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Encoding of contextual fear memory requires *de novo* proteins in the prelimbic cortex

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Abstract

Background—Despite our understanding of the significance of the prefrontal cortex in the consolidation of long-term memories (LTM), its role in the encoding of LTM remains elusive. Here we investigated the role of new protein synthesis in the mouse medial prefrontal cortex (mPFC) in encoding contextual fear memory.

Methods—Because a change in the association of mRNAs to polyribosomes is an indicator of new protein synthesis, we assessed the changes in polyribosome-associated mRNAs in the mPFC following contextual fear conditioning (CFC) in the mouse. Differential gene expression in mPFC was identified by polyribosome profiling (n = 18). The role of new protein synthesis in mPFC was determined by focal inhibition of protein synthesis (n = 131) and by intra-prelimbic cortex manipulation (n = 56) of Homer 3, a candidate identified from polyribosome profiling.

Results—We identified several mRNAs that are differentially and temporally recruited to polyribosomes in the mPFC following CFC. Inhibition of protein synthesis in the prelimbic (PL), but not in the anterior cingulate cortex (ACC) region of the mPFC immediately after CFC disrupted encoding of contextual fear memory. Intriguingly, inhibition of new protein synthesis in the PL 6 hours after CFC did not impair encoding. Furthermore, expression of Homer 3, an mRNA

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Author Contributions

SP designed the project. VR, KT, BLR, SS, JL, BK, XL carried out experiments and analyzed the results with the help of RWS, CZ and DB carried out bioinformatics analyses. SP wrote the paper with input from all the authors.

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enriched in polyribosomes following CFC, in the PL constrained encoding of contextual fear memory.

Conclusions—Our studies identify several molecular substrates of new protein synthesis in the mPFC and establish that encoding of contextual fear memories require new protein synthesis in PL subregion of mPFC.

Keywords

protein synthesis; polyribosomes; fear memory; prefrontal cortex; genomics; encoding; signaling pathways

Introduction

Several lines of evidence from rodent studies suggest that the medial prefrontal cortex (mPFC), the homologous structure of the dorsolateral prefrontal cortex in primates, plays an important role in consolidation of long-term memories (LTM) (1–8). Recent studies in rodents provided evidence that the mPFC is also involved in consolidation processes underlying some forms of learning (9–13). For instance, consolidation of learning strategies during a working memory task in the eight-arm radial maze requires synthesis of new proteins within the mouse mPFC (10). Similarly, the acquisition of inhibitory avoidance in mice requires gene expression and *de novo* proteins in the mPFC (11). Rats lacking the hippocampus were able to learn contextual fear by recruiting the mPFC (12,13). Although these studies pointed to the role of the mPFC in consolidation of LTM, it is unknown whether the mPFC has a critical role in encoding of LTM. Furthermore, the cellular and molecular mechanisms critical for memory consolidation, within the mPFC microcircuit, are poorly understood.

To address this, we examined whether new protein synthesis occurs in the mPFC during encoding of contextual fear memory since new protein synthesis is a requisite for consolidation and storage of LTM (14–16). Because the association of mRNAs to polyribosomes indicates activation of translation of specific mRNAs (17,18), analysis of polyribosomes of the mPFC should be a reliable measure of the activation of protein synthesis. Hence, we isolated and characterized polyribosomes following contextual fear conditioning (CFC) in the mouse, a behavioral paradigm that induces long-lasting contextual fear memories in the mPFC (19). Our RNAseq analysis identified several mRNAs in the mPFC that are differentially enriched in polyribosomes isolated one and six hours after CFC training. We found the prelimbic (PL) subregion of the mPFC is a critical site for new protein synthesis during the encoding of LTM and identified Homer 3 in the PL as an important regulator of the consolidation of contextual fear memories within the mPFC circuit. Together, these findings provide direct evidence of activation of translation in the mPFC and demonstrate the critical role of the PL during encoding of contextual fear memory.

Materials and Methods

Animals

8–12 week old male C57Bl6/J mice (Jackson Laboratories) were housed in groups of four on a 12 hour light/dark cycle with ad libitum access to food and water. All experiments were performed during the light part of the diurnal cycle. Housing, animal care and experimental procedures were consistent with the Guide for the Care and Use of Laboratory Animals and approved by the IACUC of the Scripps Research Institute.

