The Involvement of the Anterior Cingulate Cortex in Remote Contextual Fear Memory

Paul W. Frankland,[1,2,3]* Bruno Bontempi,[4]* Lynn E. Talton,[1] Leszek Kaczmarek,[1,5] Alcino J. Silva[1]†

Although the molecular, cellular, and systems mechanisms required for initial memory processing have been intensively investigated, those underlying permanent memory storage remain elusive. We present neuroanatomical, pharmacological, and genetic results demonstrating that the anterior cingulate cortex plays a critical role in remote memory for contextual fear conditioning. Imaging of activity-dependent genes shows that the anterior cingulate is activated by remote memory and that this activation is impaired by a null α-CaMKII mutation that blocks remote memory. Accordingly, reversible inactivation of this structure in normal mice disrupts remote memory without affecting recent memory.

The formation of new memories involves protein synthesis–dependent changes in synaptic structure and plasticity in the hippocampus (*1–3*). However, these memories are not stored permanently in the hippocampus. In humans and animals, damage to the hippocampus preferentially disrupts recently acquired memories while sparing remotely acquired memories (*4–7*); these effects indicate that remote memories eventually become independent of the hippocampus. To study the role of extrahippocampal structures in remote memory, we used contextual fear conditioning. In contextual fear conditioning, mice form an association between a distinctive context and an aversive event that takes place in that context. When placed back into the context, mice exhibit a range of conditioned fear responses, including freezing (*5*). Contextual fear conditioning is ideally suited for the study of remote memory, because a single training session produces robust lifelong memory (*8*) that can be measured using automated procedures (*9*).

To identify extrahippocampal regions involved in processing remote contextual fear memories, we tracked the expression of genes (*zif268* and c-*fos*) modulated by neuronal activity (*10*). Separate groups of mice were trained with 0 or 5 footshocks and tested either 1 day later (recent memory test) or 36 days later (remote memory test) (*11*). Although context exposure that is not reinforced may itself produce lasting memories (*12*), we observed no time-dependent changes in cortical gene expression after recent or remote memory tests in the control mice that were not shocked (fig. S1). Therefore, we analyzed gene expression in the shocked mice normalized with respect to these stable levels in controls. This allowed us to isolate changes in gene expression associated with contextual fear memory, and control for gene expression associated with motor activity, general arousal, and other nonspecific aspects of the testing procedure (*13*, *14*). We focused our analyses on the anterior cingulate cortex (ACC), prelimbic (PL) and infralimbic (IL) regions of the medial prefrontal cortex, temporal cortex (TC), and visual cortex (VC), because of the proposed role for the cortex in remote memory (*15–20*). Zif268 expression was elevated in ACC after the remote (160.5 ± 4.6%), but not the recent (89.4 ± 7.4%), memory tests (Fig. 1A), which suggests that this region is preferentially involved in processing remote contextual fear memories. After the remote, but not recent, memory test, similar pronounced increases in gene expression were observed in IL, PL, and TC (Fig. 1A). The same temporally graded pattern was observed in ACC, IL, PL, and TC with Fos expression (Fig. 1B), which indicates that these results generalize to other activity-dependent genes, and are not specific to Zif268. Note that the distinct patterns of gene expression following recent and remote memory tests indicate that gene expression is not simply a correlate of freezing (or other fear-related) behaviors, because freezing levels were similar at both time points.

