

Early remodeling of the neocortex upon episodic memory encoding

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Understanding the mechanisms by which long-term memories are formed and stored in the brain represents a central aim of neuroscience. Prevailing theory suggests that long-term memory encoding involves early plasticity within hippocampal circuits, whereas reorganization of the neocortex is thought to occur weeks to months later to subserve remote memory storage. Here we report that long-term memory encoding can elicit early transcriptional, structural, and functional remodeling of the neocortex. Parallel studies using genome-wide RNA sequencing, ultrastructural imaging, and whole-cell recording in wild-type mice suggest that contextual fear conditioning initiates a transcriptional program in the medial prefrontal cortex (mPFC) that is accompanied by rapid expansion of the synaptic active zone and postsynaptic density, enhanced dendritic spine plasticity, and increased synaptic efficacy. To address the real-time contribution of the mPFC to long-term memory encoding, we performed temporally precise optogenetic inhibition of excitatory mPFC neurons during contextual fear conditioning. Using this approach, we found that real-time inhibition of the mPFC inhibited activation of the entorhinal-hippocampal circuit and impaired the formation of long-term associative memory. These findings suggest that encoding of long-term episodic memory is associated with early remodeling of neocortical circuits, identify the prefrontal cortex as a critical regulator of encoding-induced hippocampal activation and long-term memory formation, and have important implications for understanding memory processing in healthy and diseased brain states.

learning | transcriptome | neuroplasticity | optogenetics | hippocampus

Long-term memory is essential for cognition and its disruption is central to myriad neurological disorders. Therefore, a deeper understanding of the mechanisms by which long-term memories are formed in the brain and lost in neurological disease states represents a critical objective in neuroscience. To this end, a wealth of studies has demonstrated that encoding of long-term memory involves early and rapid enhancements in neuronal activation, synaptic plasticity-related gene expression, structural synaptic plasticity, and synaptic efficacy within hippocampal circuits (1–5). Accordingly, lesions of the hippocampus or surrounding medial temporal lobe structures elicit severe memory impairment (6, 7). Over the weeks to months that follow the initial memory encoding event, repeated hippocampal–neocortical interactions slowly reorganize the synaptic architecture in the neocortex and gradually instantiate the memory trace within neocortical circuits, particularly the medial prefrontal cortex (mPFC) (5, 8–10). Collectively, these observations have given rise to the prevailing view that long-term memory encoding requires early and rapid plasticity within the hippocampus, whereas reorganization of the neocortex is limited to the late stages of memory processing to subserve remote memory storage and recall (11, 12).

Intriguingly however, neuroimaging studies from mouse to man demonstrate that the hippocampus and prefrontal cortex are coactivated during memory encoding (13–20). Indeed, the

magnitude of prefrontal activation during memory encoding has been shown to be predictive of the ability to later recall the experience (13), and prefrontal lesions are associated with impaired memory formation (15–17, 21–25). Nonetheless, the mechanisms by which the prefrontal cortex contributes to long-term memory encoding remain poorly understood. Herein, we provide evidence that long-term associative memory encoding activates a synaptic plasticity-related transcriptional program in the mPFC that is accompanied by rapid structural and functional plasticity of mPFC synaptic circuits. Using an in vivo optogenetic approach to examine the real-time contribution of mPFC activation to long-term memory encoding, we further show that excitatory mPFC neurons drive activation of the entorhinal–hippocampal circuit and regulate the formation of long-term memory. These results suggest a critical role for the prefrontal cortex in long-term memory encoding and have implications for understanding memory function in healthy and diseased brain states.

Results

To investigate the mechanisms by which the prefrontal cortex contributes to long-term episodic memory encoding, we used contextual fear conditioning, in which mice learn an association between a novel context and an event (foot shock) that occurs in that context. Single-trial contextual fear conditioning generates a temporally defined, long-lasting associative memory trace and is thus well suited for investigating the mechanisms that mediate long-term memory encoding in the brain (26). Moreover,

Significance

Prevailing theory suggests that episodic memory encoding is subserved by early remodeling of hippocampal circuits, whereas remodeling of the neocortex occurs weeks to months later to promote long-term memory storage and recall. Herein, we show that episodic memory encoding elicits early remodeling of neocortical circuits in mouse brain. Specifically, memory encoding triggered a transcriptomic program in the medial prefrontal cortex (mPFC) that was accompanied by rapid structural and functional plasticity of local synaptic circuits. Using optogenetics to examine the real-time contribution of the mPFC to memory encoding, we found that activity of excitatory mPFC neurons is required for hippocampal activation and formation of long-term memory. These data have important implications for understanding memory processing in healthy and diseased brain states.

