Rapid, experience-dependent translation of neurogranin enables memory encoding

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Experience induces de novo protein synthesis in the brain and protein synthesis is required for long-term memory. It is important to define the critical temporal window of protein synthesis and identify newly synthesized proteins required for memory formation. Using a behavioral paradigm that temporally separates the contextual exposure from the association with fear, we found that protein synthesis during the transient window of context exposure is required for contextual memory formation. Among an array of putative activity-dependent translational neuronal targets tested, we identified one candidate, a schizophrenia-associated candidate mRNA, neurogranin (Ng, encoded by the Nrgn gene) responding to novel-context exposure. The Ng mRNA was recruited to the actively translating mRNA pool upon novel-context exposure, and its protein levels were rapidly increased in the hippocampus. By specifically blocking activity-dependent translation of Ng using virus-mediated molecular perturbation, we show that experience-dependent translation of Ng in the hippocampus is required for contextual memory formation. We further interrogated the molecular mechanism underlying the experience-dependent translation of Ng, and found that fragile-X mental retardation protein (FMRP) interacts with the 3′UTR of the Nrgn mRNA and is required for activity-dependent translation of Ng in the synaptic compartment and contextual memory formation. Our results reveal that FMRP-mediated, experience-dependent, rapid enhancement of Ng translation in the hippocampus during the memory acquisition enables durable context memory encoding.

Significance

De novo protein synthesis is critical for memory formation. We found that protein synthesis during acquisition is transiently required for contextual memory formation. We identified one candidate gene, Nrgn (encoding protein neurogranin, Ng) with enhanced translation upon novel-context exposure, and found that experience-dependent translation of Ng in the hippocampus is required for contextual memory formation. Furthermore, fragile-X mental retardation protein interacts with the 3′UTR of the Nrgn mRNA, which is required for activity-dependent translation of Ng in the synaptic compartment and contextual memory formation. Together, these results indicate that experience-dependent and acute translation of Ng in the hippocampus during memory acquisition enables durable context memory encoding.

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The pool of candidate genes undergoing translation in response to neural activity is potentially extensive. For example, preceding evidence suggests that ~1,000 mRNAs are targets of the activity-dependent translation regulator, the fragile X mental retardation protein (FMRP, encoded by the Fmr1 gene) (12, 13). Many potential FMRP-interacting mRNA targets are involved in pathways important for synaptogenesis, synaptic plasticity, and implicated in neurodevelopmental and psychiatric disorders (12–14). In contrast to this vast landscape of potential targets, the number of bona fide FMRP targets that are functionally validated remains limited, as summarized in Pasciuto and Bagni (15). Studies confirming experience- (not electrical or chemical stimulation) dependent translational regulation (not transcriptionally dependent) of candidate genes are sparse, with the exception of CaMKIIα (16), using light exposure after dark rearing, and Arc (17), using repeated context exposure to induce translation after the initial gene transcription. Although the contribution of these gene targets to learning and memory has been heavily explored, the specific effect of activity-dependent protein-synthesis of any one gene product on synaptic plasticity and learning and memory remains largely unknown.

One attractive candidate that undergoes local translation in an activity-dependent manner is neurogranin (Ng; gene name, Nrgn). Neurogranin (Ng) with activity-dependent translation regulates the fragile X mental retardation protein (FMRP, encoded by Fmr1). Many potential FMRP-interacting mRNA targets are involved in pathways important for synaptogenesis, synaptic plasticity, and implicated in neurodevelopmental and psychiatric disorders (12–14). In contrast to this vast landscape of potential targets, the number of bona fide FMRP targets that are functionally validated remains limited, as summarized in Pasciuto and Bagni (15). Studies confirming experience-dependent translation of Ng during context exposure from the association with fear, we found that protein synthesis during the transient window of context exposure is required for contextual memory formation. Among an array of putative activity-dependent translational neuronal targets tested, we identified one candidate, a schizophrenia-associated candidate mRNA, neurogranin (Ng, encoded by the Nrgn gene) responding to novel-context exposure. The Ng mRNA was recruited to the actively translating mRNA pool upon novel-context exposure, and its protein levels were rapidly increased in the hippocampus. By specifically blocking activity-dependent translation of Ng using virus-mediated molecular perturbation, we show that experience-dependent translation of Ng in the hippocampus is required for contextual memory formation. We further interrogated the molecular mechanism underlying the experience-dependent translation of Ng, and found that fragile-X mental retardation protein (FMRP) interacts with the 3′UTR of the Nrgn mRNA and is required for activity-dependent translation of Ng in the synaptic compartment and contextual memory formation. Our results reveal that FMRP-mediated, experience-dependent, rapid enhancement of Ng translation in the hippocampus during the memory acquisition enables durable context memory encoding.

