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Spine-type Specific Recruitment of Newly Synthesized AMPA Receptors with Learning

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Abstract

The stabilization of long-term memories requires de novo protein synthesis. How can proteins, synthesized in the soma, act on specific synapses that participate in a given memory? We studied the dynamics of newly synthesized AMPA-type glutamate receptors (AMPA receptors) induced with learning using transgenic mice expressing the GluR1 subunit fused to green fluorescent protein (GFP-GluR1) under control of the *c-fos* promoter. We found learning-associated recruitment of newly synthesized GFP-GluR1 selectively to mushroom-type spines in adult hippocampal CA1 neurons 24 hours after fear conditioning. Our results are consistent with a “synaptic tagging” model to allow activated synapses to subsequently capture newly synthesized receptor and also demonstrate a critical functional distinction in the mushroom spines with learning.

AMPA receptors are the primary mediators of fast excitatory transmission in the mammalian brain, and play a key role in long-term potentiation (LTP) (1). There is experience-dependent synaptic trafficking of GFP-GluR1 homomers in barrel cortex and lateral amygdala of juvenile rats (12–25 days old) (2,3). These results suggest that changes in the strength of excitatory synapses contribute to learning, possibly through rapid synaptic insertion of pre-existing GluR1-containing AMPA receptors, although it is unclear whether similar mechanisms are present in the adult (4–6). Short-term memories are lost if new protein synthesis is inhibited, indicating a requirement for de novo protein synthesis to consolidate the memories into a long-lasting form (7). This has raised a fundamental question: How do new proteins, synthesized in the soma, exert their effect on specific synapses involved in synaptic or behavioral plasticity? It has been suggested that stimulation produces a “tag” at the activated synapses to allow the capture of newly synthesized plasticity-related proteins at later time points to maintain the increased synaptic strength (8). Synthesis of AMPA receptors is increased after LTP induction (9), suggesting a possible role in this process. To test this might be operating in the adult brain in vivo, we developed transgenic mice to monitor the trafficking and turnover of newly synthesized AMPA receptors induced at the time of learning in a fear conditioning paradigm.

The transgenic mice express a GFP-GluR1 fusion protein in a doxycycline (Dox) regulated (10) and neuronal activity-dependent manner (referred to as GFP-GluR1^{c-fos} Tg mice) (Fig. 1A). We used the activity-dependent *c-fos* promoter (11,12) to induce a rapid and transient expression of GFP-GluR1 in response to environmental stimuli and to focus on the molecular and cellular events specifically in those neurons activated by the behaviorally relevant events.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S4

References

Fear conditioning (13) was used in adult animals to evaluate regulated expression in the dorsal hippocampus. GFP fluorescence, immunohistochemical, and immunoblot analysis revealed that GFP-GluR1 expression was negligible in the presence of Dox, even in fear conditioned mice (Fig. 1B, C, F, G), showing successful transgene suppression by Dox. The GFP expression seen in some of the cell nuclei is derived from a cfos-nlsGFP transgene, which is expressed independently of the Dox-regulated system. To test the inducibility of the GFP- GluR1^{c-fos} Tg by behavioral training, mice were removed from Dox for 4 days and were either fear conditioned or allowed to remain in the homecage. At 24 hours after conditioning, prominent GFP-GluR1 expression was detected in the fear conditioned animals relative to homecage controls (Fig. 1D-G), showing activity-dependent expression of GFP-GluR1 in the absence of Dox. The induced somatic GFP-GluR1 was detected in approximately 25% of CA1 pyramidal neurons within 2 hours of fear conditioning and was maintained for more than 24 hours (Fig. 1F, and fig. S1). One concern is the potential for overexpressed receptors to function aberrantly. In many previous studies of recombinant GluR1 trafficking, the receptor formed exclusively homo-oligomers (2,3,14,15), while the endogenous receptor complex is primarily a heteromer (16). The induced expression of the GFP-GluR1 was approximately 3 % of endogenous GluR1 levels (Fig. 1G). GFP-GluR1 was co-immunoprecipitated with the endogenous GluR2 subunit (Fig. 1H), demonstrating the formation of the natural heteromeric forms. The dendritic transport rate and half-life of GFP-GluR1 were consistent with that determined previously for endogenous receptor in vitro (17-19) (fig. S1, S2).