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previous studies suggest that associative fear learning increases neuronal activity in the mPFC (15) and that the mPFC is critical for long-term storage and recall of associative fear memory (9). To verify that this paradigm increases neuronal activity in the mPFC, we examined expression of the neuronal activity-dependent immediate early gene, early growth response 1 (*zif268/egr-1*), in the mPFC 1 h following contextual fear conditioning (27). In accord with previous data (15), we found that fear conditioning significantly increased immediate early gene expression in the mPFC compared with control animals exposed to either the context or foot shock alone, suggesting that the mPFC exhibits associative learning-specific neural activation (Fig. S1). Additionally, as immediate early gene expression in the mPFC was significantly greater in context-exposed control animals relative to control animals exposed only to foot shock, context-exposed mice served as controls in subsequent experiments (Fig. S1). Together, these data suggest that associative memory encoding increases neuronal activity in the mPFC and further validate contextual fear conditioning as a useful paradigm by which to examine the role of the prefrontal cortex in long-term associative memory encoding.

Next, we sought to determine whether the memory encoding-induced increase in mPFC neuronal activity was associated with a shift in the mPFC transcriptome. To this end, we performed genome-wide RNA sequencing (RNA-seq) of mouse mPFC 1 h following contextual fear conditioning. Strikingly, we found that 342 genes (121 up-regulated, 221 down-regulated) were differentially expressed in the mPFC of mice that underwent fear conditioning compared with control animals (Fig. 1A and Table S1). Gene ontology and gene network analyses of differentially expressed genes revealed that genes up-regulated in the mPFC during associative memory encoding form a highly interconnected network involved in biological processes that promote synaptic plasticity and memory formation, including synthesis of cAMP, neurogenesis, long-term potentiation, and axon guidance (28) (Fig. 1B and C and Table S2). Conversely, genes down-regulated in the mPFC during associative memory encoding are implicated in biological processes that have been shown to suppress synaptic plasticity and memory function, such as proliferation of microglia, activation of phagocytes, and apoptosis (29, 30) (Fig. 1B and C and Table S2). Quantitative RT-PCR analysis of independent samples confirmed these expression changes (Fig. 1D and Table S3). These results suggest that the mPFC transcriptome is reprogrammed rapidly during associative memory encoding and that memory encoding primes the mPFC transcriptome for neuroplasticity.

Given that changes in gene expression are required to drive long-lasting forms of synaptic plasticity (28), we reasoned that the memory encoding-induced gene expression changes we observed in the mPFC might be associated with early remodeling of the local synaptic architecture. To test this hypothesis, we performed transmission electron microscopy (TEM) to analyze the ultrastructure of individual mPFC synapses 1 h following contextual fear conditioning. Specifically, we assessed the length of the active zone and postsynaptic density as well as the number of docked synaptic vesicles per synapse, as these metrics represent structural indices of synaptic strengthening and are increased under conditions associated with memory formation (31, 32). We found that associative memory encoding led to a rapid induction of ultrastructural synaptic plasticity in the mPFC, as contextual fear conditioning significantly expanded the size of the active zone and postsynaptic density (Fig. 2A and B and Fig. S2A and B) and increased the number of docked vesicles within individual mPFC synapses relative to controls (Fig. 2C and Fig. S2C). In accord with previous studies (33, 34), we found that active zone length, postsynaptic density length, and docked synaptic vesicle number were highly correlated at the level of individual synapses (Fig. 2D and Fig. S2D and E). Consistent with these observations, we found that expression of the presynaptic marker,

