GKAP orchestrates activity-dependent postsynaptic protein remodeling and homeostatic scaling

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How does chronic activity modulation lead to global remodeling of proteins at synapses and synaptic scaling? Here we report that guanylate kinase–associated protein (GKAP; also known as SAPAP), a scaffolding molecule linking NMDA receptor–PSD-95 to Shank-Homer complexes, acts in these processes. Overexcitation removes GKAP from synapses via the ubiquitin-proteasome system, whereas inactivity induces synaptic accumulation of GKAP in rat hippocampal neurons. Bidirectional changes in synaptic GKAP amounts are controlled by specific CaMKII isoforms coupled to different Ca²⁺ channels. CaMKIIα activated by the NMDA receptor phosphorylates GKAP Ser54 to induce polyubiquitination of GKAP. In contrast, CaMKIβ activation via L-type voltage-dependent calcium channels promotes GKAP recruitment by phosphorylating GKAP Ser340 and Ser384, which uncouples GKAP from myosin Va motor complex. Overexpressing GKAP turnover mutants not only hampers activity-dependent remodeling of PSD-95 and Shank but also blocks bidirectional synaptic scaling. Therefore, activity-dependent turnover of PSD proteins orchestrated by GKAP is critical for homeostatic plasticity.

Synapses are continuously modified by use and experience. This synaptic plasticity is believed to be a basis of information storage in the brain. Hebbian plasticity, such as long-term potentiation (LTP) and long-term depression, is relatively rapid and synapse-specific, and is regulated by positive-feedback mechanisms. LTP and long-term depression are mediated mainly by the trafficking of AMPA receptors (AMPARs) into and out of stimulated synapses¹. In contrast, homeostatic plasticity involves the global modification of synapses and operates over longer timescales. Homeostatic plasticity provides a global negative feedback and is crucial for stabilizing neuronal network function². Synaptic scaling is one form of homeostatic plasticity that occurs at excitatory neurons, and it results in the adjustment of the strength of all excitatory synapses up or down via modification of AMPAR amounts². Several molecules, including BDNF, CaMKII, Arc, Plk2, TNF-α, all-trans retinoic acid and mGlur-Homer1α, have been identified to be involved in synaptic scaling (reviewed in refs. 2, 3). However, the detailed signaling pathways and molecular biochemical changes at synapses associated with homeostatic synaptic scaling still need to be established²,³.

Activity-dependent protein turnover at the synapses by the ubiquitin-proteasome system (UPS) has emerged as a mechanism associated with the long-term global modification of synapses⁴. The activity-dependent changes in postsynaptic density (PSD) components occur in an ensemble fashion, with specific groups of PSD proteins accumulating or declining with similar kinetics and magnitudes. Such coordinated regulation could be explained if the UPS targets a few ‘master organizing molecules’ in the PSD that are important for recruiting other PSD components⁴. The identity of these master organizing molecules is unknown, but good candidates are two scaffold proteins, GKAP and Shank family proteins, that are among the most highly polyubiquitinated proteins in the PSD⁴. The biochemical changes at synapses accompanied by chronic activity modulation, especially those regulated by the UPS, are a potential molecular mechanism for homeostatic plasticity.

GKAP refers to a family of four scaffold proteins initially identified by their interaction with the guanylate kinase domain of PSD-95 (hence named guanylate kinase–associated protein or SAP90/PSD-95–associated protein (SAPAP) and hDLG-associated protein (DAP))⁵–⁷. In addition to interacting with PSD-95 family proteins, GKAP directly binds to other proteins including Shank⁸ and 8-kDa dynein light chain (DLC)⁹. GKAP family proteins are some of the most abundant postsynaptic scaffolding proteins in the PSDs¹⁰, and they have been shown to be essential for the recruitment and accumulation of Shank at excitatory synapses¹¹. Therefore, synaptic GKAP might contribute to synaptogenesis and dendritic spine morphogenesis by mutually reinforcing the Shank-Homer complex¹¹,¹².

CaMKII is a multifunctional protein kinase, highly enriched in the PSDs, and has a central role in synaptic plasticity, learning and memory¹³,¹⁴. Four CaMKII isoforms are expressed in mammals; CaMKIIα, CaMKIIβ, CaMKIIδ and CaMKIIγ. CaMKIIα and CaMKIIβ are predominant isoforms in the brain. They have similar, broad substrate

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specificity in vitro. However, accumulating evidence indicates that CaMKII isoforms also have isosrm-specific functions. CaMKIIα is critical for hippocampal LTP and serves as a scaffold for recruitment of proteasomes to dendritic spines. In contrast, CaMKIIβ is important for neurite extension, for maintenance of dendritic spine structure, for dendritic patterning through centrosome regulation and for proper synaptic targeting of CaMKIIα. They are also differentially regulated by excitatory activity: chronic elevated excitatory activity increased the CaMKIIα/CaMKIIβ ratio, whereas inactivity decreased the CaMKIIα/CaMKIIβ ratio. Furthermore, the general CaMKII inhibitor KN-93 or knockdown of CaMKIIβ prevented the changes in AMPAR miniature excitatory postsynaptic current (mEPSC) by activity blockade. These results suggest that CaMKII is involved in the expression of synaptic scaling. However, the regulatory targets of CaMKIIα and CaMKIIβ isoforms mediating homeostatic plasticity remain unclear.