h Hippocampus | Contextual memory | Dentate gyrus | ASD | Schizophrenia

Novel experience induces de novo protein synthesis in the brain and protein synthesis is required for long-term memory. It is important to define the critical temporal window of protein synthesis and identify newly synthesized proteins required for memory formation. Using a behavioral paradigm that temporally separates the contextual exposure from the association with fear, we found that protein synthesis during the transient window of context exposure is required for contextual memory formation. Among an array of putative activity-dependent translational neuronal targets tested, we identified one candidate, a schizophrenia-associated candidate mRNA, neurogranin (Ng, encoded by the Nrgn gene) responding to novel-context exposure. The Ng mRNA was recruited to the actively translating mRNA pool upon novel-context exposure, and its protein levels were rapidly increased in the hippocampus. By specifically blocking activity-dependent translation of Ng using virus-mediated molecular perturbation, we show that experience-dependent translation of Ng in the hippocampus is required for contextual memory formation. We further interrogated the molecular mechanism underlying the experience-dependent translation of Ng, and found that fragile-X mental retardation protein (FMRP) interacts with the 3′UTR of the Nrgn mRNA and is required for activity-dependent translation of Ng in the synaptic compartment and contextual memory formation. Our results reveal that FMRP-mediated, experience-dependent, rapid enhancement of Ng translation in the hippocampus during the memory acquisition enables durable context memory encoding.
Ng, a small neuronal protein (78 amino acids) primarily expressed in the somato-dendritic compartment (dendrite, dendritic spine, and cell body) of projection neurons in the cerebral cortex, hippocampus, striatum and some subcortical nuclei, including the amygdala (18–20). Ng belongs to the protein family, composed of small, abundant proteins that preferentially bind to the Ca$^{2+}$-free form of calmodulin (CaM). Ng is the only family member in the postsynaptic compartment in the forebrain (21). It has been hypothesized that Ng regulates the availability of CaM for Ca$^{2+}$ binding and Ca$^{2+}$ buffering capacity in neurons (22), thus critically influences Ca$^{2+}$ or Ca$^{2+}$/CaM-dependent neuronal processes, such as synaptic plasticity and, consequently, learning and memory (23–25). Ng is associated with schizophrenia (26, 27) and a rare genetic disorder with symptoms of intellectual disability, Jacobsen syndrome (28), implying an important role of Ng in cognitive function.

Previous studies have shown that Ng levels change in response to behavioral, environmental, and hormonal stimulation in rodent models, and schizophrenic and aging brains in humans (29–38), with emphasis on regulation at the transcription level. The 3’UTR of the Ngmr mRNA contains a putative dendritic targeting sequence that is potentially important for both translation and translational control of Ng (39, 40). The Ngmr mRNA is also a potential target of FMRF (12). However, it is unknown whether the expression of Ng is regulated at the translation level by experience.

Here, using a hippocampus-dependent behavioral paradigm (41), specifically separating learning of context from fear-conditioning (42), we report that there is a transient window for de novo protein synthesis during memory acquisition required for contextual memory formation. Through a screening, we identified Ng, which is up-regulated in the hippocampus during this critical window, and this up-regulation is necessary for durable encoding of the memory. We further found that FMRF interacts with the 3’UTR of the Ngmr mRNA, and the FMRF-mediated, novel-context–induced Ng up-regulation is essential for the contextual memory formation.

**Results**

**Contextual Memory Formation Requires Rapid Protein Synthesis.** To define the temporal window for de novo protein synthesis required in contextual memory formation, we adapted a contextual memory test in which contextual exposure and associative fear learning, using an electric foot shock, were separated by 24 h (42). Consistent with previous studies, associative learning was established (42), prior to the challenge in the arena in which the shock is later delivered (SI Appendix, Fig. S1), showing it was the sufficient preexposure to the context, rather than other cues caused by handling, which allowed associative learning. This approach separates the contextual memory formation and associative learning, and uses the associative component to interrogate the establishment of contextual memory. Behavioral freezing levels are then used to monitor successful association of foot shock with the context and, hence, determine whether contextual memory was formed or not. Combined with pharmacological manipulation, this procedure enabled us to probe the distinct phases of memory formation with defined temporal control. We intraperitoneally injected the protein synthesis inhibitor anisomycin (150 mg/kg) at four different time points relative to the context exposure (Fig. 1A). Consistent with previous results (42), the intraperitoneal injection of anisomycin 30 min before context exposure (~30 min from the starting point of context exposure) prevented contextual memory formation (Fig. 1B). The intraperitoneal injection immediately before context exposure (~0 min from the starting point of context exposure) also prevented contextual memory formation (Fig. 1C), suggesting that protein synthesis during the initial context exposure was required for context memory formation. In contrast, intraperitoneal injection of anisomycin immediately after (8 min after the starting point of context exposure) and 7 min after context exposure (~15 min after the starting point of context exposure) did not affect contextual memory formation (Fig. 1D and E).

Previous studies reported that anisomycin injected intraperitoneally in rats or mice reaches effective concentrations in the brain cerebrospinal fluid between 15 and 45 min to inhibit protein synthesis for more than 3 h in the hippocampus (43, 44). Considering these observations, our results show that the first phase of protein synthesis requirement ceases shortly after the context exposure, suggesting the first de novo protein synthesis required for context memory formation has a rapid onset, and is transient.