Details of Contextual fear conditioning, Polyribosome isolation and analysis, Cannula placement, RNAseq, Differential gene expression, Pathway analysis, qPCR, Histological analysis, Confocal microscopy, Immunohistochemistry and Stereotactic injection are described in Supplement 1.

Results

Differential recruitment of mRNAs to polyribosomes in mPFC during encoding of contextual fear memories

To determine the role of mPFC in encoding of contextual fear memory, we isolated and characterized polyribosomes from the mPFC following contextual fear conditioning (CFC) in the mouse (see Results and Figure S1 in Supplement 1; and Table S1 in Supplement 2). RNAseq analysis of polyribosome fractions from the mPFC (Figure 1A; Table S2 in Supplement 2) of fear conditioned (n=9) and context control animals (n=9) identified several differentially enriched mRNAs (Figure 1B, 171 mRNAs at T1 and 151 mRNAs at T2, $p < 0.05$; Tables S3 & S4 in Supplement 2). These results suggest activation of translation of specific mRNAs in the mPFC following CFC training. Interestingly, 95% of genes that are present in polyribosomes at T1 and T2 are unique suggesting a differential activation of translation in the PFC following CFC training. mRNAs of semaphorins, SLIT and plexins, key signaling molecules involved in neuronal growth, were enriched in polyribosomes isolated at the T1 time point, whereas mRNAs of ZFP, KLHL, and members of SYNGR were enriched in polyribosomes isolated at the T2 time point when compared to context alone controls suggesting temporal changes in translation of specific mRNAs in the mPFC during encoding of contextual fear memories.

To identify specific pathways that might be differentially recruited at T1 and T2, we analyzed polyribosome RNAseq data by gene ontology (GO) and KEGG pathway analysis (Figures 1C & D; Tables S5 & S6 in Supplement 2). This analysis identified 79 pathways in T1 and 57 pathways at T2 in fear conditioned animals when compared to context alone controls ($p < 0.05$). Among these, 54 pathways were unique for T1 and 32 were unique at T2 whereas both T1 and T2 shared 25 pathways (Figure S2 in Supplement 1; Tables S7–9 in Supplement 2). Axon guidance (GO:0007411), cell migration (GO: 0016477), cell motility (GO:0048870) and regulation of cell death (GO: 0010941) were specific to T1, whereas Proteasome (mmu03050), Huntington's disease (mmu05016), Parkinson's disease (mmu05012), and regulation of synaptic plasticity (GO:0048167) were specific for T2. These findings suggest that different signaling pathways are recruited in mPFC at T1 and T2 during encoding of contextual fear memory.

To confirm RNAseq data, we carried out a new set of CFC experiments and analyzed the expression of 14 candidate genes (NYP2R, Slc9a4, Homer3, TGF β 2, PKP2, SLIT3, FGFR, GABARA5, KLHL25, LMO4, MATN2, EIF3G, SYNGR3 and CAMK2G) identified from RNAseq analysis of polyribosomes isolated from mPFC. Consistent with the RNAseq data, quantitative real time PCR (qPCR) results shown in Figure S3 in Supplement 1 suggest enrichment of the 14 candidate genes (n=20 animals for each group, One way ANOVA, Tables S10 & 11 in Supplement 2, Tukeys multiple comparison test) in mPFC polyribosomes following CFC training when compared to shock alone and context alone controls.

We asked whether the change in the association of specific mRNAs to polyribosomes in CFC trained animals is accompanied by changes in their transcription and/or differential association with monosomes and polyribosomes. We analyzed expression of 14 candidate genes (described above) in total RNAs, polyribosomes and monosomes isolated from mPFC of mice following CFC training and in control mice (Figure 2A, n= 20 animals each for context alone and context + shock at T1). qPCR data show that these mRNAs are differentially enriched in the total extracts and in the polyribosomes of mice that were CFC trained when compared to context alone controls (n=20, p<0.05, Student's t test, Figures 2B, C&D, Supplementary Tables S10, 11 & S12 in Supplement 2) but not in monosomes (see Results in Supplement 1). Together, these results suggest transcriptional and translational activation of several genes in mPFC within 1 hour of CFC training.