Here we report that the bidirectional regulation of GKAP amounts at synapses is controlled by differential phosphorylation of GKAP by different CaMKII isoforms, which are activated by Ca2+ entry through different channels. Furthermore, the turnover of GKAP at synapses is required for the normal activity-dependent remodeling of PSD protein composition as well as homeostatic synaptic scaling.

RESULTS
CaMKII controls activity-dependent GKAP turnover at synapses
Chronic activity modulation of cultured hippocampal neurons induces bidirectional and reversible changes in the protein composition of PSDs. GKAP was one of major scaffolding proteins that showed an activity-dependent turnover at PSDs. Because CaMKII is regulated by activity and has a role in the activity-dependent recruitment of proteasomes, we tested whether CaMKII is involved in the regulation of GKAP. Blocking CaMKII activity with KN-93, but not with inactive analog KN-92, abolished not only the tetrodotoxin (TTX)-dependent accumulation of GKAP and PSD-95 but also the bicuculline (Bic)-induced depletion of GKAP and PSD-95 at synaptic sites (Fig. 1). KN-93 or KN-92 alone had no substantial effects on the synaptic levels of GKAP and PSD-95 (Fig. 1c–e). Thus, CaMKII activity controls both activity-dependent accumulation and removal of GKAP and PSD-95 at synapses.

How does CaMKII activity regulate both recruitment and accumulation as well as removal of GKAP at synapses, which are seemingly opposed processes? As CaMKII requires Ca2+ for activation, we addressed whether the sources of the Ca2+ influx are different, by blocking two major neuronal Ca2+ channels, NMDA receptors and L-type voltage-dependent Ca2+ channels (L-VDCCs). The NMDA receptor antagonist AP5 completely abolished Bic-induced GKAP and PSD-95 removal but had no effect on TTX-dependent accumulation of GKAP and PSD-95 at synapses (Fig. 1f,g). In contrast, the L-VDCC antagonist nimodipine (Nimo) prevented the TTX-induced accumulation of GKAP and PSD-95 but had no effect on Bic-induced depletion of GKAP and PSD-95. Nimo alone did not affect the basal number of GKAP and PSD-95 puncta substantially. Thus, CaMKII activation through the NMDA receptor promotes removal of GKAP and PSD-95 from synapses, whereas CaMKII activation mediated by L-VDCC increases the abundance of these proteins at synapses.

Figure 1 CaMKII activity is required for both Bic-induced removal and TTX-dependent accumulation of GKAP and PSD-95 at synapses. (a,b) Immunofluorescence images of GKAP and PSD-95 24 h after treatment with either TTX (2 µM) alone, TTX + KN-93 (10 µM), or TTX + KN-92 (10 µM) (a), or Bic (40 µM) alone, Bic + KN-93 (10 µM) or Bic + KN-92 (10 µM) (b). (c) Effect of KN-93 or KN-92 alone on GKAP and PSD-95 clusters. (d,e) Quantification of GKAP and PSD-95 cluster density (d) and intensities (e) 24 h after the indicated treatments. (f) Immunofluorescence images showing the effect of AP5 (100 µM) and Nimo (5 µM) on Bic- or TTX-induced changes of GKAP and PSD-95 clusters at synapses. (g) Quantification of the effect of the Ca2+ channel blockers on the density of GKAP and PSD-95 clusters. Error bars (d,e,g), s.e.m. n ≥ 20 neurons per condition. ***P < 0.001. Scale bars, 5 µm.
**Figure 2** CaMKII isoform-specific regulation of activity-dependent GkAP turnover. (a) Immunofluorescence images of cultured hippocampal culture neurons (14 d in vitro (DIV)) subjected to CaMKIα RNAi (α RNAi) or CaMKIIβ RNAi (β RNAi) and 1 d later treated with either Bic (40 µM) or TTX (2 µM) for 24 h and examined for the changes in GkAP clusters (red) by immunofluorescence staining. Transfected neurons were identified by β-galactosidase (β-Gal) immunofluorescence (green). (b) Quantification of the effect of α RNAi and β RNAi on activity-dependent turnover of endogenous GkAP, measured by the changes in the cluster density from nontransfected neighboring neurons (Non-bf) and neurons subjected to RNAi. (285–542), truncated CaMKIIβ containing only the actin-association domain (residues 285–542), n > 20 for each construct. (c) Immunofluorescence images showing the effect of a chimeric CaMKIIα with actin association domain of CaMKIIβ (α-AD) on the TTX-induced changes in GkAP. Schematics of the α-AD are shown at the top of the representative images. Hippocampal neurons were subjected to β RNAi + CaMKIIβ (wild-type; WT) or β RNAi + α-AD, treated with TTX for 24 h, and immunostained for the CaMKII (green) and GkAP (red). (d) Quantification of β RNAi rescue by α-AD on GkAP cluster density. α-WT, CaMKIIα; n > 20 for each construct. (e) Activation of CaMKII isoforms in hippocampal neurons that were untreated (C) or treated with 40 µM Bic (B) or 2 µM TTX (T) for 12 h or 24 h. Total protein extracts were examined for protein levels of autophosphorylated-CaMKII (p-CaMKII) (p-CaMKIIα) or individual CaMKII isoforms (CaMKIIα, CaMKIIβ, CaMKIIβ). Full-length blots are presented in Supplementary Figure 1. (f) Quantification of relative phospho-CaMKIIα and phospho-CaMKIIβ amounts (n = 3). Scale bars, 5 µm. All error bars, s.e.m. ***P < 0.001.