**Novel Context Induces Rapid Increase of Ng Protein Levels in the Hippocampus.** Activation of NMDARs in synaptoneuromes induces rapid changes in protein synthesis, leading to elevated protein synthesis 15- to 60-min poststimulation (45), a time window coinciding with that required for protein synthesis-dependent memory formation. Many genes are potential targets of activity-dependent translation, which are thought to be involved in memory formation (46, 47). However, little is known about which genes are translationally up-regulated in the hippocampus during the critical time window essential for context memory formation. To identify the potential targets during context exposure, we isolated a polyribosome-enriched mRNA pool (48, 49) from total hippocampal lysate of naïve animals and animals exposed to a novel context (Materials and Methods and Fig. 2 A and B). An array of candidate genes and their gene family members (SI Appendix, Table S1) were tested using qRT-PCR. The selection criteria were: (i) activity-dependent and experience-dependent protein synthesis has been shown, or suggested via functional studies; (ii) putative or proven dendritically targeted mRNA; and (iii) functionally validated FMRF targets relevant to synaptic structure and function (SI Appendix, Dataset S1). We found that, among 28 genes tested, exposure to a novel context induced an increase only of Ngmr mRNAs in the actively translating, polyribosome-enriched mRNA pool (Fig. 2C). This result showed that exposure to novel context induced the selective recruitment of the Ngmr mRNA to the polyribosome-enriched compartment.
Fig. 2. Ng is up-regulated rapidly following novel-context exposure. (A) The behavioral paradigm for RNA and protein analysis. (B) The workflow for analyzing the ribosome enriched fraction. (C) Quantification of relative levels (Fold-change Nov/Ctrl) of mRNA candidates in the hippocampal ribosome-enriched fraction from novel-context–exposed (Nov) animals to those from control animals (Ctrl). Each cDNA target was normalized to GAPDH cDNA within each sample, and then the relative fold-change was calculated. Colored symbols, individual data points; colors, different cohorts of animals; n = 7 out of three cohorts; mean ± SEM, in black lines in each data column. One-way ANOVA, F(27, 168) = 8.356, P < 0.0001; followed by Dunnett’s test, compared with Nov. (D) Representative confocal images of coronal sections obtained from novel-context–exposed (Nov) and control mice (Ctrl). (Left Top) Ng (green) and (Left Bottom) Synaptophysin (red). (Scale bar, 500 μm) (Right Top) Ng (green) in hippocampus. (Scale bar, 200 μm) White boxes show area of detail. (Right, Middle, and Bottom) Ng (green) in dentate gyrus and CA1. (Scale bar, 10 μm) (E and F) Representative Western blot images (E) and quantification (F, n = 8 pairs) of select proteins in hippocampal lysates from control (Ctrl) and novel-context–exposed (Nov) animals. In this and subsequent Western blot quantification graphs, gray symbols, individual data points; mean ± SEM, and the bar graph, One-way ANOVA; F(5, 42) = 10.33, P < 0.0001; (F) F(4, 20) = 0.54, P = 0.70; (F), F(4, 20) = 1.099, P = 0.38; followed by Dunnett’s test, compared with 100%. **P < 0.0001. (G–I) The behavioral paradigm (G), representative Western blot (H), and quantification (I) of Ng [normalized to tubulin (Tub)] in hippocampal lysates from animals exposed 0 min (n = 6), 2 min (n = 6), 8 min (n = 6), and 8 min (n = 6) to the novel context, and collected at 15 min after the initial context exposure. One-way ANOVA, F(2, 15) = 12.43, P = 0.0007; followed by Tukey’s test, compared with the no exposure control, **P < 0.01. (J–L) The behavioral paradigm (J), representative Western blot (K), and quantification (L) of select proteins (normalized to Tub) in hippocampal lysate from animals collected before (0 min, n = 6), immediately after the initial exposure (8 min, n = 6), and 15 min after the initial exposure (15 min, n = 6). One-way ANOVA, Ng, F(2, 15) = 8.37, P = 0.003; CaMKII, F(2, 15) = 0.756, P = 0.49; GAPDH (three sets of samples were used), F(2, 6) = 1.014, P = 0.42; CaM, F(2, 15) = 0.06, P = 0.94; PSD-95, F(2, 15) = 1.41, P = 0.27, compared with the no exposure control (0 min), *P < 0.05, **P < 0.01. (M–O) The behavioral paradigm (M), representative Western blot (N), and quantification (O) of Ng in hippocampal lysate from animals collected at different time points relative to the initial context exposure (n = 5 for 0, 6, 15, 30, 60, 120 min, F(5, 240) = 4.31, P = 0.02, followed by Dunnett’s test, compared with the no exposure control (0 min), *P < 0.05, **P < 0.01. (P–R) The behavioral paradigm (P), representative Western blot images (Q), and quantification (R) of Ng in hippocampal lysate from control (Ctrl, black bar) and novel-context–exposed (Nov, red bar) animals treated with either saline (sal) or anisomycin (aniso); n = 6 in each group. Two-way ANOVA, interaction F(1, 20) = 12.13, P = 0.002; followed by Tukey’s test. *P < 0.05.

Given the fact that immediate early genes (IEGs) respond to novel-context exposure and enhance their transcription and translation (50, 51), we tested several IEGs as a positive control, including Egr1, Fos, Npas4, Arc, and Homer1, both in the polysome-enriched fraction and in the total RNA input. Egr1, Arc, and Homer1 were neither significantly changed in the polysome-enriched fraction nor in the total RNA input (SI Appendix, Fig. S2 A and B). Consistent with previous findings (52), we found that Fos and Npas4 were significantly up-regulated in both the polysome-enriched fraction and in the total RNA input. Thus, transcription of Fos and Npas4 responds to context exposure rapidly and significantly. We next tested whether the increase of Nogn mRNA in the polysome-enriched compartment was due to the increase of total Nogn mRNAs. In contrast to Fos and Npas4, Ngn mRNA levels in the total RNA input was not increased by novel-context exposure (SI Appendix, Fig. S2B), which indicated that the enhancement of the Ngn mRNAs in the polysomosomal fraction was due to the recruitment of existing Ngn mRNAs into the fraction rather than enhanced transcription of Ngn.

The enhanced Ngn mRNA in the polysome fraction implies an increase in translation. We therefore analyzed the influence of context exposure on protein levels using immunofluorescence staining and Western blotting. Consistent with the specific increase of the Ngn mRNA in the hippocampal polysome pool, the increased Ng protein levels were detected in the hippocampal CA1, CA3, and dentate gyrus (DG) regions in response to novel-context exposure using immunostaining with an Ng-specific antibody (Fig. 2D). Ng protein levels, but not CamKIIa, GAPDH, or CaM protein levels, were increased in the hippocampus of the animals exposed to a novel context compared with naïve animals (Fig. 2 E and F). No significant differences of Ng levels were detected from striatal and cortical homogenates, suggesting the increase of Ng levels in hippocampus was specific (SI Appendix, Fig. S2 C–F).

