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Research

Brain region-specific activity patterns after recent or remote memory retrieval of auditory conditioned fear

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Memory is thought to be sparsely encoded throughout multiple brain regions forming unique memory trace. Although evidence has established that the amygdala is a key brain site for memory storage and retrieval of auditory conditioned fear memory, it remains elusive whether the auditory brain regions may be involved in fear memory storage or retrieval. To investigate this possibility, we systematically imaged the brain activity patterns in the lateral amygdala, MGm/PIN, and AuV/TeA using activity-dependent induction of immediate early gene *zif268* after recent and remote memory retrieval of auditory conditioned fear. Consistent with the critical role of the amygdala in fear memory, the zif268 activity in the lateral amygdala was significantly increased after both recent and remote memory retrieval. Interesting, however, the density of zif268 (+) neurons in both MGm/PIN and AuV/TeA, particularly in layers IV and VI, was increased only after remote but not recent fear memory retrieval compared to control groups. Further analysis of zif268 signals in AuV/TeA revealed that conditioned tone induced stronger zif268 induction compared to familiar tone in each individual zif268 (+) neuron after recent memory retrieval. Taken together, our results support that the lateral amygdala is a key brain site for permanent fear memory storage and suggest that MGm/PIN and AuV/TeA might play a role for remote memory storage or retrieval of auditory conditioned fear, or, alternatively, that these auditory brain regions might have a different way of processing for familiar or conditioned tone information at recent and remote time phases.

[Supplemental material is available for this article.]

Auditory fear conditioning is a form of associative learning during which initially neutral auditory stimulus (CS, conditioned stimulus) is paired with painful foot-shock (US, unconditioned stimulus) and animals learn the association between CS and US (Kapp et al. 1979; LeDoux 2000; Fanselow and Gale 2003). When the CS is presented during testing, animals exhibit conditioned fear-response behavior such as freezing, which is used as a memory index. The amygdala, especially the lateral nucleus (hereafter LA), has been known to be a key brain site where fear-conditioning memory is encoded and stored (Davis 1992; Fanselow and LeDoux 1999; Maren and Quirk 2004; Dityatev and Bolshakov 2005; Han et al. 2007, 2009). Previous studies demonstrated that synaptic plasticity such as long-term potentiation (LTP) is elicited at the synapse of LA neurons following auditory fear conditioning (Quirk et al. 1995, 1997). In addition, lesion of LA or perturbing gene expression such as transcription or translation in LA disrupts long-term memory formation for auditory fear conditioning (Bailey et al. 1999; Schafe and LeDoux 2000; Maren et al. 2003).

During auditory fear conditioning, LA receives auditory CS input signals mainly from auditory thalamus and auditory cortex. In particular, medial division of medial geniculate nucleus and adjacent posterior intralaminar nucleus (MGm/PIN) send direct synaptic projections to the lateral amygdala, while the auditory cortex serves as an indirect pathway through the auditory thalamus (LeDoux et al. 1990; Doron and LeDoux 2000). Traditionally, these structures have been viewed as passive signal relay structures. However, the emerging idea is that they may play a role for memory formation or storage of auditory fear conditioning. According to an anatomical study, the MGm/PIN may be a brain site where CS and US converge during auditory fear conditioning, since the region receives both auditory and somatosensory inputs (Bordi and LeDoux 1994). Indeed, associative neuronal activity is elicited in MGm during auditory fear conditioning (McEchron et al. 1996; Maren et al. 2001) and increasing CREB (cAMP/Ca^{2+} responsive element binding protein) in MGm/PIN enhances memory for auditory fear conditioning (Han et al. 2008). A recent study using a specific brain lesion in rat suggests that secondary auditory cortices may serve as a memory storage site for remote auditory fear memory. Specific lesion of secondary auditory cortices after memory formation impairs remote auditory conditioned fear memory (Sacco and Sacchetti 2010). In addition, a recent report showed that the disinhibition of a specific type of interneuron in the auditory cortex layers 2/3 during fear conditioning is a critical event within local cortical circuits for normal memory formation of fear conditioning with complex CS tones (Letzkus et al. 2011). Together, these results support the idea that the auditory thalamus and auditory cortex may play important roles rather than just passively relaying auditory information to the amygdala to support auditory conditioned fear memory.

The fact that neurons in lateral amygdala receive CS inputs from both auditory thalamus and auditory cortex during auditory fear conditioning has raised important questions as to how each CS input pathway contributes to fear-conditioning memory formation. A pre-training lesion shows that each individual CS pathway alone can fully support fear memory (Romanski and LeDoux

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1992), while a post-training lesion study suggests that the auditory cortico–amygdala pathway may play a more important role (Boatman and Kim 2006). The direct input from the auditory thalamus to the LA is thought to be important for fast encoding of fear-conditioning memory, while an indirect pathway from the auditory cortex to LA contributes to processing detailed and complex CS information. Despite much research, it still remains largely elusive whether auditory thalamus and cortical structures are involved in the formation or storage of auditory conditioned fear memory, respectively, and, if so, how these structures contribute to support auditory conditioned fear memory.