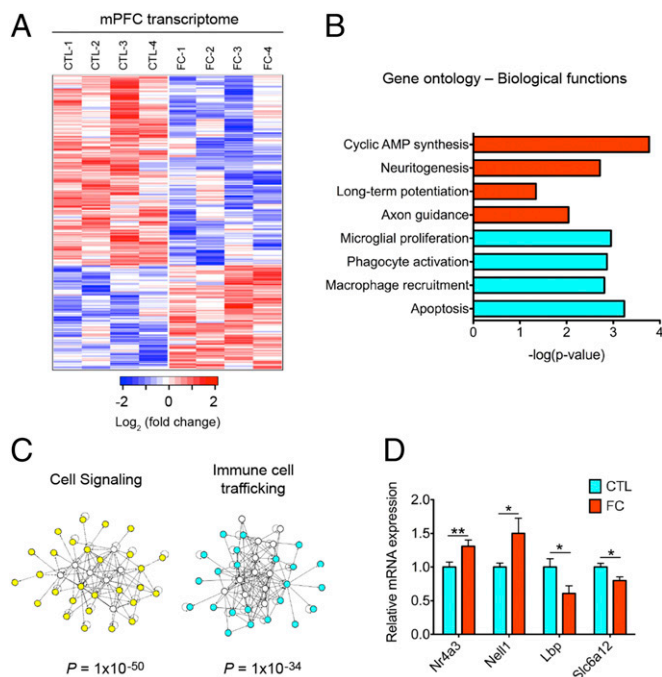


Fig. 1. Associative memory encoding primes the mPFC transcriptome for neuroplasticity. (A) Heatmap depicting 342 differentially expressed genes (DEGs) determined by genome-wide RNA sequencing (RNA-seq) of the mPFC upon control exposure (CTL) or contextual fear conditioning (FC). Rows represent DEGs, columns represent transcriptomic profiles of individual animals. Blue and red represent low and high levels of gene expression, respectively ($n = 4$ mice per group). (B) Ingenuity Gene Ontology Analysis depicting to which biological processes the DEGs contribute (red, up-regulated upon FC; blue, down-regulated upon FC). (C) Ingenuity Gene Network Analyses of DEGs up-regulated (Cell Signaling; $P = 1 \times 10^{-50}$) or down-regulated (Immune Cell Trafficking; $P = 1 \times 10^{-34}$) in the mPFC upon FC. Colored and uncolored nodes represent DEGs identified by RNA-seq and the Ingenuity network generation algorithm, respectively. (D) Expression of a subset of DEGs revealed by RNA-seq was confirmed in independent mPFC samples using quantitative RT-PCR ($n = 5-10$ mice per group). Values are normalized to expression levels of *Gapdh*. * $P \leq 0.05$; ** $P \leq 0.01$. Values represent mean \pm SEM.

synaptophysin, was significantly increased in the mPFC 1 h following contextual fear conditioning compared with controls (Fig. 2E and F and Fig. S3).

To further examine whether memory encoding elicits early plasticity within postsynaptic mPFC circuit elements, we used Golgi-Cox impregnation to investigate the morphology of individual dendritic spines on mPFC pyramidal neurons. Dendritic spines represent the postsynaptic component of excitatory synapses and exhibit morphological and functional continua, ranging from thin spines with a small head (i.e., thin spines) that are highly plastic and are hypothesized to underlie experience-dependent rewiring of neural circuits, to spines with a large, mushroom-like head (i.e., mushroom spines) that are more stable and are hypothesized to represent the physical substrates of long-term memories (35). We found that the ratio of thin spines to mushroom spines in the mPFC was significantly increased 1 h following contextual fear conditioning compared with controls (Fig. 3A and B), suggesting that associative memory encoding increases dendritic spine plasticity in mPFC circuits. Next, to determine whether memory encoding-induced structural plasticity in mPFC circuits is associated with functional alterations in excitatory synaptic strength, we performed ex vivo whole-cell patch-clamp recording of mPFC pyramidal neurons to examine miniature excitatory postsynaptic currents (mEPSCs) (36). We found that mEPSC frequency, but not