To examine the temporal features of context-induced up-regulation of Ng levels in the hippocampus, we varied the
exposure duration and the time of collection and tested the Ng levels in the hippocampus using Western blotting. A short exposure (2 min), which was not sufficient for contextual memory formation (SI Appendix, Fig. S1), did not induce changes in hippocampal Ng levels (Fig. 2 G–I), whereas a longer exposure (8 min), which was sufficient for contextual memory formation (SI Appendix, Fig. S1), led to increased Ng levels in the hippocampus (Fig. 2 G–I). This result showed that the temporal requirement of context exposure for increasing hippocampal Ng levels is consistent with the temporal requirement for contextual memory formation. Furthermore, the increase of hippocampal Ng levels after context exposure was transient, as the increase of Ng levels was detected immediately after the novel-context exposure (Fig. 2 J–L) and subsided 30–60 min after the initial exposure (Fig. 2 M–O), consistent with the temporal requirement of protein synthesis for contextual memory formation (Fig. 1).

This novel experience-induced up-regulation of Ng levels in the hippocampus requires protein synthesis. Intraperitoneal administration of anisomycin, 30 min before the novel-context exposure, blocked novel-context–induced up-regulation of hippocampal Ng protein levels (Fig. 2 P–R). Taken together, these results reveal rapid, experience-dependent up-regulation of translation of Ng, whose temporal expression pattern is consistent with the temporal profile of context exposure and protein synthesis required for contextual memory formation.

**Neural Activity Induces Elevated New Protein Synthesis of Ng.** Our results thus far suggest that enhanced translation of the Nrgn mRNA leads to increased Ng protein levels. In support of this hypothesis, the increase of Ng protein levels and the increase of Nrgn mRNA in the polyribosome were linearly correlated (SI Appendix, Fig. S3). To directly test whether neural activity elevates Ng protein synthesis, we used dissociated primary neuronal culture, which enabled direct visualization of newly synthesized targeted labeled proteins on cell biology.

Treating the neuronal culture with bicuculline (Bic), a GABA receptor blocker, for 15 min to enhance excitatory neuronal activity, induced a significant increase of Ng levels in the total cell lysate compared with control, vehicle-treated sister cultures. The protein synthesis inhibitor cycloheximide (CHX), blocked the activity-dependent up-regulation of Ng protein levels, but the transcription inhibitor Actinomycin D (ActD) did not (Fig. 3 A–F), confirming the findings from the behavioral experiment that translation but not transcription is required for activity-dependent up-regulation of Ng (Fig. 2 and SI Appendix, Fig. S2).

To directly test whether de novo protein synthesis of Ng was induced by elevated neural activity, we used puromycylation with the proximity-ligase assay (PLA) to visualize specific newly synthesized proteins (53) (Fig. 3H). First, we checked the total Ng levels using conventional immunofluorescent staining. Consistent with the Western blot results, Bic treatment induced a significant, protein synthesis–dependent increase of total Ng levels, both in the dentritic and somatic compartments, using Ng primary antibodies (Fig. 3 G and I–K). Then, using the PLA assay, we analyzed the levels of newly synthesized Ng proteins (Fig. 3 G and H). Bic treatment substantially increased the levels of newly synthesized Ng in a translation-dependent way, detected by the positive PLA signal of anti-puromycin antibodies and anti-Ng antibodies (Fig. 3 G–I, L, and M). As a negative control, the anti-Ng interaction partner, CaM, was not significantly affected in the same preparation both at the total level and at the newly synthesized level (Fig. 3 N–R). Taken together, these results indicate that increasing neuronal activity induces rapid de novo synthesis of Ng.

**Activity-Dependent Increase of Ng Is Required for Contextual Memory Formation.** Novel-context exploration enhances neural activity in the hippocampus (54–56), which is thought to be critical for forming a stable hippocampal representation of the context. We then asked whether novel-context–induced increase of Ng contributes to contextual memory formation. To address this question, we developed a molecular reagent to perturb the activity-dependent translation of Ng. The 3′ UTR of the Nrgn mRNA contains a potential dendritic targeting sequence that may also serve as an activity-dependent translational control element (40). We tested whether the activity-dependent translation of Ng is mediated by the 3′ UTR of Nrgn. We constructed a recombinant adeno-associated viral vector (AAV) as a decoy that contained the 3′ UTR of Nrgn following an eGFP ORF, under the control of a CaMKIIα promoter that drives the expression preferentially in excitatory neurons (57) (eGFP-Ng3′UTR) (Fig. 4A). A control AAV contained the 3′ UTR of Gapdh with an eGFP ORF (eGFP-G3′UTR) (Fig. 4A). Using dissociated cultures, we tested the effect of these 3′ UTR–expressing AAVs on activity-dependent regulation of Ng expression. The expression of both eGFP-Ng3′UTR and eGFP-G3′UTR did not affect the basal levels of Ng (Fig. 4 B and C). However, the expression of eGFP-Ng3′UTR blocked Bic-induced up-regulation of Ng protein levels, whereas the expression of eGFP-G3′UTR did not (Fig. 4 B and C). eGFP levels in eGFP-Ng3′UTR expressing neurons did not significantly increase when the neurons were stimulated with Bic (Fig. 4D), suggesting that the 3′ UTR of Nrgn is necessary but not sufficient to mediate the activity-dependent translation of Ng. Thus, the exogenous 3′ UTR of the Nrgn mRNA likely functioned as a competitor for the regulatory factors that control the activity-dependent translation of endogenous Ng.