To investigate the possibility that auditory thalamus and auditory cortex might be involved in the auditory fear memory storage and/or retrieval in mice, we systematically analyzed neuronal activity patterns in the auditory thalamus, especially in MGm/PIN areas, secondary auditory cortices, AuV/TeA, and LA after recent and remote fear memory retrieval. The brain region-specific activity was determined by monitoring activity-dependent induction of the immediate early gene, *zif268* (also known as *egr-1, krox-24, TIS-8*). In LA, we found that the density of zif268 (+) neurons was significantly increased compared with the control group after both recent and remote fear memory retrieval. Interestingly, the density of zif268 (+) neurons in MGm/PIN and AuV/TeA was significantly increased after remote but not recent fear memory retrieval compared with control groups. Therefore, our zif268 imaging results support that the lateral amygdala is a key brain site where fear memory is encoded and permanently stored and also suggest that MGm/PIN and AuV/TeA might be involved in supporting remote memory of auditory conditioned fear or, alternatively, that these auditory brain regions might process familiar or conditioned tone information differently depending on the passage of time.

Results

Amygdala activity after recent and remote memory retrieval

For zif268 imaging analysis, the mouse brain sections covering LA, MGm/PIN, and AuV/TeA were obtained from three experimental groups (fear conditioning, familiar tone, and naive groups). For the fear-conditioning group (hereafter FC), mice were trained with single pairing of tone and shock in a training chamber and tested 1 d (for recent fear memory) or 28 d (for remote fear memory) after training in a testing chamber. For the familiar tone group (hereafter FT), just tone without electric foot-shock was presented to the mice in a training chamber and the same tone was re-presented during testing in a testing chamber 1 d and 28 d after training (Fig. 1A). Naive animals in a home cage were included as a control to determine baseline expression of zif268. Separate groups of mice were used for recent and remote fear memory experiments for all brain regions analyzed, and zif268 immunohistochemistry was also done separately for recent and remote groups. To avoid possible bias resulting from experimental conditions, we normalized the zif268 density value from the fear-conditioning group using data from a familiar tone group in recent and remote conditions separately. Indeed, we found that zif268 staining signals in the remote group were generally weaker than in the recent group, even for the naive animals in our experimental conditions. The absolute values of zif268 density were included (Supplemental Fig. 1).

Since amygdala, especially LA, has been known to be a key brain site for fear memory encoding and storage, we first examined neuronal activity changes in LA after recent and remote fear memory retrieval. In behavior tests at both recent (1 d) and remote (28 d) time phase, mice in the FC group but not in the

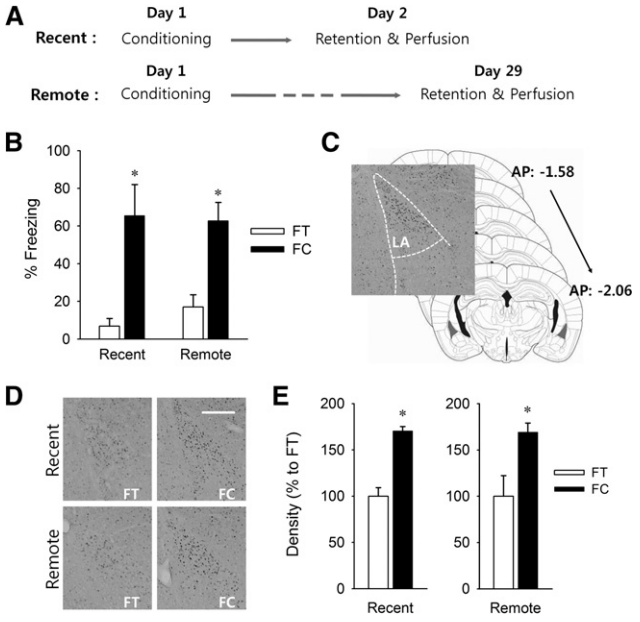

Figure 1. zif268 expression in the LA was significantly increased after both recent and remote memory retrieval of auditory conditioned fear. (*A*) Behavioral procedure of auditory fear conditioning. Fear memory was tested 1 d (recent) or 28 d (remote) after conditioning. (*B*) During a recent or remote memory retrieval test, the FC group showed robust freezing, but the FT group showed a low level of freezing. (*C*) Reconstruction of the amygdala. Five brain sections were taken from −1.58 to −2.06 (approximately bregma) along the anteroposterior (AP) direction. The density of zif268 (+) neurons was counted in LA. (*D*) Representative images of zif268 expression in the LA after the recent or remote memory retrieval. Scale bar, 300 μm. (*E*) The zif268 (+) neuronal density was significantly higher in the FC group compared with the FT group at both recent and remote time phases. The zif268 density value from the FC group was normalized to data from the FT group. All data are means ± SEM. (*) Statistically significant difference; (LA) lateral amygdala; (FT) familiar tone group; (FC) fear-conditioning group.

FT group showed high levels of freezing to the conditioned tone, indicating successful fear memory formation in the FC group (Fig. 1B) ($P < 0.001$ for recent memory test; $P < 0.05$ for remote memory test, respectively, unpaired *t*-test). To analyze neuronal activity in LA, the brain sections covering LA (1.58–2.06 mm posterior to bregma) were collected and monitored for zif268 induction (Fig. 1C). The number of zif268 (+) neurons was counted and the density of zif268 (+) neurons in a given area was measured as an index of neuronal activation from each brain section in a blind manner to make sure of unbiased cell counting. We found a significant increase of neuronal activity in LA after both recent and remote fear memory retrieval compared with the FT group. Imaging analysis showed that the density of zif268 (+) neurons in LA was significantly increased after both recent and remote memory retrieval compared with the FT group (Fig. 1D,E), suggesting that LA was persistently activated after both recent and remote auditory conditioned fear memory retrieval. Unpaired *t*-test revealed a significant effect of recent ($P < 0.01$) and remote memory retrieval ($P < 0.05$).