**CaMKII isoform-specific regulation of GkAP turnover**

To study the molecular basis of CaMKII-dependent bidirectional control of GkAP, we analyzed the roles of CaMKIIα versus CaMKIIβ, which are highly expressed in neurons. We specifically suppressed expression of CaMKIIα or CaMKIIβ by RNA interference (RNAi), using plasmid-based constructs expressing small hairpin RNAs (shRNAs), whose specificity and efficacy have been demonstrated (Supplementary Fig. 1a)16,18.

RNAi-mediated knockdown of CaMKIIα (CaMKIIα-shRNAi) did not affect amounts of synaptic GkAP substantially under normal growth conditions (control; Fig. 2a,b and Supplementary Fig. 1b,d) but completely blocked Bic-induced decrease in the amount of synaptic GkAP (Fig. 2a,b and Supplementary Fig. 1c,d). In contrast, CaMKIIβ RNAi had no effect on the increase in GkAP amount upon treatment with TTX. Overexpression of CaMKIIβ in cells subjected to CaMKIIα RNAi did not restore the Bic-induced loss of synaptic GkAP (Supplementary Fig. 1b–d). These results indicate that CaMKIIα is specifically required for the Bic-stimulated removal of GkAP from synapses. Unlike CaMKIIα RNAi, RNAi-mediated knockdown of CaMKIIβ (CaMKIIβ RNAi) significantly reduced the number of GkAP clusters (P < 0.001) to less than 40% of the amount for the control grown in untreated normal growth conditions (Fig. 2a,b). This could reflect the requirement of CaMKIIβ for GkAP accumulation in synapses or might be secondary to the loss of dendritic spines, as CaMKIIβ is implicated in synapse formation and the maintenance of dendritic spines17,18. Nonetheless, neurons subjected to CaMKIIβ RNAi still exhibited further reduction in the number of GkAP clusters (P < 0.001) after Bic treatment. However, CaMKIIβ RNAi completely blocked the increase in the amount of GkAP clusters stimulated by addition of TTX (Fig. 2a,b). Thus, CaMKIIβ is required for the increase of synaptic GkAP by TTX. To corroborate the finding, we subjected cells to CaMKIIβ RNAi and also expressed the actin-association domain of CaMKIIβ (residues 285–542) which partly rescued the CaMKIIβ RNAi-mediated loss of dendritic spines18. However, these neurons still did not exhibit a TTX-induced increase in the amount of GkAP clusters (Fig. 2b). To test the role of actin association in the differential function of CaMKII isoforms, we generated a chimeric CaMKIIα with the actin association domain grafted from CaMKIIβ (designated CaMKIIα-AD; Fig. 2c). When we transfected a vector encoding CaMKIIα-AD into neurons subjected to CaMKIIβ RNAi, the TTX-dependent increase in GkAP at synapses was restored (Fig. 2c,d and Supplementary Fig. 1e). In contrast, expression of wild-type CaMKIIα in neurons subjected to CaMKIIβ RNAi did not rescue the defects caused by knockdown of CaMKIIβ (Fig. 2d). Expression of RNAi-resistant versions of genes encoding respective CaMKII isoforms in cells subjected to RNAi restored the normal activity-dependent turnover of endogenous GkAP (Supplementary Fig. 1a,f). Thus, these data indicate that the synaptic accumulation of GkAP during inactivity requires CaMKIIβ activity, and the actin-association activity of CaMKIIβ is important for the process.
ubiquitination assays. When we incubated purified PSD-95
presented in 10 neurons (18 DIV) treated for 24 h with either DMSO (control), TTX (2 µM), Bic (40 µM), APS (100 µM), CNQX (50 µM), MG132 (50 µM), lactacystin (Lactacys, 10 µM), Bic + KN-93 (10 µM), or Bic + KN-92 (10 µM). Full-length blots are presented in Supplementary Figure 13. (e) Quantification of total protein changes resulting from the indicated treatments (n = 3). (d) Immunoblots with anti-GST and anti-GKAP antibodies of recombinant GST-GKAP (200 ng) subjected to in vitro ubiquitination in the presence of either Ca²⁺ (+) or EGTA (−), and without (−P2) or with synaptosomal fraction (+P2) as a source of ubiquitin ligase and CaMKII. No rxn, GST-GKAP without the in vitro ubiquitination reaction. (e) Immunofluorescence images of neurons treated with either Bic alone, MG132 alone, lactacystin alone, Bic + MG132, or Bic + lactacystin for 24 h, and examined for the localization of GKAP, PSD-95 and PSD-93 by immunocytochemistry. Bottom images show the formation of GKAP aggregates in soma after Bic + MG132 treatment. (f) Quantification of cluster densities of GKAP, PSD-95 and PSD-93 24 h after the indicated treatments. n ≥ 25 neurons per condition. ***P < 0.001. Scale bars, 5 µm. Error bars, s.e.m.