We then analyzed the protein levels of three candidates characterized by qPCR (NYP2R, eIF3G and TGF β 2) in mPFC of a new set of mice trained in CFC (context + shock) and their controls (context alone) by Western Blotting. Consistent with RNAseq results, immunoblotting data displayed ~ 60% increase in NPY2R protein level in mPFC of context + shock animals (163.5 $\pm$ 21.8) compared to context alone animals (100 $\pm$ 9.6) (n=9, p<0.05, Student's t test, t=2.661). We found ~ 40% increase in EIF3G protein levels in context + shock animals (144.3 $\pm$ 12.4) as compared to context alone animals (100 $\pm$ 6.8) (n=6, p<0.05, Student's t test, t=3.125). Finally, we found a two fold increase in TGF β 2 protein levels in context + shock animals (201.1 $\pm$ 24.5) when compared to context alone (100 $\pm$ 16.9) animals (n=9, p<0.005, Student's t test, t=3.394). Consistent with the RNAseq and qPCR data, Western Blotting data (Figures 2E–H; Table S12 in Supplement 2) suggest that, CFC training results in enhanced synthesis of specific proteins in mPFC.

Differential activation of protein synthesis in PL and ACC during encoding of contextual fear memory

Our RNAseq and qPCR analyses revealed activation of translation of specific mRNAs within the mPFC during encoding of contextual fear memory. To determine the role of translational activation in the mPFC during encoding, we focused on two of the subdivisions of the mPFC that are implicated in memory storage, the anterior cingulate cortex (ACC) and prefrontal cortex (PL) subdivisions. The ACC has been implicated in long-term maintenance of memories (6) whereas several studies have suggested the role of the PL in fear memory expression (34–41). It is not clear whether the ACC and/or PL have a role in encoding of contextual fear and if so what the underlying molecular mechanisms are. Based on previous

studies that described the role of the ACC and PL in learning and memory, we considered the possibility that the encoding of contextual fear requires activation of translation in the ACC and PL. Therefore, we used fluorescence imaging analysis to detect the level of phosphorylation of Elf2 α within the PL and ACC within the first hour following CFC training. Prior studies have shown that a decrease in the ratio of Elf2 α phosphorylation to total Elf2 α is associated with an increase in protein synthesis (42). Hence, the ratio between phosphorylated to total Elf2 α will be a reliable measure of translational activation. One hour after CFC training, we isolated brains from fear conditioned and control mice, and imaged the distribution of phosphorylated and total Elf2 α proteins in PL and ACC using specific antibodies (Figure S4 in Supplement 1). Confocal projection images of phosphorylated and total Elf2 α levels in PL and ACC cortex depicted in Figure 3 show a statistically significant decrease in phosphorylated to total Elf2 α ratio in PL [$t(72) = 2.065$, $p < 0.05$], but not in the ACC [$t(96) = 1.743$, $p > 0.05$]. This indicates an increase in protein synthesis in PL within the first hour following CFC training.

New protein synthesis in PL is required for encoding of contextual fear memories

To understand this differential protein synthesis in the mPFC during encoding of contextual fear memory, we systematically blocked protein synthesis in the PL and ACC by microinfusion of the protein synthesis inhibitor, Anisomycin, (10, 43, 44) at the end of the CFC training session and evaluated the strength of the contextual fear memory by analyzing their freezing response 24 hours later (Figure 4A). Saline injected and sham mice were used as controls (Figure S5 in Supplement 1). We included a group of mice implanted with guide-cannulae above the PL subdivision to detect any effect on freezing due to the intracranial injection per se.

We microinfused Anisomycin (ANI) in the PL subregion and measured the freezing response. During the test (context without shock), overall, mice injected with ANI (64 $\mu\text{g}/0.2 \mu\text{l}/\text{side}$) exhibited reduced fear to the context in which they were trained as compared to the saline-treated mice (Figure 4B, C, D & H). A one way ANOVA performed on the % freezing measured over the 5 minutes of testing revealed the groups statistical differ, $F(2,40) = 5.88$, $p < 0.01$. Post-hoc Tukey HSD test revealed the saline and sham groups did not differ in their % freezing ($p = 0.75$) and that both sham ($p < 0.01$) and saline ($p < 0.05$) groups were statistically different from the ANI group. Note that the behavior of all three groups was identical before and after the three shocks of the CFC training session (Figure 4B). A repeated-measure ANOVA performed on % freezing measured at 1 minute bins of the 5 minute test session revealed a significant main effect of treatment group, $F(2,40) = 6.34$, $p < 0.01$. There was a significant Group X Time interaction, $F(8,160) = 2.59$, $p < 0.05$ and individual analysis indicated the sham and saline groups did not differ in their percent freezing, indicating no effect due to the injection (Figure 4C&D). Individual comparisons revealed that the percent of freezing of the groups was comparable during the first 2 minutes and over the remaining 3 minutes, the ANI-treated mice showed significant reduced freezing as compared to the two control groups (3rd minute, $p < 0.001$; 4th minute, $p < 0.01$; 5th minute, $p < 0.01$).