Auditory thalamus activity after recent and remote fear memory retrieval

Next, to determine the possible involvement of the auditory thalamus, particularly medial division of medial geniculate nucleus and adjacent posterior intralaminar nucleus MGm/PIN in recent and remote auditory fear memory storage or retrieval, the

neuronal activation in MGm/PIN was monitored by the activity-dependent expression of immediate early gene, *zif268* after recent and remote fear memory retrieval. By counting the number of zif268 (+) neurons in a given MGm/PIN area, the density of zif268 (+) neurons in MGm/PIN was measured in a blind manner as done in LA analysis. Brain sections (2.92–3.28 mm posterior to bregma) covering MGm/PIN were included for cell counting (Fig. 2A). After recent fear memory retrieval, mice in the FC group showed similar levels of zif268 (+) neuronal density with the FT group ($P = 0.558$, unpaired *t*-test) (Fig. 2B,C). To exclude the possibility that this result was a zif268-specific effect, another immediate early gene, *c-Fos*, was used to monitor the neuronal activation in MGm/PIN after recent fear memory retrieval. The c-Fos imaging data showed similar activity patterns with zif268, so unpaired *t*-test comparisons showed no difference in the c-Fos (+) neuronal density between the FC and FT groups after recent memory test ($P = 0.781$), confirming that it was not a zif268-specific effect (Fig. 2D).

Interestingly, however, after remote fear memory retrieval, mice in the FC group showed significantly higher density of zif268 (+) neurons in MGm/PIN compared with mice in the FT group ($P < 0.05$, unpaired *t*-test), suggesting potentially memory-related activity in MGm/PIN after remote fear memory retrieval (Fig. 2B,C). Of note is that MGm/PIN was also activated by the presentation of a familiar tone at the remote time point, but the density of zif268 (+) neurons was significantly lower compared with the FC group.

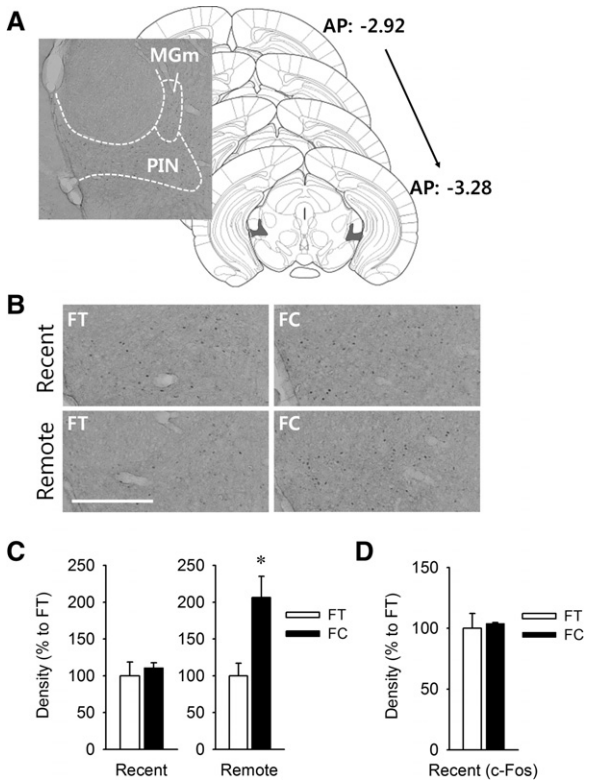

Figure 2. The density of zif268 (+) neurons in MGm/PIN was significantly increased after remote but not recent memory retrieval of auditory conditioned fear. (*A*) Reconstruction of auditory thalamus. MGm/PIN was analyzed for zif268 expression in the four brain sections from −2.92 to −3.28 (approximately bregma). (*B*) Representative images of zif268 expression in the MGm/PIN. Scale bar, 200 μm. (C) After recent fear memory retrieval, the density of zif268 (+) neurons in the MGm/PIN was not significantly changed between the FC and FT groups. In contrast, after the remote memory retrieval, the FC group showed significantly increased density of zif268 (+) neurons compared with the FT group. (*D*) When the c-Fos expression was analyzed in the MGm/PIN after the recent memory retrieval, the density of c-Fos (+) neurons was not significantly changed between FC and FT groups, consistent with zif268 expression. All data are means ± SEM. (*) Statistically significant difference; (FT) familiar tone group; (FC) fear-conditioning group.

To further examine zif268 activity induced by tone processing in MGm/PIN, we also monitored zif268 signals in MGm/PIN in response to simple presentation of a novel tone or unfamiliar tone (UFT). We performed a new set of experiments including UFT, FT, and FC groups for recent and remote testing as described above, but this time we included the UFT group. We added these new imaging data to the previous data for further zif268 imaging analysis (Supplemental Fig. 2A,B). Since immunohistochemistry was done simultaneously on the brain sections collected from all UFT group animals, and the zif268 density values from different UFT groups were not statistically different, the density values in the UFT groups were combined into a single value for the following analysis. The zif268 imaging data from the UFT and FC groups were normalized to data from the FT group for recent and remote testing separately as above. We again found that the zif268 (+) neuronal density was significantly increased after remote fear memory retrieval compared with the UFT and FT groups ($F_{(3,17)} = 7.395$; $P = 0.002$, one-way ANOVA) (Supplemental Fig. 2B). Bonferroni post hoc comparisons revealed that the density of zif268 (+) neurons was significantly higher in the FC group ($P < 0.05$) than in the UFT and FT groups (Supplemental Fig. 2B). However, the UFT and FT groups showed no significant difference in the levels of zif268 (+) neuronal density (bonferroni post hoc comparisons, $P > 0.05$). At a recent time phase, all experimental groups, UFT, FT, and FC, showed similar levels of zif268 (+) neuronal density ($F_{(2,17)} = 0.3835$; $P = 0.688$, one-way ANOVA) (Supplemental Fig. 2A).