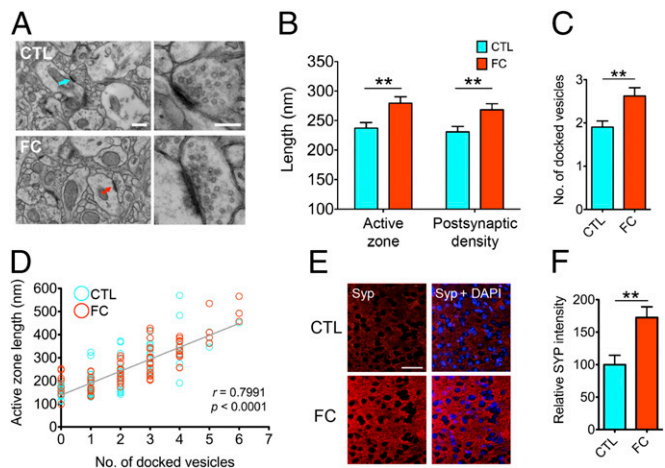


Fig. 2. Early remodeling of mPFC synapse ultrastructure upon associative memory encoding. (A) Representative transmission electron micrographs depicting mPFC synapse ultrastructure upon CTL or FC. (Scale bar, 500 nm.) Arrows indicate magnified synapses shown at *Right*. (Scale bar, 200 nm.) (B and C) Quantification of active zone and postsynaptic density length (B; $n = 77$ –92 synapses from four mice per group) and docked synaptic vesicle number (C; $n = 69$ –92 synapses from four mice per group) in mPFC synapses upon CTL or FC. (D) Active zone length was correlated with docked synaptic vesicle number across treatment groups. Blue and red circles indicate synapses in CTL and FC groups, respectively (Pearson $r = 0.7991$; $P \leq 0.0001$). (E) Representative immunohistochemical images depicting synaptophysin immunoreactivity in the mPFC upon CTL or FC. (Scale bar, 50 μm .) (F) Quantification of E ($n = 6$ mice per group). ** $P \leq 0.01$. Values represent mean \pm SEM.

amplitude, was significantly increased at excitatory mPFC synapses 1 h following contextual fear conditioning relative to controls (Fig. 3C), suggesting that associative memory encoding rapidly enhances the efficacy of excitatory presynaptic transmission in the mPFC. Finally, using parallel TEM (Fig. S4A and B) and Golgi–Cox impregnation (Fig. S4C), we found that despite the observed structural and functional plasticity induced in existing mPFC circuits following associative memory encoding, total synapse density in the mPFC remained unchanged. Collectively, these data suggest that mPFC activation during long-term associative memory encoding is associated with early transcriptional, structural, and functional remodeling of the existing mPFC circuitry.

Next, we sought to determine the causal role of early mPFC activation in long-term memory encoding. As the mPFC represents a hub of information processing in the brain (37–41), the mPFC may play a privileged role in regulating hippocampal-dependent memory encoding. However, whether the mPFC regulates hippocampal activation during the encoding of long-term memories is not known. To test this possibility, we first confirmed that contextual fear conditioning increases neuronal activity in the entorhinal cortex and hippocampus. Consistent with previous data (10, 42), we found that Zif268 expression was significantly increased in the entorhinal cortex (Fig. S5A and C) and hippocampal area cornu ammonis 1 (CA1) (Fig. S5B and D) 1 h following context exposure or contextual fear conditioning. Next, we used an *in vivo* optogenetic approach to permit real-time manipulation of excitatory mPFC neuronal activity in freely behaving mice. Specifically, mPFC excitatory neurons were transduced bilaterally with an adeno-associated virus serotype 5 (AAV₅) encoding a third-generation halorhodopsin (eNpHR3.0) fused to enhanced yellow fluorescent protein (EYFP) under transcriptional regulation of the Ca²⁺/calmodulin-dependent protein kinase II α (CaMKII α) promoter (CaMKII α -eNpHR3.0-EYFP), followed by implantation of a fiber optic in the mPFC to permit real-time optogenetic silencing of local excitatory