The results presented above suggest that processing of remote contextual fear memory involves coordinated activation of multiple cortical regions. We thus examined cortical activation in mutant mice (α-CaMKII$^{+/-}$), which have specific deficits in remote memory (*16*). Both the magnitude and the specificity of the contextual fear memory phenotype in the α-CaMKII$^{+/-}$ mice make them an ideal tool to examine neural systems for remote memory. Concurrent with their wild-type (WT) littermates (*11*), α-CaMKII$^{+/-}$ mice were contextually conditioned and tested either 1 or 36 days after training. Contextual fear was dramatically reduced in the α-CaMKII$^{+/-}$ mice at the longer retention delay when we used either freezing or activity suppression as a measure (*16*) (Fig. 2). The pronounced increase in Zif268 expression in ACC after the remote memory test was absent in the mutants (Fig. 1A). Rather, Zif268 expression was similar in mutants after the recent (82.1 ± 4.7%) and remote memory tests (93.2 ± 10.6%). The increased Zif268 expression associated with remote memory was also blocked in IL, PL, and TC (Fig. 1A), which indicated that cortical activation associated with remote memory may be widely blocked in the α-CaMKII$^{+/-}$ mice. Fos expression associated with remote memory was similarly blocked in ACC, IL, and PL in α-CaMKII$^{+/-}$ mice (Fig. 1B). It is thought that consolidation occurs in a reactivation-dependent manner, either during online (e.g., retrieval tests) or offline (e.g., sleep) situations (*19–22*). Reactivation may lead to the gradual refinement of the memory network and integration of that network with related, preexisting memories (*19*). Therefore, to assay synaptic remodeling underlying these processes during memory recall, we examined expression of growth-associated protein 43 (GAP-43), a marker of synaptogenesis (*23*), in ACC (*11*). GAP-43 expression was elevated after the remote memory test in WT (recent 11.5 ± 1.7, remote 19.2 ± 2.1; $P < 0.05$), but not α-CaMKII$^{+/-}$ (recent 10.2 ± 0.9, remote 11.2 ± 2.1; $P > 0.05$), mice. These results indicate that the α-CaMKII$^{+/-}$ mutation may impede cortical reorganization necessary for consolidation.

The CA1 region of the hippocampus is strongly activated during acquisition and recall of contextual fear conditioning (*13*), which suggests that this region has a critical role in processing contextual fear memories. In WT mice, gene expression was elevated in CA1 after the recent (Zif268: 126.1 ± 3.9%; Fos: 174.2 ± 6.3%), but not remote (Zif268: 79.9 ± 5.9%; Fos: 93.5 ± 11.4%), memory test (Fig. 1). These data suggest that consolidation involves the gradual disengagement of CA1 (*13*), coupled with progressive recruitment of cortical regions. Furthermore, because Zif268 expression was reduced below control levels after the remote memory test in WT mice, activity in CA1 may be inhibited

[1]Departments of Neurobiology, Psychiatry, Psychology and Brain Research Institute, UCLA, 695 Charles Young Drive South, Los Angeles, CA 90095–1761, USA. [2]Department of Integrative Biology, Hospital for Sick Children Research Institute, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. [3]Department of Physiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8. [4]Laboratoire de Neurosciences Cognitives, CNRS UMR 5106, Avenue des Facultés, 33405 Talence, France. [5]Nencki Institute, Pasteura 3, 02–093, Warsaw, Poland.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: silvaa@mednet.ucla.edu.