To rule out that this observed reduction of freezing is due to an impairment of the acquisition of contextual fear by Anisomycin treatment, a naïve cohort of mice was implanted with bilateral PL cannulae and then received CFC training. Percent freezing responses were measured 1 hour after Anisomycin infusion in PL. As depicted in Figure S6 in Supplement 1, we found no statistically significant difference ($p > 0.05$) between saline and ANI injected groups, indicating that protein synthesis is not required for the acquisition of contextual fear memory.

We asked whether protein synthesis has a critical role in the ACC subregion of the mPFC during encoding of contextual fear memories. Unlike the PL, microinfusion of ANI within the ACC did not affect the percentage of freezing to the context following CFC training (Figure 4E, F, G & I; Figure S5 in Supplement 1). A one way ANOVA performed on the % freezing measured over the 5 minutes of test revealed that the three groups did not statistically differ, $F(2,16) = 0.11$, $p > 0.05$. Additionally, repeated-measure ANOVA on % freezing during each of the 1-minute bins of the test yielded no main effect of the treatment group. Together, these findings indicate that the encoding of contextual fear memory requires *de novo* proteins within the PL subdivision of the mPFC.

Temporal regulation of translation at PL in encoding of contextual fear memories

As shown in Figure 4, new protein synthesis in the PL but not in the ACC is required for encoding contextual fear memory. Our RNAseq analysis revealed a second wave of induction of translation in the mPFC 6 hours following CFC training (Figure 1), suggesting that the new protein synthesis is recurrently required in mPFC for the memory encoding. This raised the question whether continuous protein synthesis in the PL is required for encoding of contextual fear memory. To address this, we inhibited protein synthesis by injecting ANI into the PL 6 hours after the completion of CFC training. The results presented in Figure 5 showed that ANI-treated mice, overall, exhibited a comparable freezing level to that of saline-treated mice (57.9% vs. 47.9%, $t(13) = -1.05$, $p = 0.31$), indicating that the protein synthesis induced in the PL within the 6 hours following CFC training is not required for encoding of contextual fear memories. Together, these analyses confirmed spatial and temporal regulation of translation within the mPFC during encoding of contextual fear memories.

Homer 3 mRNA levels in PL are critical for encoding contextual fear memory

Our RNAseq analyses revealed several mRNAs the translation of which is activated within the first hour following CFC training. From this population of mRNAs, we chose Homer 3, a postsynaptic density associated protein, to investigate its putative role in the consolidation of contextual fear memories within the PL cortex because it was shown that Homer 1a in the hippocampus is essential for contextual fear conditioning (45,46) and that Homer 1a transcripts are rapidly upregulated after contextual fear conditioning (47). Furthermore, it is not known whether Homer 3 is regulated during learning and whether it plays a critical role in encoding of contextual fear memory. As shown in Figure 2, our qPCR data confirmed RNAseq data that Homer 3 mRNAs are enriched in polyribosomes within the first hour following CFC training. To determine the significance of enrichment of Homer 3 mRNAs in polyribosomes in mPFC, we asked whether ANI injection disrupts loading of Homer 3 to

polyribosomes. ANI or saline was injected in the PL. An hour later, we isolated mPFCs and quantified Homer 3 mRNA levels in total extracts, polyribosomes and monosomes. qPCR results shown in Figure S7 in Supplement 1 revealed that ANI, not saline injection, caused a significant decrease in Homer 3 mRNA loaded onto polyribosomes when compared to monosomes or total extracts ($n=12$, $P<0.05$, Student's t test).