Using these AAVs, we then tested whether activity-dependent up-regulation of endogenous Ng in the hippocampus is necessary for hippocampus-dependent memory formation. Given that the DG region in the hippocampus is important for parsing novel contextual information (58, 59), and that novel-context exposure elevates the Ng levels in the DG (Fig. 2G), we targeted eGFP-Ng3′UTR or eGFP-G3′UTR AAVs into the DG region bilaterally using stereotoxic injections (SI Appendix, Fig. S4A). Five days after AAV injection, the animals were subjected to behavioral tasks. Basal locomotor activity and anxiety were not different between animals with eGFP-Ng3′UTR or eGFP-G3′UTR, tested in the open-field exploration and elevated plus maze (SI Appendix, Fig. S4 B–E). Using different cohorts of injected animals, we tested contextual memory formation (Fig. 4E). The expression of eGFP-G3′UTR in the DG did not affect contextual memory formation (Fig. 4F). However, the expression of eGFP-Ng3′UTR in the DG blocked contextual memory formation (Fig. 4F). These results are consistent with the hypothesis that activity-dependent translation of endogenous Ng is necessary for contextual memory formation. As a decoy construct, the 3′ UTR of the Nrgn mRNA may influence the activity-dependent translation of other genes. To test whether the learning deficits seen in eGFP-Ng3′UTR–expressing animals resulted from specific interference with Ng levels, we built a rescue construct, expressing Ng-eGFP ORF to achieve a higher level of Ng in the background of the exogenous Ng3′UTR (Ng-eGFP-Ng3′UTR), and a functionally null mutant (NgΔIO-eGFP-Ng3′UTR) (21) was used as a control (Fig. 4G).

Using the dissociated culture, we confirmed that the expression of Ng-eGFP-Ng3′UTR and NgΔIO-eGFP-Ng3′UTR blocked Bic-induced up-regulation of endogenous Ng levels (Fig. 4 H and J); and at the same time, the exogenous expression cassette allowed additional expression of Ng-eGFP or NgΔIO-eGFP (Fig. 4H). We bilaterally injected Ng-eGFP-Ng3′UTR or NgΔIO-eGFP-Ng3′UTR expressing AAVs into the DG region of the hippocampus (SI Appendix, Fig. S4F). Mobility and anxiety levels were indistinguishable between mice injected with either virus (SI Appendix, Fig. S4 G–J). Expression of NgΔIO-eGFP in the Ng3′UTR expressing background did not rescue the behavioral deficit in contextual memory formation, whereas expression of Ng-eGFP in the Ng3′UTR expressing background did rescue the memory deficit (Fig. 4J). Therefore, elevated Ng levels sufficiently rescued the contextual memory deficit caused by expressing Ng3′UTR, meaning that: (i) even if the translation of other transcripts can be influenced by the expression of Ng3′UTR, they are not critical for contextual memory formation;
or (ii) their expression can be rescued by introducing exogenous Ng. Collectively, these results indicate that activity-dependent translation of Ng is necessary for contextual memory formation.

**FMRP Interacts with the 3′ UTR of the Ng3 mRNA.** To further investigate the molecular mechanism underlying activity-dependent up-regulation of Ng translation, we made deletions of the 3′ UTR of Ng3, and tested its effect on activity-dependent translation of Ng and contextual memory formation. We generated an AAV containing the nucleotides 389–577 of the Ng3 mRNA (eGFP-Ng3 UTR 389–577) (Fig. 5A), a small portion of the 5′-end of the 3′ UTR of the Ng3 mRNA consisting of a cis-element for putative dendritic targeting and translational regulation (40). Expressing this small portion of the 3′ UTR of the Ng3 mRNA was sufficient to block the Bic-induced increase of Ng in dissociated neuron culture, similar to the full-length 3′ UTR of the Ng3 mRNA (Fig. 5B and C). In addition, as was the case with eGFP-Ng3 UTR, expressing eGFP-Ng3 UTR 389–577 in the hippocampus prevented contextual fear-memory formation (Fig. 5D and E). Thus, we narrowed down the critical element of the Ng3 UTR 389–577 for activity-dependent translation of Ng, which contributes to contextual memory formation. To identify the interaction partners that might contribute to activity-dependent translation of Ng, we generated in vitro-transcribed biotinylated RNA transcripts of the 3′ UTR of the Gapdh mRNA (G3 UTR), the 3′ UTR of the Ng3 mRNA (Ng3 UTR), and 389–577 of the 3′ UTR of the Ng3 mRNA (Ng3 UTR 389–577), to affinity-purify potential interacting proteins from the hippocampal lysate.
The overexpression of 3′-UTR of Gapdh is essential for conditional memory formation. (A) Diagrams of expression cassettes in AAV vectors for expressing eGFP followed by the 3′-UTR of Gapdh (eGFP-G3′UTR) and the 3′-UTR of Nrgn (eGFP-Ng3′UTR) under the control of the CaMKII promoter. (B) Representative Western blot images of Ng and Tub from neuronal cultures infected with eGFP-G3′UTR or eGFP-Ng3′UTR, with (+) or without (−) Bic treatment. (C) Quantification of percentage of Ng from neuronal cultures infected with eGFP-G3′UTR or eGFP-Ng3′UTR, treated with Bic, normalized to vehicle- or eGFP-treated sister neuronal lysates, n = 7 from four independent cultures. One-way ANOVA, F(2, 15) = 11.24, P = 0.001, followed by Dunnett’s test, compared with control, **p < 0.01. (D) Quantification of percentage change of eGFP from neuronal cultures infected with eGFP-G3′UTR or eGFP-Ng3′UTR, treated with Bic, normalized to vehicle-treated sister neuronal lysates, n = 6 from three independent cultures. One-way ANOVA, F(2, 15) = 11.24, P = 0.001, followed by Dunnett’s test, compared with control, P > 0.05. (E) The schematics for the contextual memory test after viral infusion. (F) Quantification of effects of the expression of eGFP-G3′UTR and eGFP-Ng3′UTR in DG on percentage of freezing during recall from control (Ctrl, black) and preexposed (Ctrl, red) mice; eGFP-G3′UTR (n = 5, Ctrl; n = 7, Ctxt); eGFP-Ng3′UTR (n = 7, Ctrl; n = 8, Ctxt). Two-way ANOVA, F(1, 23) = 6.102, P = 0.02, followed by Tukey’s test, **p < 0.01. (G) Diagrams of expression cassettes in AAV vectors for expressing the 3′UTR of Nrgn (eGFP-Ng3′UTR) fused to Ng-eGFP (Ng-eGFP-Ng3′UTR) or Ng-eGFP-Ng3′UTR under the control of the CaMKII promoter. (H) Representative Western blot images of Ng and Tub from neuronal cultures infected with eGFP-G3′UTR, Ng-eGFP-Ng3′UTR or Ng-eGFP-Ng3′UTR, with (+) or without (−) Bic treatment. (I) Quantification of percentage of Ng from neuronal cultures infected with eGFP-G3′UTR, Ng-eGFP-Ng3′UTR or Ng-eGFP-Ng3′UTR, treated with Bic, normalized to vehicle-treated sister neuronal lysates, n = 6 from three independent cultures. One-way ANOVA, F(3, 24) = 10.38, P = 0.001, followed by Dunnett’s test, **p < 0.001. (J) Quantification of effects of the expression of Ng-eGFP-Ng3′UTR and Ng-eGFP-Ng3′UTR in the hippocampus DG region on percentage of freezing during recall from control (Ctrl, black) and preexposed (Ctrl, red) mice; Ng-eGFP-Ng3′UTR (n = 4, Ctrl; n = 7, Ctxt); Ng-eGFP-Ng3′UTR (n = 5, Ctrl; n = 10, Ctxt). Two-way ANOVA, F(1, 22) = 7.57, P = 0.01, followed by Tukey’s test, **p < 0.01, ***p < 0.001, n.s. nonsignificant.
Given that FMRP proteins are globally absent in the Fmr1 KO mice, it is unclear whether the memory deficit in the Fmr1 KO mice was due to specific disruption in the hippocampus. We next asked whether FMRP in the hippocampal DG region is specifically required for memory formation via regulating Ng translation. We generated AAV-expressing shRNA that target the Fmr1 gene (Fig. 7C, shFmr1), using a published targeting sequence (62). A control AAV-expressing shRNA against luciferase (shLuc) was used as control (Fig. 7C). Expressing shFmr1 in cortical neurons effectively decreased FMRP levels (Fig. 7D and SI Appendix, Fig. S6A), and inhibited activity-dependent Ng up-regulation in the dissociated neuron culture (Fig. 7D and E).