The experience of shock itself during auditory fear conditioning might increase the response of MGm/PIN to a tone. To test this possibility, we included the shock-alone group (hereafter termed SA) as a control, in which mice received shock alone in the training chamber, and the next day a tone was given to the mice in the testing chamber. We found that zif268 (+) neuronal density in the SA group was increased by a tone presentation, but it was significantly lower compared with the remote FC group ($F_{(3,17)} = 7.395$; $P = 0.002$, one-way ANOVA) (Supplemental Fig. 2B), indicating that the increase of zif268 (+) neuronal density in MGm/PIN after remote fear memory retrieval was not due to a shock effect. Bonferroni post hoc comparisons revealed that the density of zif268 (+) neurons was significantly higher in the FC group ($P < 0.05$) than in the SA, UFT, and FT groups (which did not differ, $P > 0.05$).

Taken together, these results showed that the density of zif268 (+) neurons in MGm/PIN was increased only after remote but not recent fear memory retrieval, compared with control groups, and it was not due to a simple neuronal response to a novel or familiar tone or shock effect.

Auditory cortex activity after recent and remote fear memory retrieval

Auditory cortex serves as another CS input pathway during auditory fear conditioning, delivering auditory sensory information to the amygdala. Using the rat brain, a recent study (Sacco and Sacchetti 2010) suggests that secondary auditory cortices may support remote fear memory storage or retrieval. Based on this finding, we investigated neuronal activity patterns following recent and remote fear memory test in the mouse secondary auditory cortical areas, AuV and TeA, which are two major secondary auditory cortical areas projecting to lateral amygdala in the mouse

brain. Brain sections covering most parts of AuV and TeA (1.94–4.22 mm posterior to bregma) were included for zif268 imaging analysis (Fig. 3A). Unbiased cell counting of zif268 (+) neurons was performed in the AuV and TeA, respectively. First we analyzed the activity in AuV and TeA after recent fear memory retrieval. We found similar neuronal activity patterns in AuV and TeA after recent fear memory retrieval with the activity patterns in the auditory thalamus. No difference in the density of zif268 (+) neurons in both AuV and TeA was found between FC and FT groups ($P = 0.859$ in AuV and $P = 0.902$ in TeA, respectively, unpaired *t*-test). (Fig. 3B,C). Thus, it is likely that the increase of zif268 activity shown in AuV and TeA in the FC group after recent memory retrieval might not reflect memory-related components, but perhaps the neuronal response to familiar tone processing.

Next, we examined the neuronal activity in AuV and TeA after remote fear memory retrieval. The density of zif268 (+) neurons in the AuV and TeA of the FC group was significantly increased after remote fear memory retrieval compared with the FT group ($P < 0.05$ in AuV and $P < 0.01$ in TeA, respectively, unpaired *t*-test) (Fig. 3B,C). Although both AuV and TeA showed the increase of zif268 neuronal density compared with the FT group, we found a more dramatic increase in TeA than in AuV. The density of zif268 (+) neurons in AuV and TeA was also increased by familiar tone presentation compared with naive group, but the increase was much smaller compared with the increase found in the FC group (Supplemental Fig. 1). Therefore, as shown in MGm/PIN, the zif268 (+) neuronal density in AuV and TeA were specifically increased after remote but not recent fear memory retrieval, compared with the control group, consistent with previous data from a rat study (Sacco and Sacchetti 2010).

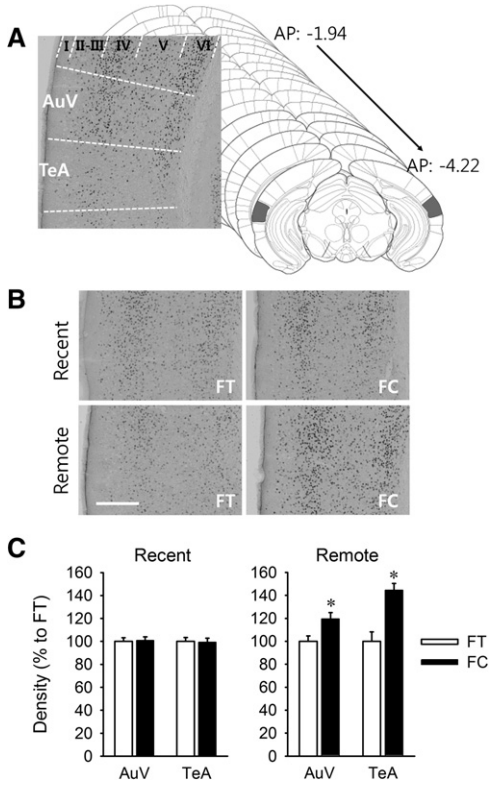

Figure 3. The density of zif268 (+) neurons in the secondary auditory cortices was increased after remote but not recent memory retrieval of auditory conditioned fear. (*A*) Reconstruction of secondary auditory cortices. AuV and TeA were analyzed in the 13 and 20 brain sections, respectively, covering from −1.94 to −4.22 (approximately bregma). (*B*) Representative images of zif268 expression in the AuV and TeA. Scale bar, 300 μm. (*C*) Only after remote memory retrieval, the FC group showed significantly higher density of zif268 (+) neurons than the FT group in the AuV and TeA, but not after recent memory retrieval. The increase of zif268 (+) neuronal density was more remarkable in the TeA than in the AuV. All data are means ± SEM. (*) Statistically significant difference; (FT) familiar tone group; (FC) fear-conditioning group.