In contextual fear conditioning, animals learn the association between a specific environment (context) and an aversive stimulus such as a footshock. Brief training produces a stable and long-lasting memory that requires the hippocampus (13). We used fear conditioning in the GFP-GluR1^{c-fos} Tg mice to test for learning-associated synaptic trafficking of newly synthesized AMPARs in the adult hippocampus. Mice were removed from Dox, fear conditioned to elicit a contextual long-term memory, and to induce synthesis of GFP-GluR1 in the activated neurons (FC24h; Fig. 2A). Control mice received either context exposure without foot-shocks (CT24h; Fig. 2A) or unpaired context-shock training, which failed to produce a contextual fear memory (UP24h; Fig. 2A and fig. S3). Mice were returned to their homecage and treated with high Dox (6g/kg mouse chow) to rapidly suppress further expression of GFP-GluR1. Twenty-four hours after training, brain slices were fixed and stained with anti-GFP antibody, without permeabilization, to detect surface GFP-GluR1 expression (fig. S4). A subset of neurons in the CA1 pyramidal cell layer of the dorsal hippocampus was stained with the lipophilic fluorescent dye, DiI, to visualize the entire dendritic arbor and spine distribution on individual neurons (Fig. 2B-D). Consistent with previous studies examining endogenous c-Fos induction (20), all group of mice showed a similar number of GFP-GluR1⁺ CA1 neurons (Fig. 1F). Levels of GFP-GluR1 expression were also similar in the fear-conditioned and context-exposure group (Fig. 2E). This design allows us to induce the receptor at the time of learning, and to follow its distribution to synapses at later time points specifically in behaviorally activated neurons.

Dendritic spines are morphologically diverse but typically categorized into thin, stubby, and mushroom type (21,22) (Fig. 2D). We counted the proportion of surface GFP immuno-positive spines (GFP-GluR1⁺ spines) on apical and basal dendrites in the trained (FC24h) and two control groups (CT24h and UP24h), and analyzed the data separately for each spine type (Fig. 2F). There were no significant differences in GFP-GluR1⁺ thin or stubby spines between groups. In contrast, for mushroom spines, we found a significant increase in the proportion of GFP-GluR1⁺ spines in the fear-conditioned group compared to the context-only and unpaired-shock group. The increased labeling returned to control levels by 72 hours post training (FC72h). While GFP-GluR1 is delivered to approximately 50% of all spines, independent of behavioral contingencies or spine type, the presence of the shock reinforcer in the fear-

conditioned group allows increased capture of receptor specifically in the mushroom-type spines.

Fear memory can be attenuated by extinction training, repeated exposure to an unreinforced conditioned stimulus. Extinction is thought to involve new learning rather than loss of the original memory, although the underlying mechanism remains elusive (23). We tested whether memory extinction alters the distribution of pre-existing GFP-GluR1 in neurons that were activated during the original fear conditioning. GFP-GluR1^{c-fos} Tg mice were withdrawn from Dox, fear conditioned, and returned to their homecage for 2 days on a high Dox diet. Conditioned mice received either 3 trials of extinction training (FC72h+EXT; Fig. 3A) or were left undisturbed in the homecage (FC72h; Fig. 3A). We confirmed a markedly reduced freezing response after extinction training (Fig. 3B). At 72 hours after fear conditioning, brain slices were prepared and the distribution of GFP-GluR1 was analyzed (Fig. 3C). For thin and stubby spines, extinction training did not affect the proportion of GFP-GluR1⁺ spines, while the proportion of GFP-GluR1⁺ mushroom spines was significantly higher in the extinction group than in the control group. A western blot revealed that extinction training on Dox did not induce additional GFP-GluR1 expression (Fig. 3D).

We also tested whether fear conditioning induced changes in spine density, as is seen in some strong learning paradigms (24,25). We analyzed the proportion of each spine type and total spine density in behaviorally activated neurons. There were no significant changes in total spine density (Fig. 4A) or in spine-type distribution (Fig. 4B) in any group.

Our results demonstrate that fear conditioning alters trafficking of newly synthesized AMPARs in the adult hippocampus and that the change is spine-type specific. One of the most remarkable features of dendritic spines is their morphological diversity, with the mushroom spines considered to represent the most mature and stable spine morphology (22,26). At 24-hours following fear conditioning, these spines show an increased ability to incorporate and/or retain newly synthesized AMPARs. The change itself is not persistent and returns to baseline untrained levels by 72 hours. This may represent a subsequent change in receptor composition, for example replacement of GluR1-containing receptors with GluR2/GluR3 receptors (27, 28), or it may indicate a time-limited role for alterations of receptor composition in memory. Surprisingly, extinction training leads to a prolonged elevation in the proportion of GFP-GluR1⁺ mushroom spines, even though it also leads to a reduction in the behavioral expression of the fear memory response. This could represent a redistribution of AMPARs to an alternate subset of synapses associated with extinction learning. Alternately, this could result from the stabilization of GFP-GluR1 in the synapses labeled with fear conditioning. While little is known about the functional importance in the morphologically distinct spine classes in vivo (22,26), the current results demonstrate a unique role for the mushroom spines in learning-associated changes in receptor trafficking and suggest they may be critical sites for behaviorally relevant plasticity. The learning-associated increase in GFP-GluR1 recruitment was detected in ~23% of mushroom spines, representing ~3% of all spines. Such changes are consistent with a sparse subset of synapses contributing to any given memory trace.

What role this receptor trafficking plays in the fear-conditioning paradigm is unclear. The trafficking changes require the same temporal pairing of context with shock required to produce the behavioral memory and likely represent an associative synaptic event. In fear conditioning, the hippocampus is generally thought to encode a representation of the context with the associative changes for fear occurring in the amygdala (13). However, electrophysiological studies indicate that the shock causes a partial remapping of the hippocampal representation (29) and the receptor trafficking changes may alter those synapses that participate in this incorporation of the unconditioned stimulus signal into the hippocampal representation.