To test whether the increased Homer 3 mRNA levels induced by CFC training will result in increased Homer 3 protein levels, we isolated proteins from the mPFC and analyzed by Western Blotting the changes in Homer 3 protein levels. Autoradiogram and quantification shown in Figure 6 revealed an increase in Homer 3 protein levels in the mPFC as compared to Control mice (Figures 6A & B, $n=8$; % increase over control = 28.06 ± 4.48 ; $p<0.05$, Student's t test) 1 hour following CFC training.

To elucidate the role of Homer 3 upregulation in encoding of contextual fear memories, we performed loss of function studies by knocking down Homer 3 mRNA in the PL using siRNAs (Figure 6C; Results and Figures S7–9 in Supplement 1; Tables S13 & S14 in Supplement 2). We carried out CFC training after 72 hours of bilateral injection of Homer 3 siRNAs and control siRNAs (Figure 6D). During training, both groups displayed comparable behavior (mobility and freezing) before and following the shocks, indicating that Homer 3 siRNA injection did not produce any deficit in the locomotor activity or the ability of mice to perceive the shocks (Figure 6E).

When tested 24 hours after training with CFC (context without shock, Figure 6F & G; Figure S10 in Supplement 1), mice injected with Homer 3 siRNA exhibited reduced fear (overall freezing during the 5 minute test session) as compared to the Control group (36.1% vs. 50.3%, $t(28) = 2.25$, $p < 0.05$). A repeated-measure ANOVA performed on the 1 minute bins of the 5 minute test session revealed that the two groups differ in their percent of freezing, $F(1,28) = 4.97$, $p < 0.05$. The Group X Time interaction was not significant. Individual comparison indicated that the Control and Homer 3 KD groups did not differ in their percent of freezing during the first 2 minutes and that over the remaining 3 minutes, the Homer 3 KD mice showed significantly reduced freezing as compared to the Control group (3rd minute, $p < 0.05$; 4th minute, $p < 0.05$; 5th minute, $p < 0.05$). This pattern of freezing mirrors what we observed with ANI administration within the PL subdivision. Together, these results support a fundamental role of Homer 3 within the PL cortex during the encoding of contextual fear memory.

Discussion

According to the original theory of consolidation, memory is initially stored in the hippocampus before being permanently stored within the neocortex (6, 16,48). Recent findings suggest that in some forms of learning parallel traces are formed between the hippocampus and the cortex and there might be a gradual disengagement of the hippocampus (73). Unlike the hippocampus, the mechanism by which the mPFC plays a critical role in initial encoding of long-term memories is not understood. To address this we examined whether encoding of contextual fear memory requires new protein synthesis in mPFC. We systematically analyzed mRNAs associated with polyribosomes, an approach

several researchers have used to study translational activation of mRNAs (71,72). Using this approach we identified several mRNAs whose translation are temporally regulated in mPFC during CFC. We next established a critical role for translation in mPFC in the encoding of contextual fear memory.

A critical role for Prelimbic cortex in encoding of contextual fear memory

Prior studies suggest a major role for mPFC in the permanent storage of memories. For example, the ACC subregion of mPFC plays a critical role in the maintenance of LTM (6) whereas the PL subregion of mPFC is critical for the expression of contextual fear (34–41). These studies implied that subregions of mPFC have a specific role in expression and maintenance of contextual fear memory. Consistent with this idea, our findings suggest that the PL subregion plays a critical role during encoding of contextual fear. Specifically, inhibition of new protein synthesis in the PL immediately after CFC training reduced the percentage of freezing during the test conducted 24 hours later, whereas similar manipulations in the ACC did not significantly affect freezing. Several studies have reported the importance of PL in the expression of fear whereas infralimbic cortex (IL) in the extinction of fear (74). The specific roles of these subregions in encoding, expression, retrieval and extinction as well as their underlying molecular mechanisms remain to be unraveled.

Importantly, the requirement of new protein synthesis in the PL is temporally regulated. There is a specific time window during encoding that is sensitive to the disruption of protein synthesis in the PL. Polyribosome profiling revealed a second wave of translational activation in the mPFC at the 6-hour time point. However, protein synthesis inhibition in the PL at 6 hours following CFC training did not impair CFC learning. This apparent discrepancy can be explained by the possibility that the 1st wave of protein synthesis in the PL is critical for encoding of contextual fear memory, whereas the 2nd wave of protein synthesis at other subregions of the mPFC, namely the ACC, is important for the storage of this memory as proposed by Frankland and colleagues (6).