Region and layer-specific activation of secondary auditory cortex

In order to determine whether there were any specific regions within secondary auditory cortices that showed significant activation after fear memory retrieval, we further analyzed zif268 imaging data in AuV and TeA. First, we monitored the population distribution of the zif268 (+) neurons along the AP (anterioposterior) axis (1.94–4.22 mm posterior to bregma). After recent fear memory retrieval, imaging analysis revealed no significant differences between the FC and FT groups, although we found a slight decrease of zif268 (+) neuronal density in the FC group at a very posterior part (around 3.6–4.2 mm posterior to bregma) compared with the FT group (Fig. 4A). Next, the same analysis was done with brain sections obtained from a remote fear memory retrieval condition. The results indicated that the density of zif268 (+) neurons was significantly higher in the FC group than in the FT group throughout almost all brain areas along the AP axis that we examined (Fig. 4A). Of note is that the basal expression level of zif268 protein in AuV and TeA measured from naive animals was much lower at anterior parts (around 1.94–3.0 mm posterior to bregma) compared with posterior parts (3.0–4.22 posterior to bregma) of secondary auditory cortices, resulting in more dramatic changes in zif268 (+) neuronal density at anterior parts (1.94–3.0 mm posterior to bregma).

Next, we were interested in examining whether there was any layer specificity in neuronal activity in AuV and TeA after auditory conditioned fear memory retrieval. The six layers were reanalyzed for zif268 induction after recent and remote fear memory test. Since we found that posterior parts of the AuV/TeA showed a high level of zif268 background signals (Fig. 4A), we chose brain sections from the anterior parts (2.3–2.78 mm posterior to bregma) for the layer analysis. After recent fear memory retrieval, no differences were found in zif268 (+) neuronal density between the FC and FT groups in all layers of AuV and TeA, except for layers V and VI in TeA, where we found a slight increase of zif268 density ($P = 0.048$ in layer V and $P = 0.064$ in layer VI, respectively, unpaired *t*-test) (Fig. 4B).

When examined after remote fear memory retrieval, zif268 density was specifically increased in layers IV and VI in both the AuV and TeA of the FC group compared with the FT group with more dramatic changes in TeA ($P < 0.01$ in AuV layer IV and $P < 0.05$ in AuV layer VI; $P < 0.001$ in TeA layer IV; and $P < 0.001$ in TeA layer VI, respectively, unpaired *t*-test) (Fig. 4B). This result is inconsistent with previous study using rat (Sacco and Sacchetti 2010) reporting that the increase of neuronal activity was most significant in layers II–III and IV of secondary auditory cortices after remote memory retrieval. This discrepancy might be due to the difference in experimental systems and behavior protocol.

zif268 signal-intensity analysis

We often found that the individual zif268 (+) neurons in the AuV and TeA areas showed different zif268 induction levels, measured by zif268 immunohistochemical signal intensity. Within whole zif268 (+) neuronal populations, some neurons showed much stronger zif268 signal intensity than others. Since the density of zif268 (+) neurons was increased in AuV and TeA after remote fear memory retrieval, we wondered how fear memory retrieval would change the distribution of neuronal populations with

different levels of zif268 signal intensity in AuV and TeA areas. To address this issue, we analyzed zif268 signal intensity and its distributions in AuV and TeA neuronal populations after recent and remote fear memory test. The signal intensity of zif268 was determined by measuring the relative value of pixel intensity with the image analysis software (NIS Elements software, see Materials and Methods). The brain sections obtained from three experimental groups (FC, FT, and naive groups) used for zif268 cell counting were reanalyzed for intensity analysis. First, we examined zif268 signal-intensity distributions in the AuV and TeA after recent fear memory retrieval. Cumulative distribution curve showed a normal distribution pattern of zif268 signal intensity within whole zif268 (+) neuronal populations in all experimental groups. We found that the distribution curve was shifted toward stronger zif268 signal intensity in response to conditioned tone presentation during recent fear memory retrieval compared with familiar tone presentation (Fig. 4C), indicating that a conditioned tone generally induced stronger zif268 protein expressions than a familiar tone in individual zif268 (+) neurons after recent fear memory retrieval, but without significant changes in the total number of zif268 (+) neurons. Next, we examined zif268 signal-intensity distributions after remote fear memory retrieval. Interestingly, after remote the fear memory test, the size of neuronal population with relatively stronger zif268 signal intensity in the FC group was increased, while the size of relatively weaker zif268 (+) neuronal population was not significantly changed compared with the FT group, indicating that the density of zif268 (+) neurons after remote fear memory retrieval was increased due to the increase of the size of the stronger zif268 (+) neuronal population. This result suggests that this newly incorporated strongly activated neuronal population in AuV and TeA after remote fear memory retrieval might represent memory-specific ensemble of neurons in secondary auditory cortices.