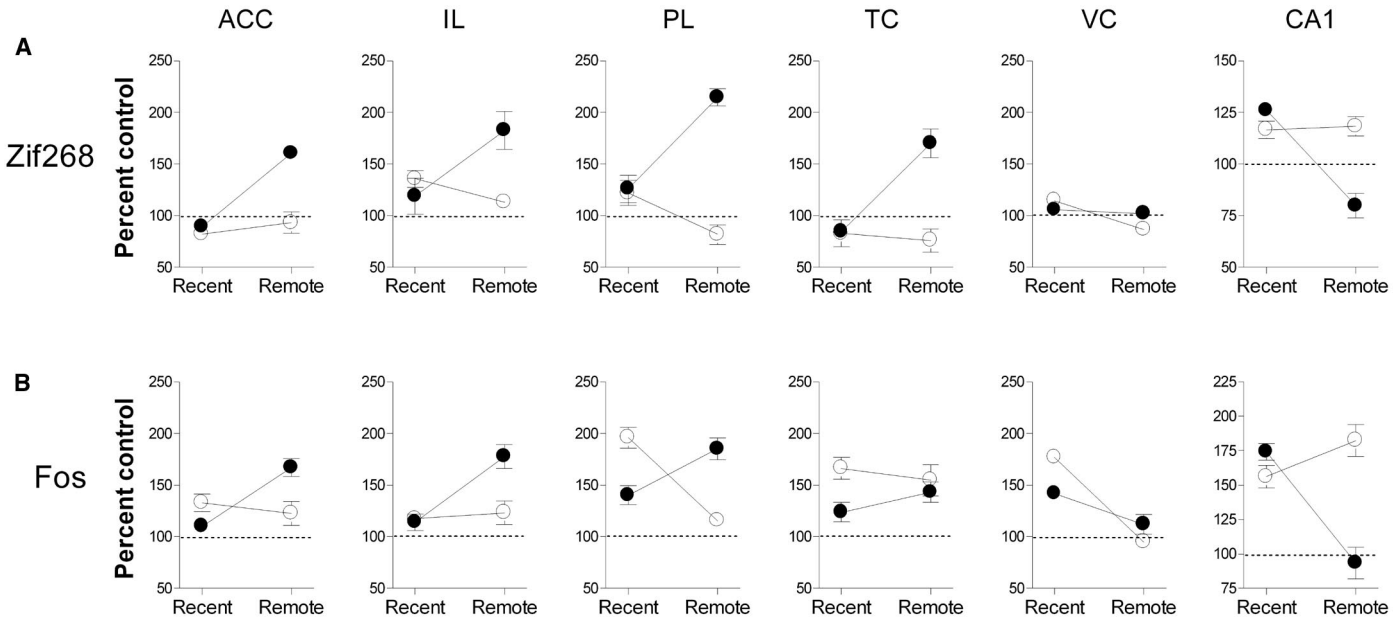

Fig. 1. Expression of activity-dependent genes after recent or remote memory tests. In order to isolate changes associated with memory, gene expression in the shocked groups is expressed as a percentage relative to controls that were not shocked. Changes in gene expression in different brain regions are shown for WT (black circles) and α-CaMKII$^{+/-}$ (open circles) mice after recent or remote memory tests. (**A**) Zif268 expression was elevated in WT, but not α-CaMKII$^{+/-}$ mice, after the remote memory test in ACC [Genotype × Delay interaction $F(1,28) = 16.82$, $P < 0.05$], IL [$F(1,28) = 9.82$, $P < 0.05$], PL [$F(1,28) = 33.73$, $P < 0.05$], and TC [$F(1,28) = 15.52$, $P < 0.05$], but not VC [$F(1,28) = 0.52$, $P > 0.05$]. In the CA1 region of the hippocampus, Zif268 expression was elevated after the recent, but not remote, memory tests in WT mice. In contrast, Zif268 expression was elevated at both time points in α-CaMKII$^{+/-}$ mice [$F(1,28) = 25.93$, $P < 0.05$]. (**B**) Changes in Fos expression were qualitatively similar to those in Zif268 for WT and α-CaMKII$^{+/-}$ mice. Fos expression was elevated in WT, but not α-CaMKII$^{+/-}$ mice, after the remote memory test in ACC [$F(1,28) = 14.03$, $P < 0.05$], IL [$F(1,28) = 9.69$, $P < 0.05$], PL [$F(1,28) = 46.37$, $P < 0.05$], but not TC [$F(1,28) = 1.83$, $P > 0.05$] nor VC [$F(1,28) = 1.39$, $P > 0.05$]. In CA1, Fos expression was elevated after the recent, but not remote, memory tests in WT mice. In contrast, Fos expression was elevated at both time points in α-CaMKII$^{+/-}$ mice [$F(1,28) = 30.78$, $P < 0.05$].

during processing of remote memories. In contrast, Zif268 levels remained elevated after the remote memory test in CaMKII$^{+/-}$ mice, which do not express behavioral memory at this time point. Therefore, the absence of this inhibitory feedback in the mutants may allow new encoding to occur.

Reduced cortical activation of Zif268 and Fos in the α-CaMKII$^{+/-}$ mice after remote memory tests most likely reflects differences in the organization of memory in these mutants. However, it is possible that the α-CaMKII$^{+/-}$ mutation disrupts regulation of these activity-dependent genes. We therefore conducted additional control experiments (*11*). First, we examined gene expression associated with training (Fig. 3A). Consistent with the observation that α-CaMKII$^{+/-}$ mice acquire contextual fear conditioning normally, we found that Zif268 and Fos expression was similar in WT and α-CaMKII$^{+/-}$ mice across different cortical regions. Furthermore, gene expression in the cortex was similar in WT and α-CaMKII$^{+/-}$ mice after removal from their home cage (Fig. 3B) and in controls that were not shocked (fig. S1). Therefore, the regulation of Zif268 and Fos appears to be normal in the α-CaMKII$^{+/-}$ mice under conditions associated with either low or high levels of neural activation, which suggests that changes in gene expression observed after remote memory tests are a consequence of changes in mnemonic processing in these mutants.