Molecular substrates of translation in mPFC

Long-lasting synaptic plasticity and the accompanying morphological changes of synapses, which underlie learning and memory, require new protein synthesis (33,49,50,51,52). Recently a number of experiments have provided information about the processes that underlie translational control of synaptic plasticity during learning and memory (33,49,53–58). Hence, identification of mRNAs that are actively translated and the temporal regulation of translation during systems consolidation of LTM have been a long sought goal in the research on LTM. In the present investigation, we have identified several mRNAs that are enriched in polyribosomes in the mPFC following CFC. We have identified 171 mRNAs and 151 mRNAs enriched 1 hour and 6 hours following CFC training. These two pools of mRNAs overlap only <5% suggesting two waves of activation of translation during encoding. Identification of these mRNAs provided a new molecular framework in the mPFC for encoding and consolidation of memory. Biological pathway analysis of these mRNAs allowed identifying signaling pathways that are differentially recruited at these two time points. For example, pathways that regulate axonal guidance and cell migration are recruited

within the first hour following CFC training. By contrast, pathways involved in synaptic plasticity, Huntington Disease, and Parkinson's disease are recruited six hours after CFC training (Tables S5–S9 in Supplement 2). This differential expression of signaling pathways suggests that specific cellular and synaptic changes in the mPFC are associated with encoding of contextual fear memory.

Homer 3, a novel regulator of encoding of contextual fear memories in PL mPFC

To understand the functional significance of the molecular substrates of translation during encoding that we identified, we focused on a post-synaptic density protein, Homer 3. The Homer family consists of three proteins, Homer 1, 2 and 3. They act as adaptors for many post-synaptic density proteins and bind to the intracellular tail of group 1 of metabotropic glutamatergic receptors (mGluRs). Homer 1a has been shown to play an important role in contextual fear conditioning (45–67). Recent studies have suggested that Homer 3 interacts with amyloid precursor protein (59–61) and inhibits the production of A β (59–61). In Purkinjee neurons, Homer 3 interacts with mGluR1alpha and this interaction is regulated by phosphorylation (62). Our findings (RNAseq, qPCR and Western) revealed that a specific member of the Homer family of proteins, Homer 3, is upregulated in the mPFC within the first hour following CFC training. The inhibition of function of Homer 3 by specific knockdown of Homer 3 mRNAs in the PL subdivision resulted in a significant reduction in freezing suggesting that Homer 3 contributed to the encoding of contextual fear. This memory phenotype is consistent with the role of the Homer family of proteins in regulating synaptic plasticity and memory. At this point we do not have a better understanding of the other molecules that interact with Homer 3 in the PL. Based on the previous findings that Homer 3 interacts with mGluR1alpha, a reduction in Homer 3 levels might have led to alterations in mGluR1 signaling in the PL resulting in a deficit in encoding of contextual fear. Consistent with this possibility, several studies have suggested that mGluR1 signaling is critical in synaptic plasticity and LTM (51,63–69).

Our study established a new role for the PL cortex in the encoding of contextual fear and revealed complex temporal and spatial regulation of translation in the mPFC. It remains to be determined how translation in subregions of mPFC such as prelimbic, infralimbic and anterior cingulate cortex and the hippocampus are coordinated for long-term memory storage, how different molecular substrates of translation are recruited during consolidation, and the molecular mechanisms that govern their recruitment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Polyribosome analysis reveals activation of translation in medial prefrontal cortex (mPFC) during encoding of contextual fear memories

A: Overall strategy to isolate and analyze polyribosomes. Following contextual fear conditioning (CFC), the mPFC was isolated to prepare polyribosomes for RNAseq and qPCR analyses. B: Differential gene expression analysis (DEGs) of polyribosomal RNAs from mPFC at 1 hour after (T1) and 6 hours after (T2) of CFC training. Venn diagram presents mRNAs shared between the two time points. C and D: Enrichment analysis result of differentially expressed genes between “Control” and “Fear conditioned” groups in the mPFC at 1 hour after (C) and 6 hours after (D) CFC training. Nodes represent significantly enriched ($p < 0.05$) GO biological process functions and edges are the overlap between two gene sets.