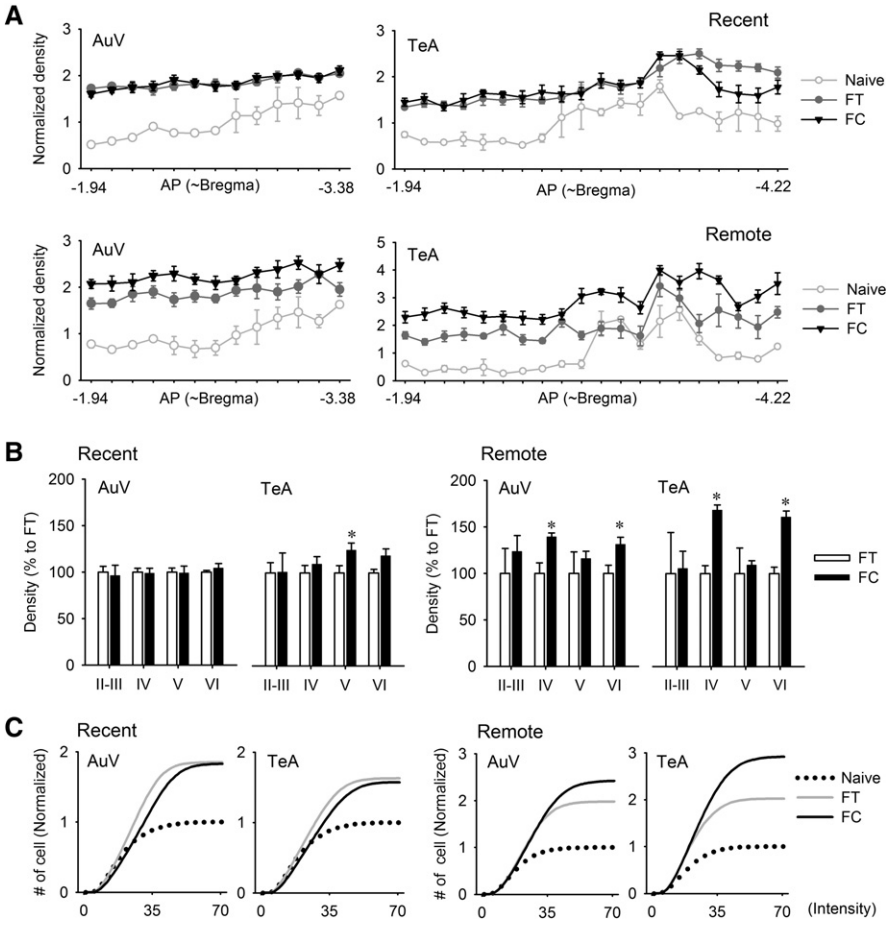

Figure 4. Detailed analysis of zif268 expression patterns in the secondary auditory cortices. (*A*) The density distributions of the zif268 (+) neuronal populations along the anteroposterior axis in the AuV and TeA after recent (*top*) or remote (*bottom*) memory retrieval. The density values from the FT and FC groups were normalized to the mean value from the naive group. (*B*) Cortical layer-specific expression of zif268 in AuV and TeA. The result indicated that the difference of zif268 (+) neuronal density between the FC and FT groups was most significant in the layers IV and VI after the remote memory retrieval. The density value of zif268 (+) neurons from the FC group was normalized to data from the FT group. (*C*) Accumulated intensity distributions of zif268 signals in zif268 (+) neuronal populations in the AuV and TeA following recent and remote memory retrieval. After the recent memory retrieval, the distribution curve of the FC group was shifted with the right side toward the direction of stronger zif268 signals compared with the FT group in both AuV and TeA, without significant change in the total number of zif268 (+) neurons. In contrast, after remote memory retrieval, the distribution curve of the FC group was shifted upward compared with the FT group, indicating that the size of neuronal populations with relatively stronger zif268 signals was specifically increased following remote memory retrieval, resulting in an increased total number of zif268 (+) neurons in the FC group compared with the FT group. The number of cells was normalized to the naive group. All data are means ± SEM. (*) Statistically significant difference; (FT) familiar tone group; (FC) fear-conditioning group.

Discussion

Using zif268 imaging, we aimed to investigate brain activity patterns in three brain regions important for auditory fear memory, LA, auditory thalamus, and auditory cortex after auditory fear memory retrieval. Specifically, we monitored the density of immediate early gene, *zif268*-induced neurons in LA, MGm/PIN, and AuV/TeA after recent and remote auditory fear memory retrieval. To isolate brain activities potentially related to fear memory from the activities related to auditory processing or sensory perception, we compared zif268 activity induced by the presentation of the tone paired with the foot-shock (FC group) with the novel (UFT group) or previously perceived tone (FT group). Importantly, zif268 (+) neuronal density in LA was significantly increased after both recent and remote fear memory retrieval compared with control group. This result supports that LA is the essential brain site for permanent storage of fear memory. However, MGm/PIN and AuV/TeA showed different activity patterns from LA. In both MGm/PIN and AuV/TeA, zif268 (+) neuronal density was specifically increased after remote but not recent fear memory retrieval compared with control groups.