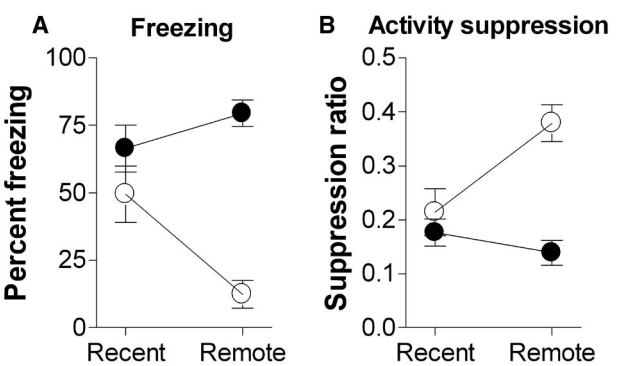

Fig. 2. Contextual fear memory in α-CaMKII$^{+/-}$ mice (open circles) versus WT mice (black circles). To assess memory in α-CaMKII$^{+/-}$ mice and WT mice, two behavioral indices of conditioned fear were measured in the same mice: (**A**) freezing; (**B**) activity suppression (*9*). Whereas WT mice exhibited robust levels of freezing and activity suppression in both the recent (1 day after training) and remote (36 days after training) retention tests, freezing [Genotype × Delay interaction, $F(1,28) = 10.62$, $P < 0.05$] and activity suppression [Genotype × Delay interaction $F(1,28) = 9.69$, $P < 0.05$] were markedly reduced at the longer retention delay in the α-CaMKII$^{+/-}$ mice.

The results presented above indicate that specific cortical sites are activated by remote memory processes and that this activation is absent in mice with remote memory deficits. To directly test whether these cortical sites are required for remote memory, we examined the effects of transient inactivation using lidocaine (*11*). Because remote memories are likely stored in distributed cortical networks (*19*, *24*), they may be resistant to focal disruption (*25*). However, executive structures like PL and ACC, which are both robustly activated by remote contextual memory, are thought to play an integrative role in memory (*26*–*28*) and may therefore be amenable for targeted disruption. Lidocaine infusions into ACC disrupted contextual fear memory at remote (18 and 36 days), but not recent (1 or 3 days) time points (Fig. 4A). Similar infusions into the neighboring PL had no effect on either 1-day-old or 36-day-old contextual fear memories (Fig. 4B). Although the imaging data show that a broad cortical network is activated by remote memory, these inactivation results

