, suggesting that it plays an essential role in acquisition, consolidation or retrieval in contextual fear memory [21]. The medial hypothalamus (MH) contains groups of neurons which command defensive behavior, and is highly activated following predator exposure or predator-derived odors [22] and $GABA_A$ receptor antagonist-induced hyperactivity in the ventromedial hypothalamus (VMH) enhanced fear behavior [23]. Therefore, lesion of hypothalamic dorsal premammillary nucleus (PMd) severely reduces the defensive responses, and pharmacological β-adrenergic blocking of PMd abolishes contextual conditioned responses [24,25].

The immediate early genes, c-*fos* and Egr1, and α-actinin are rapidly induced in the piriform cortex by Pavlovian fear conditioning [2]. Although the major afferents to the piriform originate from olfactory bulb, it has also been shown to receive multimodal input from other cortical areas and project to the basolateral amygdala (BLA) [2], indicating possible role of the piriform cortex in an associative memory. The piriform cortical layer II, which is composed of pyramidal cells, presumably receives excitatory input from the lateral olfactory tract (LOT) fibers and inhibitory input from interneuronal layer I, and makes excitatory synaptic contacts with other pyramidal cells [26].

Amygdala plays a modulatory role in memory consolidation, whereas the hippocampus is more likely a locus of memory processing [27]. Our data showed Syt13 mRNA expression levels were increased in lateral amygdala, but not in hippocampus. This result suggests that Syt13 can contribute to the modulatory role of amygdala distinct from hippocampal function in fear memory processing. Also, lateral amygdala, which showed significant changes in Syt13 mRNA expression, is the primary input station within amygdalar cirucuit for conditioned sensory information via cortical and thalamic routes [28]. Therefore, increased Syt13 mRNA expression in lateral amygdala seems to primarily reflect increased information flux and contribute to the synaptic potentiation within amygdalar circuit.

Unlike other synaptotagmins, Syt13 has previously been shown to have two distinct properties [3,4]. Syt13 lacks extracellular N-terminus and critical residues responsible for calcium binding. Because of its distinctive characteristics as well as widespread distribution in brain, Syt13 has been suggested to be involved in constitutive vesicular transport [4], and recent reports indicate that Syt4 is involved in the releases of brain-derived neurotrophic factor (BDNF) [29] and oxytocin [30]. At present, it is not known what it regulates and how it modulates synaptic plasticity, nevertheless, the increased expression of Syt13 in our present experiment is highly likely to alter synaptic strength and maintain an abiding tension, thereby providing fear memory storage and freezing behavior under aversive environments.

In conclusion, Syt13 mRNA expressions in various brain regions were increased following electrical footshock during contextual fear conditioning, and its expressions were maintained and reached the peak after re-exposure to experimental context. Since fear conditioned mice showed consistently altered expression of Syt13 mRNA, unlike IEGs showing rapid turn-on and off, it is highly likely that Syt13 contributes to synaptic modification in fear memory processing in a long-lasting manner. Based on this basic information about spatiotemporal regulation of Syt13 mRNA expression within brain in contextual fear memory process, subcellular localization and detailed functional mechanisms of Syt13 in synaptic plasticity should further be explored, and this would be our directions for future studies exploring its role in the fear memory.

Acknowledgments

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