neuronal activity (Fig. S6). Stereotaxic injection of the CaMKII α -eNpHR3.0-EYFP vector into mPFC resulted in mPFC-specific expression (Fig. 4A), and within mPFC, eNpHR3.0-EYFP was expressed in CaMKII α -positive excitatory neurons (Fig. 4B). Single-cell recordings from acute mPFC slices confirmed that optical (593 nm) inhibition dampened action potential spiking in excitatory neurons expressing eNpHR3.0-EYFP (Fig. 4C). Next, we found that real-time optogenetic inhibition of the mPFC during contextual fear conditioning (Fig. 4D) significantly reduced Zif268 expression in the mPFC of mice expressing eNpHR3.0-EYFP compared with mice expressing EYFP alone (Fig. 4E and F), confirming that optogenetic inhibition prevented mPFC activation during long-term associative memory encoding. Interestingly, optical inhibition also reduced the expression of synaptophysin in the mPFC of mice expressing eNpHR3.0-EYFP, but not EYFP alone (Fig. 4E and F), suggesting that early plasticity of mPFC circuits during associative memory encoding requires excitatory neuronal activity. To determine whether the mPFC regulates hippocampal circuit activation during memory encoding, we next examined the effect of real-time optogenetic mPFC inhibition on hippocampal area CA1, an area critical for long-term memory formation (7). Remarkably, we found that optogenetic inactivation of the mPFC during memory encoding significantly reduced the expression of Zif268 and synaptophysin in hippocampal area CA1 of mice expressing eNpHR3.0-EYFP compared with mice expressing EYFP alone (Fig. 4G and H), suggesting that the mPFC is

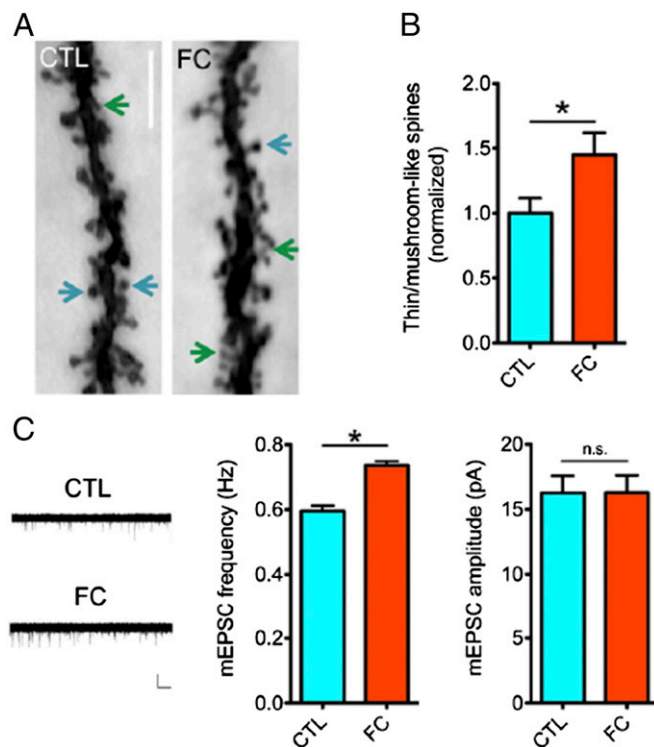


Fig. 3. Associative memory encoding enhances dendritic spine plasticity and synaptic efficacy in the mPFC. (A) Representative Golgi–Cox impregnated dendritic spines present on layer II/III mPFC pyramidal neurons upon CTL or FC. Green and blue arrows indicate thin and mushroom-shaped spines, respectively. (Scale bar, 5 μm .) (B) Quantification of A ($n = 716$ –753 spines from three to four mice per group). (C) Representative traces of miniature excitatory postsynaptic currents (mEPSCs) recorded from layer II/III mPFC pyramidal neurons upon CTL or FC. (Scale bar, 40 pA, 2.5 s). Quantification of mEPSC frequency and amplitude in mPFC pyramidal neurons upon CTL or FC ($n = 10$ –11 neurons per group). * $P \leq 0.05$; n.s., not significant. Values represent mean \pm SEM.