To test whether this hippocampal, FMRP-regulated Ng up-regulation is essential for context memory formation, we further developed AAVs that overexpress Ng-eGFP either with the background of shFmr1 or of shLuc as control (Fig. 7F). We bi-laterally injected AAVs into the DG region of the hippocampus using stereotaxic injection, and tested contextual formation 14 d after the viral infusion (Fig. 7G). We found that expression of shFmr1 in the DG region of the hippocampus inhibited contextual memory formation, and this impairment of context memory can be partially rescued by overexpression of Ng-eGFP (Fig. 7H). shLuc and overexpression of Ng-eGFP with shLuc did not significantly affect context memory formation (Fig. 7H). Taken together, these results further support that FMRP-dependent de novo protein synthesis of Ng in the hippocampus upon novel-context exposure is critical for context memory formation. **

Discussion

Our present results confirm that novel-context–induced de novo protein synthesis is required for contextual memory formation. The rapid and transient time window flanks the contextual exposure and may thus be involved in memory encoding. Novel-context exposure recruits Ng mRNA into the actively translating mRNA pool and induces de novo Ng protein levels in the hippocampus. Using an AAV-mediated vector, we tested if FMRP translation of Ng requires the 3′UTR of Ng to interfere with activity-dependent up-regulation of Ng and rescue, we showed that novel-context–dependent up-regulation of Ng is necessary for contextual memory formation. To understand the molecular mechanism, we used the biotinylated in vitro-transcribed RNA of the 3′UTR of Nrgn, and identified protein interaction partners. We found that FMRP interacts with the 3′UTR of Nrgn, among other proteins. Further analyses showed that FMRP is required for novel-context–dependent up-regulation of Ng in the hippocampus critical for contextual memory formation. Taken together, our data show that novel-context exposure induces rapid, activity-dependent translation of Ng in the hippocampus via an FMRP-dependent mechanism, necessary for durable contextual memory encoding.

Our study highlights: (i) our understanding of the immediate temporal requirement of activity-dependent translation in learning and memory; (ii) the functional contribution of activity-dependent translation of a target gene, Nrgn, in learning and memory; and (iii) the involvement of FMRP-dependent mechanism underlying the activity-dependent translation of Ng critical for contextual memory encoding.

Importantly, the experimental design that we adopted separates contextual memory formation from associative learning (42, 63) and allows the interrogation of contextual memory using the associative cue. This is different from a conventional contextual fear-conditioning paradigm, in which the context exposure and the fear association were presented concurrently. Using...
the conventional contextual fear-conditioning paradigm, Ryan et al. (9) showed that posttraining blockade of protein synthesis did not affect the implementation of the memory trace, but the memory recall using the natural cue was impaired. Our results reveal that protein synthesis inhibition after context exposure does not affect memory performance when the context exposure was separated from the associative cue (Fig. 1), suggesting that the protein synthesis after context exposure in the classic contextual fear-conditioning paradigm may be essential for strengthening the associative component of the task, but not necessary for the contextual memory encoding per se. The immediate de novo protein synthesis requirement for contextual memory formation (Fig. 1) suggests that de novo protein synthesis during the acquisition phase may play an important role in gating, facilitating, and durably encoding the memory.