Previous studies using rats showed that lesion of secondary auditory cortices impairs fear memory retrieval only when it is

done in a remote time phase but not a recent time phase (Sacco and Sacchetti 2010). Our imaging data in the mouse brain is consistent with this previous finding, in that neuronal activity in AuV and TeA measured by the density of zif268-induced neurons was increased only after remote but not recent memory retrieval, compared with the control FT group in our study. Thus, the increased zif268 activity in AuV and TeA after remote fear memory retrieval is likely to reflect brain activity related to the remote memory storage or retrieval of auditory conditioned fear. As shown in the lesion study of rat secondary auditory cortex, the specific lesion or functional interruption of MGm/PIN might reveal the functional significance of this region for fear memory storage or retrieval. However, although the previous study used the lesion of MGm/PIN to show that the post-training lesion of MGm/PIN at early phase after conditioning has no effect on the memory in the rat fear-potentiated startle behavior paradigm (Campeau and Davis 1995; but see Boatman and Kim 2006), specific lesion study of MGm/PIN might be technically challenging. As far as we know, MGm/PIN lesion has never been reported at a remote time phase after fear memory formation. The lesion of MGm/PIN would also affect the function of secondary auditory cortices, because the secondary auditory cortices anatomically receive synaptic input from MGm/PIN (Campeau and Davis 1995), making it hard to dissociate pure MGm/PIN lesion effects on behavior, if any. In addition, MGm/PIN constitutes one of the main auditory sensory-processing brain regions, so it is likely that the nonselective lesion or functional disruption of this brain structure would interfere with some aspects of the auditory perception process, which will make it difficult to specify fear memory-related activity in the MGm/PIN region. Specific targeting and manipulation of a subset of neurons that were specifically activated by fear-conditioned tone but not by familiar tone would help prove whether MGm/PIN and AuV/TeA are involved in remote fear memory storage or retrieval. Nevertheless, based on our zif268 imaging data, it is more likely that increased zif268 neuronal density in MGm/PIN and AuV/TeA after remote fear memory retrieval might represent brain activities related to the storage or retrieval of remote auditory fear memory. However, it is also possible that the increased zif268 activity in these auditory brain regions after remote memory retrieval might reflect a different way of processing either conditioned tone associated with foot-shock or the tone never associated with foot-shock at different time phases.

We found that zif268 (+) neuronal density in MGm/PIN and AuV/TeA after recent fear memory retrieval was not different from the one after a novel or familiar tone presentation. This result more likely suggests that the zif268 activity in these auditory brain regions after recent fear memory retrieval might reflect tone processing but not memory processing-related neuronal activity. However, there are reports supporting the critical role of MGm/PIN for fear-conditioning memory formation. Western blot and immunohistochemistry show that the zif268 protein expression is induced in the MGm/PIN at an early time phase after auditory fear conditioning (Overeem et al. 2010). In addition, the intrathalamic infusion of U0126, a MEK inhibitor, prior to training impairs long-term memory for auditory fear conditioning (Apergis-Schoute et al. 2005). When examined right after training of the auditory fear conditioning, the expression of the phosphorylated form of CREB (pCREB) in MGm/PIN is specifically increased in response to associative tone-shock pairing (Han et al. 2008). In addition, increasing CREB in the MGm/PIN region before training enhances recent memory for auditory conditioned fear (Han et al. 2008). One possible explanation is that MGm/PIN might play important roles in the fast encoding of fear memory and its consolidation process at an early time phase after the training of auditory fear conditioning, but not for the fear memory storage or retrieval at a recent time phase. However, we cannot completely rule out the possibility that the zif268 activity after recent fear memory retrieval in auditory brain regions, especially in MGm/PIN, might represent brain activity related to recent fear memory storage or retrieval. The specific and fast manipulation method of neuronal activity will be required to clearly prove whether these auditory brain regions may participate in the storage or retrieval of recent auditory fear memory.

Little is known about cortical layer-specific memory encoding and storage in auditory fear conditioning. In our zif268 analysis in secondary auditory cortices, we found the most significant increase of zif268 density in layers IV and VI after remote fear memory retrieval. This result is inconsistent with the result from previous studies in the rat brain, reporting the most significant increase of zif268 labeling in layers II–III and IV (Sacco and Sacchetti 2010). This discrepancy might result from different behavior protocols used in each study and probably reflect species difference in functional neural circuit organization in the mouse and rat brains. For instance, we gave the mice auditory CS with 2.8 kHz and 85 dB for 30 sec and single pairing of CS and US was used for auditory fear conditioning in this study. In contrast, in previous studies using rat, auditory CS with 1.0 kHz and 85 dB was presented for 6 sec and seven auditory CSs were delivered. According to anatomical and physiological characterization of the organization of mammalian cortical layers, certain types of neurons in cortical layer VI are known to have reciprocal synaptic connections with neurons in layer IV and sensory thalamic nucleus (Briggs 2010). These corticocortical and corticothalamic neural circuits might contribute to the storage and retrieval of remote memory of auditory conditioned fear.

In general, the total number or density of immediate early gene-induced neurons in a given brain region has been used as an index of neuronal activity. In our study, when examined after recent fear memory retrieval, the density of zif268 (+) neurons in AuV and TeA showed no significant increase by conditioned tone compared with familiar tone presentation. To search for a potential difference in activity patterns other than the total number of zif268 (+) neurons between recent FC and FT groups in AuV and TeA, we examined the intensity distributions of relative zif268 signals within whole zif268 (+) neuronal populations. Interestingly, although the density of zif268 (+) neurons was not significantly changed, the intensity of zif268 signals was significantly enhanced in almost every individual zif268 (+) neuron after recent memory retrieval compared with the familiar tone, shifting intensity distribution curve toward the stronger intensity side in the FC group. After remote fear memory retrieval, however, the size of the neuronal population with relatively higher levels of zif268 signal intensity was increased by fear memory retrieval compared with familiar tone presentation, while the size of neuronal population with relatively lower levels of zif268 signal intensity was not changed. Although it has not been determined yet what information zif268 signal intensity might represent, it is possible that the induced level of zif268 protein in secondary auditory cortices may represent the salience value of auditory sensory stimuli.