Figure 2. CFC training produce changes in transcription and translation of specific genes in mPFC

A: Overall strategy to analyze transcriptional and translational changes within 1 hour of CFC training. B, C, & D: qPCR analysis of expression of 14 selected genes in total, polyribosome and monosome RNAs following CFC training. Differential gene expression in total (B), polyribosome (C) and monosome (D) RNAs are shown in bar graphs. E: Total proteins were isolated from mPFC following CFC training and from controls. Representative autoradiographs of Western Blot analysis are shown. F, G, and H: Bar graphs showing quantitation of Western Blot data. Number of animals used for each experiment is indicated in the bar graphs. Data was normalized to alpha tubulin levels. Error bars are SEM. * $p < 0.05$.

Figure 3. CFC training induces decrease in Elf2α phosphorylation in the mPFC

A: Two representative fluorescent images of immunohistochemical analysis of phospho (eIF2α-P) and total eIF2α (eIF2α-T) expressions in the PL and ACC region of mouse mPFC. Adult mouse brains (Context + Shock and Context alone) were isolated one hour after training and brain slices that contain both PL/ACC were prepared. These slices were then stained with phospho-eIF2α (Ser51) (Green), eIF2α (Red) and Hoechst (Blue). Confocal projection images of representative examples in the PL and ACC of mouse mPFC are shown. White scale bar, 20 μm. B & C: Quantitation of immunohistochemical analysis in PL (B) and ACC (C) of mouse mPFC (n=4 for all). Error bars are SEM. * $p < 0.05$.

Figure 4. Targeted pharmacological inhibition of protein synthesis at PL and ACC subregions of mPFC

A: Experimental design. Following contextual fear conditioning, Anisomycin (ANI) or saline was infused into either the PL or ACC and % of freezing was measured 24 hours after training. B: Performance of mice infused with ANI or saline into the PL during training. C: Mice injected with ANI showed statistically significant differences in the level of freezing by the third minute until the end of the test, 3 minutes: $t(28) = p < 0.01$; 4 minutes: $p < 0.01$; 5 minutes: $p < 0.05$. D: Bar graphs showing impairment in contextual fear memory in ANI infused mice compared with saline-infused animals when tested 24 hours after CFC training. E: Performance of mice infused with ANI or saline into the ACC during training. F: ANI injected and control group did not have any significant effect on the freezing levels during the 5 minutes of contextual fear memory test. Numbers of animals used in the experiments are indicated in the bar graphs. G: ANI or saline injected immediately after CFC training into the ACC (Saline, $n = 8$; ANI, $n = 6$) did not have any significant effect on the freezing levels during the contextual fear memory test. H: Photomicrograph shows cannula

placements within the mPFC/PL (Scale bar: 1 mm.). I: Photomicrograph shows cannula placements within the mPFC/ACC (Scale bar: 1 mm.). Error bars are SEM. ** $p < 0.01$; *** $p < 0.001$.

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Figure 5. Inhibition of protein synthesis six hours after training did not affect encoding of contextual fear memories

A: Schematic of the procedure. Following 6 hours after contextual fear conditioning, Anisomycin (ANI) or saline was infused into the PL and the % of freezing was measured 24 hours after training. B: Performance of mice infused with ANI or saline into PL during training. C: Changes in % of freezing in 5 minutes during the contextual fear memory test after 24 hours of training. D: Bar graphs showing % of freezing in Saline and ANI injected mice. Numbers of animals used in the experiments are indicated in the bar graphs. C: Error bars are SEM.

Figure 6. Homer 3 in prelimbic mPFC is critical for the encoding of contextual fear

A: Autoradiogram showing an increase in Homer 3 protein levels in mPFC following CFC training. B: Quantitation of data shown in “A”. Data was normalized to alpha tubulin levels. C: siRNA knockdown of Homer 3 mRNA in primary cortical neuron cultures. D: Design of the experiment. Following 72 hours of micro-infusion of Homer 3 siRNA/control siRNA to PL, mice were trained in CFC. The % of freezing was measured 24 hours after training. E: Performance of the Homer 3 siRNA and control siRNA injected mice during training. F: Changes in % of freezing in 5 minutes during the contextual fear memory test after 24 hours of training. G: Bar graphs showing % of freezing in Saline and ANI injected mice. Numbers of animals used in the experiments are indicated in the bar graphs. Error bars are SEM. * $p < 0.05$