We next examined the effect of Bic and TTX on the activation of the two CaMKII isoforms in hippocampal neurons. Bic increased the activation of both CaMKIIα and CaMKIIβ by more than sixfold compared to untreated control condition (Fig. 2e), which we determined by phospho-CaMKII antibody that recognizes autophosphorylation of both isoforms (CaMKIIα phospho- (p)Thr286 and CaMKIIβ pThr287). In contrast, suppressing activity of hippocampal neurons by TTX increased the autophosphorylation of CaMKIIα by more than threefold after 12 h without substantial changes in phosphorylated CaMKIIα amounts (Fig. 2f). After a 24-h TTX treatment, CaMKIIβ again exhibited more than threefold greater autophosphorylation than CaMKIIα did. Neither Bic nor TTX substantially changed the total amount of either CaMKII isoform. These results indicate that CaMKIIβ is a dominantly active form of CaMKII isoforms during chronic inactivity.

CaMKII activity promotes GKAP degradation by the UPS

We found that increasing activity removed GKAP from synapses (Fig. 1b,d,e), which is likely due to degradation of GKAP by the UPS. Bic treatment (24 h) substantially decreased total GKAP amount to ~20% of that in the control (Fig. 3a). Incubation with proteasome inhibitors, MG132 or lactacystin (Lactacys) increased total GKAP amount (>180%), similarly to TTX treatment. Total GKAP amount increased to ~150% of baseline after 24 h TTX treatment as well as after AP5 or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) treatments. In contrast, total GluR2 and GluR3 protein amounts were not substantially affected by any of these treatments (Fig. 3a). Incubation with KN-93, but not KN-92, prevented the Bic-induced decrease in GKAP amounts (Fig. 3b,c), indicating the involvement of CaMKII in GKAP degradation.

Although these data suggest that GKAP undergoes UPS-dependent degradation, it is unclear whether GKAP is directly ubiquitinated in a CaMKII-regulated manner. To address this, we performed in vitro ubiquitination assays. When we incubated purified recombinant glutathione-S transferase fused to full-length GKAP (GST-GKAP) with ubiquitin and P2 fraction (synaptosome-enriched fraction) of rat forebrain as a source of E2 and E3 enzymes (Fig. 3d), electrophoresis revealed the appearance multiple
GST-GKAP protein bands of higher molecular weights and a decrease in the amount of GST-GKAP. We did not observe the shift to higher molecular weight in the absence of Ca$^{2+}$ ion and without the addition of the P2 fraction. The molecular-weight shifts were inhibited by the substitution of normal ubiquitin with a triple lysine mutant of ubiquitin (3KR-Ub), which cannot catalyze polyubiquitination chain elongation. This result indicates that the higher-molecular-weight GST-GKAP bands represent bona fide polyubiquitinated GKAP. Finally, blocking CaMKII activity in the P2 fraction by KN-93 addition of Ca$^{2+}$ and CaM stimulated phosphorylation of multiple bands in the PSD fraction (Fig. 3e). The stimulation of endogenous CaMKII activity present in the PSD. We examined phosphorylation of GKAP, Shank and NR2B by subsequent immunoprecipitation of these proteins under denaturing conditions to minimize coprecipitation of interacting proteins. Addition of Ca$^{2+}$ and CaM stimulated phosphorylation of multiple bands in the PSD fraction (Fig. 4a). The stimulation of endogenous CaMKII activity strongly increased incorporation of $[^{32}P]$ATP into GKAP and Shank, as well as into the known CaMKII substrate NR2B28. These data show that GKAP can be phosphorylated by CaMKII in the PSD.

GKAP is a substrate of CaMKII

To determine whether GKAP phosphorylated by CaMKII directly, we first performed the in situ phosphorylation assay of PSDs26, in which we incubated purified PSDs with $[^{32}P]$ATP under conditions that either promote (by adding Ca$^{2+}$ and CaM) or suppress (by adding EGTA) endogenous CaMKII activity present in the PSD. We examined phosphorylation of GKAP, Shank and NR2B by subsequent immunoprecipitation of these proteins under denaturing conditions to minimize coprecipitation of interacting proteins. Addition of Ca$^{2+}$ and CaM stimulated phosphorylation of multiple bands in the PSD fraction (Fig. 4a). The stimulation of endogenous CaMKII activity strongly increased incorporation of $[^{32}P]$ATP into GKAP and Shank, as well as into the known CaMKII substrate NR2B28. These data show that GKAP can be phosphorylated by CaMKII in the PSD.

To determine whether CaMKII phosphorylates GKAP directly, we performed in vitro phosphorylation of purified GST-fusion proteins of various domains of GKAP using purified rat brain CaMKII (Fig. 4b); for domain structure of GKAP, see Fig. 4c). The repeat regions of GKAP that bound to PSD-95 (R1–R2, R1–R3 and R3–R5), as well as the DLC-binding domain of GKAP (DLC-BD), were directly phosphorylated by CaMKII in a Ca$^{2+}$ and CaM–dependent manner. The guanylate kinase domain of PSD-95 that binds to GKAP was not.
Supplementary Fig. 2b

CaMKII is required for the disruption of the GKAP–PSD-95 complex.