Using activity-dependent induction of immediate early gene, *zif268*, we investigated brain activity patterns in LA, MGm/PIN, and AuV/TeA regions in the mouse brain after the retrieval of recent or remote auditory fear memory by comparing the zif268 activity from the fear memory retrieval group with the one from control groups such as familiar tone or novel tone presentation or shock-alone groups. We found that the density of zif268-induced neurons in the lateral nucleus of amygdala was persistently increased after both recent and remote fear memory retrieval, while the density of zif268-induced neurons in MGm/PIN and AuV/TeA was increased only after remote but not recent auditory conditioned fear memory retrieval.

Our results support the idea that LA serves as a key brain site for the encoding and permanent storage of auditory fear memory. Our findings also suggest that MGm/PIN and AuV/TeA might be involved in the storage or retrieval process of remote auditory fear memory, or alternatively, the different zif268 activity patterns in these auditory brain regions after recent and remote fear memory retrieval might reflect a different way of processing for familiar or conditioned tone information at recent and remote time phases.

Materials and Methods

Mice

Adult F1 hybrid (C57Bl/6 X 129) mice were group housed (four mice per cage) on a 12-h light/dark cycle at a constant temperature of 22 ± 1°C. Food and water were available ad libitum throughout the experiment. All procedures were approved by the Animal Ethics Committee at KAIST.

Auditory fear conditioning

Male mice aged 2–3 mo were individually housed for a week before the training. All animals were handled for four consecutive days including the last 2 d of context habituation (5 min) in the retention chamber, and randomly assigned to the recent or remote group. Each group included three subgroups: (1) fear-conditioning group (FC, tone + foot shock, $n = 6$ for recent test, and $n = 5$ for remote test); (2) familiar tone group (FT, all procedures were the same as the FC group except the US presentation, $n = 7$ for recent and $n = 6$ for remote); (3) naive (home cage animals, $n = 3$ each for recent and remote). For further study in MGm/PIN, two subgroups were additionally included: (4) unfamiliar tone group (UFT, novel tone presentation, $n = 7$); (5) shock-alone group (SA, all procedures were the same as the FC group except there was no CS tone during training, $n = 3$). Training consisted of placing mice in a conditioning chamber and, 2 min later, presenting a tone (2.8 kHz, 85 dB, 30 sec) that coterminated with a shock (2 sec, 0.5 mA). Mice remained in the chamber for an additional 30 sec. Retention test for auditory fear memory occurred 24 h (recent group) or 28 d (remote group) after the conditioning. Mice were placed in a context-shifted chamber, and 2 min later the tone CS was presented for 3 min. The freezing during the CS was assessed via automated procedures (Coulbourn).

Immunohistochemistry

One and one-half hours after the completion of the retention test, mice were perfused transcardially with 4% paraformaldehyde. Dissected brains were stored overnight in the 4% paraformaldehyde and transferred to 30% sucrose for 3 d. Brains were sliced coronally (40 μm) on a cryostat and prepared for immunohistochemistry using anti-zif268 (1:5000 dilution, Santa cruz) or anti-Fos (1:20000 dilution, Calbiochem) primary rabbit polyclonal antibody. A biotinylated goat anti-rabbit antibody (1:2000 dilution, Vector Laboratories) was used as a secondary antibody. Staining was visualized using the avidin-biotin peroxidase method (Vectastain Elite ABC kit, Vector Laboratories) coupled to diaminobenzidine (DAB, Sigma) as a chromogen. Stained sections were rearranged along the anterior–posterior (AP) direction. Finally, the sections were transferred to gelatin-coated slides, dehydrated, and coverslipped.

Analysis of immediate early gene expression

The order of sections was carefully reconfirmed under the microscope and each section was assigned to a corresponding AP value (approximately bregma, Franklin and Paxinos 2007). The AP-distance between consecutive sections was 120 μm, because every third section was chosen. The analyzed area covered three regions: (1) LA in the amygdala (AP: $-1.52 \sim -2.06$, five sections); (2) MGm and PIN in the auditory thalamus (AP: $-2.92 \sim 3.28$, four sections); and (3) AuV and TeA in the auditory cortex (AP: $-1.94 \sim -4.22$, 19 sections).

Quantitative analysis of zif268 (+) nuclei was performed using a computerized cell-counting software (NIS Elements software, Nikon) by three experimenters in a blind manner. All images were acquired under the same image setting. The number of zif268-positive nuclei was counted bilaterally and the density was calculated as the number of cells per 100 μm^2.

For intensity analysis of auditory cortex, mean intensity of each zif268 (+) neuron was acquired. The difference of intensity from darkest to brightest cell was divided into 70 bins. All counted neurons in each individual mouse brain were distributed by the mean intensity of each cell into the 70 bins. The distribution of intensity was averaged for each group. Figure 4C showed the accumulated distribution of the intensity.

Statistical analysis

Student *t*-test or one-way ANOVA was used for freezing behavior analysis and comparisons of zif268 (+) neuronal density between experimental groups.

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