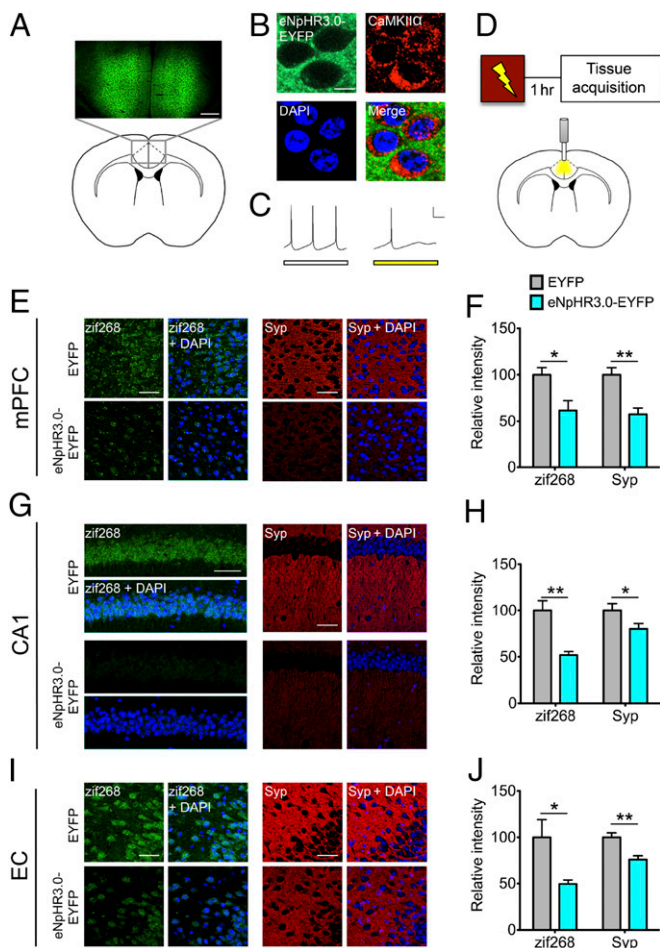


Fig. 4. Optogenetic silencing of excitatory mPFC neurons impairs entorhinal-hippocampal circuit activation during associative memory encoding. (A) AAV₅-CaMKII α -eNpHR3.0-EYFP was injected into bilateral mPFC to selectively transduce mPFC excitatory neurons and permit optogenetic inhibition thereof. (Scale bar, 500 μ m.) (B) Representative immunohistochemical images depicting CaMKII α expression in mPFC neurons transduced with CaMKII α -eNpHR3.0-EYFP. (Scale bar, 10 μ m.) (C) Ex vivo optical (593 nm) inhibition of action potential spiking (50 pA current injection) in a representative mPFC pyramidal neuron expressing eNpHR3.0-EYFP. (Scale bar, 20 mV, 100 ms.) (D) Schematic of the experimental paradigm used for in vivo mPFC optogenetic inhibition experiments shown in E–J. Animals injected with AAV₅-CaMKII α -eNpHR3.0-EYFP or AAV₅-CaMKII α -EYFP control virus received continuous optogenetic inhibition of excitatory mPFC neurons during FC. One hour later, tissue was harvested for analysis. (E–J) Representative immunohistochemical images depicting Zif268 and synaptophysin immunoreactivity in the mPFC (E and F), hippocampal area CA1 (CA1; G and H), and entorhinal cortex (EC; I and J) of mice expressing eNpHR3.0-EYFP or EYFP alone following continuous optogenetic inhibition of mPFC during FC. (Scale bars, 50 μ m.) (F) Quantification of E ($n = 3$ –4 mice per group). (H) Quantification of G ($n = 3$ –4 mice per group). (J) Quantification of I ($n = 3$ –4 mice per group). * $P \leq 0.05$; ** $P \leq 0.01$. Values represent mean \pm SEM.

a critical regulator of hippocampal activation during long-term memory encoding. Consistent with its role as the major information gateway linking the mPFC and hippocampus, we further found that mice expressing eNpHR3.0-EYFP exhibited significantly decreased expression of Zif268 and synaptophysin in the entorhinal cortex compared with control mice expressing EYFP alone (Fig. 4 I and J). Together, these data suggest that activity of excitatory mPFC neurons is required for entorhinal-hippocampal circuit activation during long-term associative memory encoding.