In 95% of cells; had no effect on coclustering (cluster formation was observed α contrast, coexpression of kinase-dead mutant of CaMKII mutant (cluster formation was observed in 60% of cells; α extent than that observed with expression of the CaMKII α cluster formation was observed in only 8% cells); in these cells, distri- sition of a constitutively active form of CaMKII staining without any cluster formation (data not shown). Formation of (ref. 5) (cluster formation was observed in 95% cells examined; 1660 Fig. 4 d overexpressed together in monkey fibroblast-like cell line, COS-7 interaction with PSD-95 (ref. 5), we hypothesized that CaMKII phos- phorylation of this region (Fig. 4b) would disrupt the GKAP−PSD-95 domain was greatly diminished (Fig. 4b), GKAP interaction with PSD-95 guanylate kinase domain of PSD-95 (Supplementary Fig. 2a,b). However, when we mutated both Ser54 and Ser201 to alanine (S54A,S201A) or aspartic acid (S54D,S201D), GKAP interaction with PSD-95 guanylate kinase domain was greatly diminished (Supplementary Fig. 2b). Double mutations of Ser54 and Ser83 had no effect on PSD-95 interaction, confirming the specificity (Supplementary Fig. 2b). In contrast, none of these mutations affected GKAP interaction with DLC2. Furthermore, the S54D,S201D double mutant did not cluster with PSD-95 in COS cells (cluster formation was observed in < 1% of cells; Fig. 4d). These results suggest that Ser54 and Ser201 have a critical role in GKAP interaction with PSD-95 and phosphorylation of these residues impairs PSD-95 binding.

This conclusion was supported by coimmunoprecipitation experiments (Fig. 4e). The amount of GKAP S54A,S201A and S54D,S201D mutants precipitating with Myc–PSD-95 was greatly reduced compared to that for wild-type GKAP (Fig. 4e). In contrast, the amount phosphorylated, showing the specificity of the reaction. These results suggest that GKAP is a substrate of CaMKII in the PSD.

CaMKII phosphorylation attenuates GKAP binding to PSD-95

Because the N-terminal repeat region of GKAP mediates direct interaction with PSD-95 (ref. 5), we hypothesized that CaMKII phos- phorylation of this region (Fig. 4b) would disrupt the GKAP–PSD-95 interaction. GKAP and PSD-95 formed co-clusters when overexpressed together in monkey fibroblast-like cell line, COS-7 (ref. 5) (cluster formation was observed in 95% cells examined; Fig. 4d). When expressed alone, GKAP and PSD-95 exhibited diffuse staining without any cluster formation (data not shown). Formation of GKAP–PSD-95 clusters was almost completely prevented by expression of a constitutively active form of CaMKIIα (T286D mutant; cluster formation was observed in only 8% cells); in these cells, distribution of GKAP and PSD-95 was largely diffuse (Fig. 4d). Expression of wild-type CaMKIIα also inhibited coclustering, though to a lesser extent than that observed with expression of the CaMKIIα T286D mutant (cluster formation was observed in 60% of cells; Fig. 4d). In contrast, coexpression of kinase-dead mutant of CaMKIIα (K42R) had no effect on coclustering (cluster formation was observed in 95% of cells; Fig. 4d), indicating that protein kinase activity of CaMKII is required for the disruption of the GKAP–PSD-95 complex. Thus, CaMKIIα activity can regulate the association of GKAP and PSD-95 in cells.
Figure 6 Role of MVa-DLC and CaMKII phosphorylation in GKAP accumulation at synapses. (a) Representative images of cultured hippocampal neurons (14 DIV) subjected to Mva RNAi and examined for endogenous GKAP by immunofluorescence staining. β-Gal was used as a transfection marker. (b) Quantification of GKAP cluster density after Mva RNAi. Control was Zn-T3 RNAi. ∆∆∆P < 0.001 (n = 20 per group). (c) Effect of Mva RNAi on the activity-dependent changes in GKAP. Arrowheads in a and c indicate GKAP clusters from nontransfected neighboring neurons. (d) Quantification of activity-dependent changes of GKAP cluster density (left) and intensity (right) in neurons subjected to Mva RNAi. n > 20 per group. ∆∆∆P < 0.001, **P < 0.01, n.s., not significant. (e) Immunoblots showing the effect of CaMKII-phosphorylation site mutations in the DLC-binding domain of GKAP on the interaction of GKAP with DLC. Myc-DLC2 was coexpressed in COS cells with either HA-tagged wild-type (WT), S340A,S384A, S340D,S384D or ADLC BD. DLC2 was immunoprecipitated (IP) with anti-HA antibody and the amounts of co-precipitating GKAP were determined by anti-HA blotting. Fulllength blots are presented in Supplementary Figure 13. (f) Immunofluorescence images of neurons expressing either HA-tagged WT, S340A,S384A or S340D,S384D and treated with Bic or TTX for 36 h. Representative images (HA staining) of GKAP S340A,S384A and S340D,S384D compared to WT GKAP. (g) Quantification of activity-dependent changes of S340A,S384A and S340D,S384D, measured by HA staining. n > 20 neurons per condition. ∆∆∆P < 0.001, **P < 0.01, n.s., not significant. Scale bars, 5 μm. Error bars, s.e.m.