LTP-induction is known to increase AMPAR synthesis (9). By coupling expression of GFP-GluR1 to neuronal activity, we specifically examined this pool of newly synthesized receptor. In this paradigm, the earliest possible time point for the delivery of GFP-GluR1 to spines is offset from the behavioral training by several hours (see fig. S1). This suggests that at the time of learning there are changes in some spines that allow the capture of newly synthesized AMPARs at later time points. This type of “synaptic tagging” has been demonstrated to play a role in the maintenance of LTP (8). The current experiments demonstrate a similar mechanism operating during behavioral learning in the adult brain, which may contribute to the stabilization of long-term memory. They also implicate GluR1-containing AMPARs as one of the cargo molecules selectively delivered to tagged synapse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Basic features of GFP-GluR1^{c-fos} Tg mice. **(A)** Schematic representation of the transgenic system. The *c-fos* promoter was used to drive rapid and transient expression of the tetracycline-regulated transactivator (tTA) in activated neurons. The tTA in turn activates transcription of a tetO promoter-linked GFP-GluR1 in a Dox-regulated manner. **(B-E)** Confocal microscopy images of intrinsic GFP fluorescence (upper two panels) and GFP immunoreactivity (lower 3 color panels) in the hippocampal slices. Nuclei were stained with TO-PRO-3 (blue). Or, stratum oriens; Py, stratum pyramidale; Ra, stratum radiatum; Mo, molecular layer; Gr, granule cell layer; DG, dentate gyrus. **(F)** Proportion of CA1 neurons with cytoplasmic GFP immunoreactive signals. Data in this and all subsequent figures are represented as mean $\pm$ s.e.m. ON Dox: $n = 5$ mice for home and FC24h. OFF Dox: $n = 5$ for home; $n = 4$ for FC1h, FC4h, FC24h, CT24h, and UP24h; $n = 3$ for FC4h and FC6h. **(G)** Western blot analysis showing the expression of GFP-GluR1 and endogenous GluR1 in the hippocampus. **(H)** Co-immunoprecipitation of GFP-GluR1 and endogenous GluR2 in the hippocampus.

Fig. 2. Preferential recruitment of newly synthesized GFP-GluR1 to mushroom spines 24 hours after fear-conditioned learning. **(A)** Experimental design. **(B)** Confocal image of a hippocampal slice labeled with anti-GFP antibody (green) and DiI (red). **(C)** Confocal image showing surface GFP-GluR1 localization on apical dendrite after fear conditioning. Some spines were GFP positive (arrow heads) but some were negative, showing an uneven synaptic trafficking of newly synthesized GFP-GluR1. **(D)** Representative images of spine morphology and surface GFP-GluR1 localization. Scale bar, 1 μ m. **(E)** Relative immunoreactivity from western blot analysis (CT24h: $n = 4$, FC24h: $n = 3$). **(F)** The percentage of GFP-GluR1⁺ mushroom spines was significantly higher (apical: $F_{2,16} = 8.86$, $p = 0.0026$, basal: $F_{2,16} = 15.43$, $p = 0.0002$) in

FC24h group (apical: $n = 7$ mice, 1927 spines, basal: $n = 7$ mice, 1295 spines) as compared to the CT24h group (apical: $n = 6$ mice, 1626 spines, basal: $n = 6$ mice, 1292 spines) and UP24h group (apical: $n = 6$ mice, 2240 spines, basal: $n = 6$ mice, 1974 spines). The proportion was significantly decreased (apical: $p = 0.0056$, basal: $p = 0.0010$, t -test) at 72 hours after conditioning (apical: $n = 6$ mice, 1233 spines, basal: $n = 6$ mice, 1138 spines). $*p < 0.01$.

Fig. 3. Distribution of pre-existing GFP-GluR1 following memory extinction training. **(A)** Experimental design. **(B)** The percentage of time spent freezing before fear conditioning (basal), after fear conditioning (after FC), and during three trials of extinction training (EXT1~3). $n = 7$. **(C)** The proportion of GFP-GluR1⁺ mushroom spines was significantly higher (apical: $p = 0.045$, basal: $p = 0.033$, t -test) in the extinction trained group (apical: $n = 7$ mice, 1870 spines, basal: $n = 6$ mice, 1845 spines) compared to the control group (FC72h, the same data as in Fig. 2F). **(D)** Relative immunoreactivity from western blot analysis ($n = 3$ for each condition).

Fig. 4.

Spine remodeling following behavioral trainings. **(A)** No significant changes were observed in spine density (apical: $F_{4,27} = 0.72$, $p = 0.58$, basal: $F_{4,27} = 0.88$, $p = 0.49$). $n = 6$ mice for UP24h, CT24h, and FC72h. $n = 7$ for FC24h and FC72h+EXT. **(B)** No significant changes were observed in spine-type distribution. $n = 6$ mice for UP24h, CT24h, and FC72h. $n = 7$ for FC24h and FC72h+EXT.