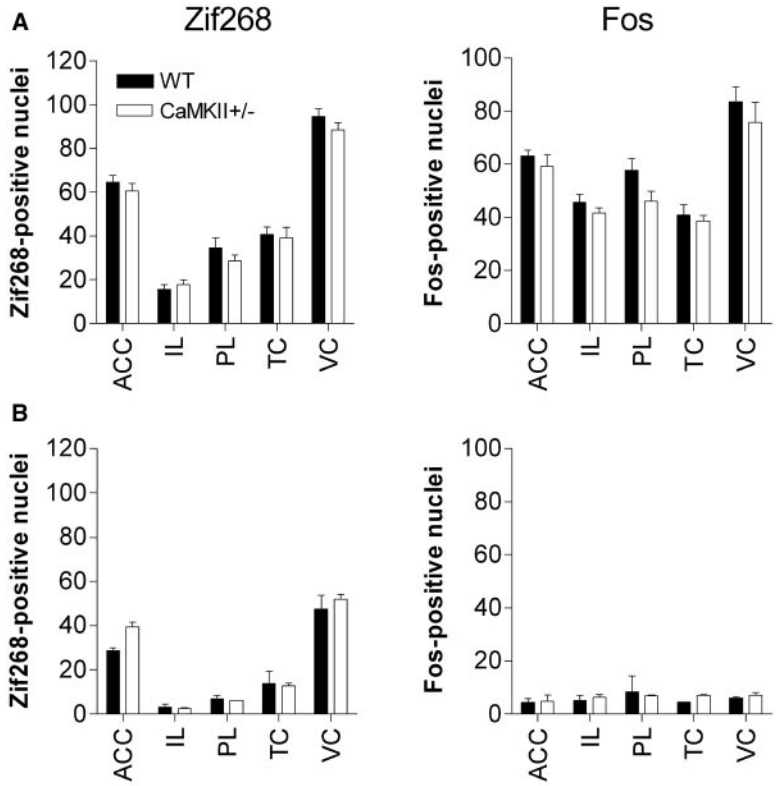

Fig. 3. Regulation of Zif268 and Fos in the cortex is normal in α-CaMKII$^{+/-}$ mice. The number of Zif268- and Fos-positive nuclei are shown for WT (black bars) and α-CaMKII$^{+/-}$ (white bars) mice. (**A**) Gene expression induced in the cortex after acquisition of contextual fear conditioning. Training-associated expression of Zif268 or Fos was similar in WT and α-CaMKII$^{+/-}$ mice in each of these regions (P values > 0.05). (**B**) Gene expression in the cortex in the home cage condition. There were no differences in Zif268 or Fos expression between WT and α-CaMKII$^{+/-}$ mice in each of these regions (P values > 0.05).

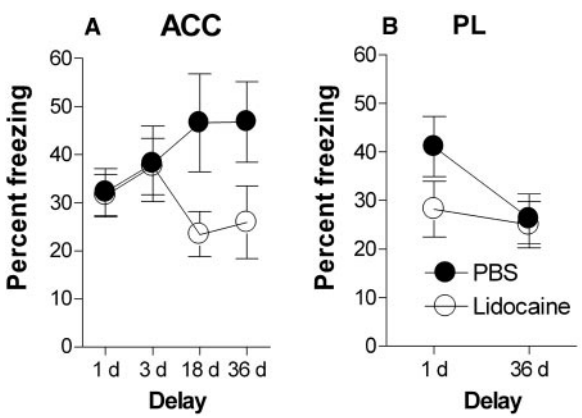

Fig. 4. Targeted pharmacological inactivation of ACC and PL. (**A**) Lidocaine-induced inactivation of ACC disrupts retrieval of remote, but not recent, contextual fear memories. Planned comparisons indicated that freezing levels were reduced in lidocaine-infused mice in the retention tests on the 18th or 36th day, but not the 1st or 3rd day after training (P values < 0.05). (**B**) Lidocaine-induced inactivation of PL did not disrupt retrieval of contextual fear memories. Planned comparisons indicated that freezing levels in phosphate-buffered saline (PBS)– and lidocaine-infused mice were not different (P values > 0.05).

identify ACC (but not PL) as an essential node within this network for processing remote memory.

Modeling, neuropsychological, and neurophysiological studies have suggested a central role for hippocampal-cortical networks in memory consolidation (*19*, *20*, *22*). Interactions between the hippocampus and cortex following initial learning lead to the gradual establishment of enduring memories in distributed cortical networks that are independent of the hippocampus. Here, we used brain imaging to identify cortical regions involved in processing fear memories. Our data show that processing fear memories involves the activation of multiple association cortical regions, consistent with the proposal that enduring memories are stored in distributed cortical networks. Cortical activation was greater after remote, rather than recent, memory tests, consistent with an increasingly important role for the cortex over time. In mice with specific deficits in remote memory, the pronounced cortical activation associated with remote memory was absent. These data suggest that CaMKII is necessary for the maturation and elaboration of cortical circuits underlying remote memory. In normal mice, imaging and inactivation experiments identified the ACC as a critical node in a broader cortical network processing remote memory. During memory encoding, ACC is thought to play an integrative role in cognitive control processes [e.g., attention, conflict monitoring (*26*, *27*, *29*)]. It remains to be determined whether the ACC is a site for memory storage per se or whether it mediates analogous processes for integrating multiple cortical representations underlying remote memories.

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Materials and Methods
Fig. S1
References and Notes

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