Phosphorylation of Ser54 is critical for GKAP ubiquitination

To determine CaMKII phosphorylation site(s) in GKAP that are critical for induction of polyubiquitination, we tested various GKAP mutants for in vitro ubiquitination. The GKAP S54A mutant was not polyubiquitinated regardless of the presence of Ca2+ (Fig. 5a). Deletion of the entire R1 that contains Ser54 (mutant designated ΔR1) also did not exhibit polyubiquitination. In contrast, the phosphomimic GKAP S54D mutant showed strong polyubiquitination, even in the absence of Ca2+ (Fig. 5a). The polyubiquitination of the GKAP S54D mutant was effectively blocked by 3KR-Ub but was not affected by the presence of CaMKII inhibitors, KN-93 or autocamtide-2 related inhibitory peptide (Fig. 5b). Therefore, polyubiquitination of the GKAP S54D mutant does not require Ca2+ and CaMKII activity, indicating that phosphorylation of Ser54 is necessary and sufficient to induce polyubiquitination of GKAP. Phosphomimic or phosphorylation-defective mutations in the DLC-BD (S340D,S384D and S340A,S384A double mutants) exhibited normal Ca2+-dependent polyubiquitination (Fig. 5c), confirming the specificity of S54D mutation for the polyubiquitination of GKAP.

To confirm that the phosphorylation of Ser54 is involved in the Bic-dependent polyubiquitination of GKAP in neurons, we immunoprecipitated GKAP under the denaturing conditions and probed for the presence of phosphorylated Ser54 (pSer54) using an antibody that showed >100-fold specificity to the pSer54-containing peptide over the nonphosphorylated peptide (Supplementary Fig. 3a).

Staining with the anti-pSer54 antibody exhibited multiple immunoreactive GKAP bands of higher molecular weights (resembling the pattern of polyubiquinated GKAP) only in samples immunoprecipitated from Bic-treated neurons (Bic alone and Bic plus MG132), and not from samples treated with TTX, TTX plus MG132 or MG132 alone (Supplementary Fig. 3b). These results demonstrate that chronic Bic treatment promotes specific phosphorylation of Ser54 and the polyubiquitination of GKAP in hippocampal neurons. In addition, these results predicted that the S54A mutant would show a defect in Bic-induced decrease in GKAP amounts. Indeed, when expressed in neurons, GKAP S54A did not show Bic-dependent reduction (Fig. 5d–f). In contrast, GKAP S54A clearly showed normal activity-dependent turnover similar to that of endogenous GKAP. Collectively, these results highlight the importance of Ser54 phosphorylation in polyubiquitination and activity-dependent degradation of GKAP.

Myosin Va transports GKAP to synapses

To unravel the molecular mechanisms behind the stabilization and recruitment of GKAP during inactivity, we examined the importance of GKAP association with an actin-based motor protein myosin Va (Mvα), which has been proposed as a potential mechanism for the transport of GKAP to synapses. To test this possibility, we first addressed the importance of GKAP interaction with the DLC, which provides a bridge to Mvα. DLC forms a dimer and binds to various targets via two conserved amino sequences, (K/R)xTQT or GIQVD (Supplementary Fig. 4a).Peptides based on the two sequences bind the same binding pocket in the DLC and prevent DLC interactions. To prevent DLC...
interaction with GKAP, we used a synthetic peptide that has a DLC-binding consensus KETQT sequence fused to the antennapedia peptide sequence, which facilitates neuronal uptake of the peptide (designated Antp-KETQT). Incubation of neurons with Antp-KETQT peptide for 18 h greatly reduced GKAP puncta density to less than 25% of control amount (Supplementary Fig. 4b,d). In contrast, the numbers of NR1 and synaptophysin puncta were not substantially affected by Antp-KETQT, indicating that synapse numbers were not changed. Control Antp-Kv1.4 peptide (Antp fused to the C-terminal sequence of Kv1.4) did not affect the cluster numbers of all examined proteins. Both Antp peptides did not change total protein levels of GKAP, PSD-95 and GluR2 (Supplementary Fig. 4e), indicating that the reduction in GKAP cluster numbers was not due to the reduction of total GKAP amount. To corroborate the data, we used a mutant GKAP that has a deletion in DLC-BD (designated ΔDLC-BD). The ΔDLC-BD mutant was largely confined to the dendritic shaft, indicating that synaptic targeting of ΔDLC-BD mutant was severely impaired (Supplementary Fig. 4f). Taken together, these results suggest that DLC interaction is required for the proper targeting of GKAP to synapses.

We next tested the role of MVa in GKAP transport to synapse by using RNAi-mediated knockdown of MVa (Mva RNAi). In COS cells, Mva RNAi specifically reduced MVa amounts but did not affect PSD-95 or GKAP (Supplementary Fig. 5a). When introduced into neurons, Mva RNAi reduced Mva staining intensities to >50% in soma and >85% in dendrites compared to nontransfected neighboring neurons (Supplementary Fig. 5b,c), showing effective knockdown of endogenous Mva. In neurons, Mva RNAi significantly (P < 0.001) reduced the number of GKAP clusters (Fig. 6a,b) and increased GKAP staining in the soma (Supplementary Fig. 5d,e). Mva knockdown completely abolished the TTX-induced increase of GKAP amounts at synapses, while it exhibited no effect on Bic-dependent reduction of synaptic GKAP clusters (Fig. 6c,d). These results strongly suggest that GKAP is actively recruited to synapses via MVa motor proteins.