Finally, to directly examine the effect of real-time optogenetic mPFC silencing on long-term memory formation, separate cohorts of mice were injected bilaterally with either the eNpHR3.0-EYFP or EYFP vector, implanted with a fiber optic in the mPFC, and received continuous optogenetic inhibition of mPFC excitatory activity during contextual fear conditioning as described above. One day and 30 d after training, mice were returned to the experimental context to assess recent and remote long-term memory, respectively (Fig. 5A). Interestingly, we found that mice expressing eNpHR3.0-EYFP spent significantly less time freezing during both the recent (Fig. 5B) and remote (Fig. 5C) memory tests compared with EYFP controls, suggesting that optogenetic inhibition of excitatory mPFC neurons during memory encoding impaired long-term memory formation. Importantly, neither basal locomotor activity (Fig. 5D) nor sensitivity to foot shock (Fig. 5E) differed significantly between the groups. These results suggest that real-time silencing of mPFC excitatory neurons during associative memory encoding is sufficient to impair the formation of long-term associative memory.

Discussion

Pioneering work on the mechanisms of memory suggests that episodic memory encoding requires early and rapid alterations in gene expression and synaptic efficacy within the hippocampus, whereas reorganization of neocortical circuits occurs weeks to months later to subservise long-term memory storage and recall (12). Intriguingly however, neuroimaging studies from mouse to man consistently report coactivation of the hippocampus and prefrontal cortex during memory encoding (13–17), thus raising the possibility that the neocortex may play a heretofore undefined role in the early stages of memory processing in the brain. In the present work, genome-wide RNA-seq analysis revealed that long-term associative memory encoding triggers a synaptic plasticity-related transcriptional program in the mPFC. Consistent with these gene expression changes, analysis of synapse ultrastructure, dendritic spine morphology, and synaptic transmission showed that memory encoding induced early remodeling of the mPFC synaptic architecture and enhanced local synaptic efficacy. Finally, using in vivo optogenetic silencing, we found that real-time inactivation of mPFC excitatory neurons during associative memory encoding impaired entorhinal-hippocampal circuit activation and the formation of long-term associative memory. Collectively, our results provide, to our knowledge, the first evidence that episodic memory encoding can induce early transcriptional, structural, and functional remodeling of the neocortex and identify the mPFC as a critical regulator of entorhinal-hippocampal circuit activation and the formation of long-term memory.

Whereas decades of investigation in humans and animal models have established a central role for the hippocampus in episodic memory encoding (1), a large body of literature suggests that the prefrontal cortex may also represent an integral component of the neural memory system. For instance, positron emission tomography and functional magnetic resonance imaging studies in humans and nonhuman primates demonstrate that the prefrontal cortex is activated during working memory and associative memory-encoding paradigms and that the magnitude of prefrontal activation at the time of encoding is predictive of the ability to subsequently remember an event (13–20). In accord with these findings, prefrontal lesions have been shown to impair episodic memory formation across species (15–17, 21–25). Interestingly however, mechanistic investigation of the role of the prefrontal cortex in memory processing has largely focused on its role in memory consolidation and retrieval. In this regard, data in animal models suggest that glucose utilization (8), activity-dependent immediate early gene expression (9, 10), and dendritic spine density (5) are increased in the prefrontal cortex following consolidation of remote, but not recent, long-term memory and

likely to provide novel insights into the mechanisms of memory function in the healthy brain and its impairment in a variety of neurological disorders.

Materials and Methods

Wild-type (B6SJL; Taconic Farms) male mice were used at 2.5 ± 0.5 mo of age for experimentation. All animal work was approved by the Committee for Animal Care of the Division of Comparative Medicine at the Massachusetts Institute of Technology. Materials and methods regarding behavioral analysis, immunohistochemistry, genome-wide RNA-seq, quantitative

RT-PCR, electron microscopy, Golgi-Cox impregnation, electrophysiology, optogenetics, and statistical analysis are described in detail in *SI Materials and Methods*.

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