Previous studies on FMRP have suggested that there is a pool of mRNAs containing hundreds of candidates that may undergo activity-dependent translation in response to behavioral stimulation (12, 15, 64, 65). However, little is known in terms of the extent, temporal dynamics, and the functional consequence of the activity-dependent translation of the candidate genes. Although some experiments have been done using reporter systems to monitor the activity-dependent translation of candidate genes (66, 67), studies of the endogenous translational targets under behavioral stimulation have been sparse. We detected a significant increase of Ng protein levels in total hippocampal lysate 15 min after the onset of the novel-context exposure, whereas other candidate genes, such as CaMKIIa and PSD-95, in the total protein levels were not increased in the hippocampus at the same time point (Fig. 2 J–L), suggesting activity-dependent translation of Ng in the hippocampus is an early responder to novel-context exposure. This also indicates that the regulation of the expression of the FMRP targets may not be homogeneous, and different genes or pools of genes can be regulated via different stimulation and in different temporal and spatial domains.

![Fig. 6. FMRP is required for novel-context–induced up-regulation of Ng and stimulation-dependent recruitment of Nrgn mRNA to polysomes in synaptoneurosomes.](Image)

![Fig. 7. FMRP-mediated, novel-context–induced up-regulation of Ng is required for contextual memory formation.](Image)
Our pull-down studies show that the 3′ UTR of Nrgn interacts with protein complexes that contain FMRP and FXR2P, and previous studies have identified the Nrgn transcript as an FMRP-bound transcript using high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (12), suggesting a direct interaction between FMRP and the Nrgn transcript. Although the discrete interaction site has not been previously described, we narrowed it down to Ng3′ UTR 389–577, which can pull down FMRP and FXR2P and block memory formation. It is known that FMRP can repress mRNA translation, which can be released in an activity-dependent manner (68, 69), and absence of FMRP can lead to a general enhancement of the de novo protein synthesis rate and an absence of dependence in protein synthesis (70). Given the presence of FXR2P in the complex, it is also possible that the interaction of FMRP-FXR2P with the 3′-UTR of the Nrgn transcript drives the activity-dependent translation of Ng, as seen in other studies (13).

De novo protein synthesis, in particular local protein synthesis upon synaptic activity, has been hypothesized to be important for synaptic plasticity and memory formation (71, 72). De novo protein synthesis is different from IEG expression in that no transcription is required (except, perhaps for Arc), and the targets include eukaryote proteins that directly impact synaptic function. Absence of FMRP from animal models causes an array of learning and behavioral deficits (73), and the deficit can be restored by resetting certain signaling pathways, or prevent expression of elevated gene expression (14, 74). Our results highlight Nrgn as one important FMRP target for regulating cellular function essential for memory formation. On the other hand, given that the rescue is only partial, these results suggest that other factors influenced by decrease of FMRP also contribute to function essential for memory formation. Absence of FMRP from animal models causes an array of learning and behavioral deficits (73), and the deficit can be restored by resetting certain signaling pathways, or prevent expression of elevated gene expression (14, 74). Our results highlight Nrgn as one important FMRP target for regulating cellular function essential for memory formation. On the other hand, given that the rescue is only partial, these results suggest that other factors influenced by decrease of FMRP also contribute to function essential for memory formation.

Taking these data together, we propose that activity-dependent local translation of Ng induced by novel experience via an FMRP-dependent mechanism enables durable memory encoding.

Materials and Methods

For commercially available resources, see SI Appendix, Table S2. All animals were maintained in a vivarium with a light-dark cycle (7:00 AM–7:00 PM). Animal care and handling were performed according to NIH guidelines and with the approval of the Massachusetts Institute of Technology institutional animal care and use committee and Division of Comparative Medicine. AAV constructs were cloned in an AAV with AAV2 ITRs (76). AAV 2/9 se- rotype AAV vectors were produced as previously described (77, 78). For the context memory test with preexposure (42), C57BL/6Ncr (7- to 9-wk-old male mice) were habituated to the room. Animals were exposed to the context for the indicated time. Twenty-four hours later, animals were placed in the chamber, given an immediate shock, and removed from the chamber after a total of 1 min. Thirty minutes later, animals were reexposed to the chamber for 3 min to assay freezing. At least two cohorts of the animals in each experiment were blinded to the experimenter. Animal IDs were used for data analysis.

Mice were rapidly decapitated and submerged in liquid nitrogen for 4 s to rapidly cool brain tissue. Hippocampi were dissected on ice within 90 s and homogenized, and polyribosome enrichment was performed as described previously (Fig. 2B (48, 49). qRT-PCR primer sequences and references are given in SI Appendix, Table S1. For Western blot, animals were killed by cervical dislocation in a separate room from the behavioral room. Brain regions were rapidly dissected while ice and flash-frozen in liquid nitrogen for further Western blot analysis.

In situ PLA was performed using the DuoLink II kit (Sigma) according to the instructions of the manufacturer. Coverslips were mounted with fluoresecence mounting medium (Dako) to subject to confocal microscopy. C57BL/6Ncr mice (8-wk-old) were anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde in PBS. After postfixation, brains were frozen in 15% (w/v) sucrose. Slices were processed according to the standard procedure for immunohistochemistry. Images were taken on either a Zeiss 710 or Zeiss 810 confocal microscope with a 5× or a 63× objective, and processed in Imaris and Adobe Photoshop.

The RNA pull-down assay was based on (13). MS was performed independently two times and proteins found in both samples were marked in bold (SI Appendix, Dataset S1). Polyribosome profiling and mRNA quantification from stimulated synaptoneurosomes procedures were described previously (45, 60, 80). Detailed materials and methods are in SI Appendix.

Statistical analysis was performed using Prism (Graphpad). Group differences were determined using either one-way or two-way ANOVA with the appropriate post hoc test. A one-sample t test was used for comparison of a group of data with a fixed value. Significance threshold was set at P = 0.05.