As the Mva-DLC interaction is important for GKAP transport to synapses, we next examined the role of CaMKII in this process. The DLC-BD of GKAP contained two potential CaMKII phosphorylation sites (Ser340 and Ser384; Fig. 4c), which may regulate GKAP-DLC interaction. We tested this possibility by coimmunoprecipitation experiments after expressing the phosphomimic (S340D,S384D) and phosphorylation-defective (S340A,S384A) GKAP double mutants with DLC in COS cells. Both wild-type GKAP and GKAP S340A,S384A mutant immunoprecipitated with DLC (Fig. 6e). In contrast, GKAP S340D,S384D and GKAP ΔDLC-BD mutants did not immunoprecipitate with DLC, suggesting that CaMKII phosphorylation of Ser340 and Ser384 prevents the interaction of GKAP with DLC. Thus, in terms of DLC interaction, GKAP S340A,S384A and GKAP S340D,S384D are dissociation and association mutants, respectively. When GKAP S340A,S384A and GKAP S340D,S384D mutants were expressed in neurons, they showed severely impaired synaptic targeting, as evidenced by a large reduction in their
GKAP turnover is critical for bidirectional homeostatic synaptic scaling.

(a) Hippocampal neurons (10 DIV) were transfected with plasmids encoding HA-tagged wild-type (WT) GKAP or GKAP ∆R1 (sequences subcloned in pIRE2-EGFP). One day after transfection, neurons were treated with either Bic (40 μM) or TTX (2 μM), and incubated further 48 h before patch-clamp recording. Representative recording traces from hippocampal neurons transfected with plasmids encoding wild-type GKAP WT or ∆R1 in control, TTX and Bic condition. (b) Average mEPSC frequency of each group. ** P < 0.01 (t-test), n > 12 per condition. (c) Cumulative probability (CP) distribution of mEPSC amplitudes from all events in wild-type GKAP and GKAP ∆R1-expressing neurons. WT; n = 1,486, 1,111, and 1,578 for control, Bic, and TTX, respectively. ∆R1; n = 1,276, 1,023, and 1,438 for control, Bic, and TTX, respectively. P < 0.001 (Kolmogorov-Smirnov test) for WT. (d) Average mEPSC frequency of each group. (e) Effect of GKAP RNAi on average mEPSC frequency. ** P < 0.01, n > 15 per condition. Error bars, s.e.m.

GKAP turnover is required for homeostatic synaptic scaling

To examine the importance of GKAP turnover in other synaptic protein turnover and homeostatic synaptic scaling, we used a GKAP turnover mutant that is defective in the bidirectional changes (∆R1). When transfected into neurons, GKAP ∆R1 showed synaptic targeting similar to that of the transfected wild-type GKAP (Fig. 7a,b and Supplementary Fig. 6a). Also, total expression of ∆R1 was very similar to that of wild-type GKAP (data not shown). However, neither Bic nor TTX treatment substantially changed the amount of ∆R1 at synapses (Fig. 7a,b). Therefore, GKAP ∆R1 is defective in bidirectional activity-dependent turnover at synapses.

To address the importance of GKAP turnover in activity-dependent remodeling of PSD proteins, we overexpressed GKAP ∆R1 in hippocampal neurons and examined its effect on two key scaffold proteins in the PSD, PSD-95 and Shank. We used wild-type GKAP as control because it showed normal activity-dependent turnover (Fig. 7a,b). Neurons overexpressing wild-type GKAP showed Bic- and TTX-dependent changes in amounts of PSD-95 and Shank at synapses, which were indistinguishable from the amounts in neighboring nontransfected neurons (Fig. 7a,c,d and Supplementary Fig. 6b). In contrast, both Bic- and TTX-driven changes in synaptic PSD-95 and Shank were abolished in neurons expressing the GKAP ∆R1 mutant (Fig. 7a,c,d). In addition, the GKAP ∆R1 mutant also blocked phosphorylation of Ser295 of PSD-95 (Fig. 7e,f), which has been shown to be important for the synaptic accumulation of PSD-95 during inactivity31. Corroborating this conclusion, overexpression of the degradation mutant, S54A, specifically blocked the Bic-induced reduction in synaptic PSD-95 amounts, whereas it did not affect TTX-induced increase in PSD-95 (Supplementary Fig. 6c,d). Furthermore, GKAP targeting mutants, S340A, S348A and S340D, S384D, impaired normal activity-dependent turnover of PSD-95 in both directions (Supplementary Fig. 6e,f). Therefore, these results indicate that GKAP turnover is critical for the activity-dependent turnover of PSD-95 and Shank at synapses.

We then examined activity-dependent changes of surface AMPARs. Chronic activity modulation led to bidirectional changes in surface expression of AMPARs, which are associated with synaptic scaling. Neurons overexpressing wild-type GKAP exhibited similar activity-dependent changes (Fig. 7g,h). In contrast, overexpression of the GKAP ∆R1 mutant eliminated the activity-dependent changes in the surface expression of both GluA1 and GluA2 in both Bic- and TTX-treated neurons (Fig. 7g,h).