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1. Alberini CM (2008) The role of protein synthesis during the labile phases of memory: Revisiting the skepticism. Neurobiol Learn Mem 90:234–246.
2. Davis HP, Squire LR (1984) Protein synthesis and memory: A review. Psychol Bull 96:518–559.
3. Flexner JB, Flexner LB, Stellar E (1963) Memory in mice as affected by intraeerebral puromycin. Science 141:57–59.
4. Alberini CM, Ledoux JE (2013) Memory reconsolidation. Cell 145:247–261.
5. Bekinschtein P, et al. (2007) Persistence of long-term memory storage requires a late protein synthesis- and BDNF-dependent phase in the hippocampus.
6. Bourtchouladze R, et al. (1998) Different training procedures recruit either one or two memory types. Science 280:1007–1013.
7. Quevedo J, et al. (1999) Two time windows of anisomycin-induced amnesia for inhibitory avoidance training in rats. Learn Mem 6:600–607.
8. Flood JF, Bennett EL, Orme E, Rosenzweig MR (1975) Relation of memory formation to controlled amounts of brain protein synthesis. Physiol Behav 10:97–102.
9. Ryan TJ, Roy DS, Pignatelli M, Arons A, Tonegawa S (2015) Memory. Engram cells retain memory under retrograde amnesia. Science 348:1007–1013.
10. Schafe GE, LeDoux JE (2000) Memory consolidation of auditory Pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. J Neurosci 20:939–946.
11. Barondes SH, Cohen HD (1968) Memory impairment after subcutaneous injection of acetylcysteine. Science 160:556–557.
12. Damell JC, et al. (2011) FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell 146:247–261.
13. Fernández E, et al. (2015) FXR2P exerts a positive translational control and is required for the activity-dependent increase of PSD95 expression. J Neurosci 35:9402–9408.
14. Sidorov MS, Auerbach BD, Bear MF (2013) Fragile X mental retardation protein and synaptic plasticity. Mol Brain 6:55.
15. Pascuito E, Bagini C (2014) SnapShot: FMRP mRNA targets and diseases. Cell 158:1446–1446.e1.
16. Wu L, et al. (1998) CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. Neuron 21:1129–1139.
17. Jakkamsetti V, et al. (2013) Experience-induced Arc/Arg1.3 primes CA1 pyramidal neurons for metabotropic glutamate receptor-dependent long-term synaptic depression. J Neurosci 30:70–79.
18. Neuner-Jehle M, Denizot JP, Mallet J (1996) Neurogranin is locally concentrated in rat cortical and hippocampal neurons. Brain Res 733:149–154.
19. Represa A, Deloulme JC, Sensenbrenner M, Ben-Ari Y, Baudier J (1990) Neurogranin: \(\text{RC3/neurogranin, a postsynaptic calpain}\) result in the targeting of a brain calcium-modulated signal. Mol Neurobiol 7:131-163.

22. Zhabotinsky AM, Camp RN, Epstein IR, Lisman JE (2006) Role of the neurogranin concentrated in spines in the induction of long-term potentiation. J Neurosci 26: 7337-7347.

23. Huang K-P, et al. (2004) Neurogranin/RC3 enhances long-term potentiation and learning by promoting calcium-mediated signaling. J Neurosci 24:10606-10609.

24. Pak JH, et al. (2000) Involvement of neurogranin in the modulation of calcium/ calmodulin-dependent protein kinase II, synaptic plasticity, and spatial learning: A study with knockout mice. Proc Natl Acad Sci USA 97:11232-11237.

25. Zhong S, Cherry T, Bies CE, Florence MA, Gerges NZ (2009) Neurogranin enhances synaptic strength through its interaction with the antiapoptotic protein bcl-2. J Neurosci 29: 8207-8219.

26. Ruano D, et al. (2008) Association of the gene encoding neurogranin with schizophrenia in males. J Psychiatr Res 42:125-133.

27. Stefansson H, et al. (2009) Common variants conferring risk of schizophrenia. Nature 455:164-168.

28. Colden CD, et al. (2009) Chromosomal microarray mapping suggests a role for B5X and Neurogranin in neurocognitive and behavioral defects in the 11q terminal deletion disorder (Jacobson syndrome). Neurogenetics 10:89-95.

29. Bernal J, Rodriguez-Pena A, Iniguez MA, Ibarrola N, Munoz A (1992) Influence of thyroid hormone on brain gene expression. Acta Med Austriaca 19:32-35.

30. Broadbelt K, Ramprasaad A, Jones LB (2006) Evidence of altered neurogranin immunoreactivity in areas 9 and 32 of schizophrenic prefrontal cortex. Schizophr Res 87: 6-11.

31. Buado B, et al. (2010) A high-fat diet induces lower expression of retinoid receptors and their target genes GAP-43/neurogranin and RC3/neurogranin in the rat brain. Br J Nutr 103:1720-1729.

32. Enderlin V, et al. (1997) Aging decreases the abundance of retinoic acid (RAR) and thyroid hormone receptors in rat brain. Mol Brain Res 42:629-632.

33. Golini RS, et al. (2012) Daily patterns of clock and cognition-related factors are modified in the hippocampus of vitamin A-deficient rats. Hippocampus 22: 546-551.

34. Huang FL, Huang K-P, Wu W, Boucheron C (2006) Environmental enrichment enhances neurogranin expression and hippocampal learning and memory but fails to rescue the impairments of neurogranin null mutant mice. J Neurosci 26:6320-6327.

35. Kovash-Kling WJ, et al. (2003) Increased expression of neurogranin via alterations in thyroid receptor/retinoid X receptor signaling. J Neurochem 121:302-313.

36. McMamara RK, Huot RL, Lenox RH, Plotsky PM (2002) Postnatal maternal separation modifies in the 3′ untranslated region of alpha-CaMKII regulate its dendritic targeting. J Neurosci 19:20729-20737.

37. Miller S, et al. (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron 36:507-519.

38. Heiman M, et al. (2008) A translational profiling approach for the molecular characterization of CNS cell types. Cell 135:738-748.