To examine the importance of GKAP turnover in synaptic scaling we directly measured mEPSCs in cultured hippocampal neurons (Fig. 8). The amplitude of basal mEPSCs was unaffected by overexpression of either wild-type GKAP or GKAP ∆R1, compared to untransfected neurons in the same culture (Fig. 8a,b). Treatment with TTX caused an increase in mEPSC amplitude in wild-type GKAP–expressing cells as well as in untransfected cells (Fig. 8b; P < 0.01). Conversely, Bic treatment reduced mEPSC amplitude in both wild-type GKAP–expressing and nontransfected neurons (Fig. 8a,b; P < 0.01). Also, the cumulative frequency plots of mEPSC amplitudes from wild-type GKAP–transfected neurons showed a leftward shift with Bic treatment and rightward shift with TTX treatment (Fig. 8c), consistent with synaptic scaling (P < 0.001, Kolmogorov-Smirnov test). In sharp contrast, neurons expressing GKAP ∆R1 did not show either TTX- or Bic-induced changes in the average mEPSC amplitude (Fig. 8b) or shifts in mEPSC amplitude distribution (Fig. 8c). Thus overexpression of GKAP ∆R1 blocked synaptic scaling in response to both hyperactivity and hypoactivity, just as it blocked the normal turnover of PSD-95 and Shank in synapses and prevented changes in synaptic cluster numbers under the control condition (Fig. 6f,g; P > 0.001). The GKAP S340D, S384D mutant showed the greatest impairment (P < 0.001 compared to S340A, S384A). Neither mutant showed the TTX-driven increase in synaptic cluster numbers (Fig. 6f,g; P > 0.23). These data indicate that both DLC interaction and CaMKII phosphorylation of DLC binding region are required for the proper GKAP targeting to synapses and accumulation of GKAP during inactivity.
surface AMPARs. We did not observe substantial changes in the frequency of mEPSCs under any of these conditions (Fig. 8d). Consistent with these data, the GKAP S54A mutant also blocked the bidirectional downscaling of mEPSCs, and GKAP S340A,S384A and GKAP S340D,S384D mutants did not support TTX-induced upscaling of mEPSCs (Supplementary Fig. 6g,h).

To confirm the importance of GKAP in synaptic scaling, we designed shRNA sequences targeting the mRNA encoding GKAP. The specificity and efficacy of these shRNAs are shown in Supplementary Figure 7. RNAi-mediated knockdown of GKAP (GKAP RNAi; by shRNA #3-1) did not influence the amplitude of basal mEPSCs substantially. However, GKAP RNAi completely abolished the bidirectional synaptic scaling, whereas untransfected neighboring neurons clearly showed activity-dependent synaptic scaling (Fig. 8e). Notably, GKAP RNAi greatly reduced the mEPSC frequency (Fig. 8f), suggesting that GKAP RNAi reduced the number of synapses. GKAP RNAi also abolished activity-dependent turnover of PSD-95 and Shank (Supplementary Fig. 7e–g). The immunostaining and electrophysiological data indicate that activity-dependent turnover of GKAP is critical for regulation of synaptic protein composition and homeostatic synaptic scaling.

Finally, we addressed the relative importance of GKAP turnover in synaptic scaling with respect to that of other known players in synaptic scaling. For this, we examined two proteins, Arc and Plk2 (also known as SNK), whose expression is induced by overexcitatiow32–36. Overexpression of ΔR1 affected picrotoxin (PTX)-induced expression of neither Arc nor Plk2 (Supplementary Fig. 8a–d), indicating that ΔR1 did not impair the signaling pathway(s) leading to the induction of these proteins. Considering that ΔR1 overexpression effectively blocked synaptic scaling (Fig. 8), these results suggested that GKAP turnover is necessary for Arc and Plk2 to exert their effect on synaptic scaling. To corroborate this notion, we examined the effect of ΔR1 on the Plk2-mediated removal or degradation of PSD-95 from synapses36. Overexpression of Plk2 removed PSD-95 from synapse in the proximal dendrites of transfected neurons (Supplementary Fig. 8e–g). Overexpression of wild-type GKAP and Plk2 did not influence PSD-95 removal substantially. In contrast, overexpression of ΔR1 with Plk2 abolished Plk2-mediated removal of PSD-95, and the S54A mutant exhibited the same effect (Supplementary Fig. 8e–g). These results suggest that GKAP removal at synapses is prerequisite for Arc and Plk2 action and additionally support the importance of activity-dependent GKAP turnover in synaptic scaling.

DISCUSSION

Activity modifies the protein composition of the PSD. Synaptic scaffolding proteins are particularly well-suited for the ‘driver’ role in PSD remodeling as their change can influence the levels of multiple interacting proteins. GKAP is a central member of the axis of major scaffolds in the PSD, consisting of PSD-95–GKAP–Shank10,37. However, compared to PSD-95 and Shank, which have various roles in synaptic function including glutamate receptor trafficking, synapse formation and spine morphogenesis37,38, little is known about the cell biology and function of GKAP. Here we defined a critical role for GKAP in activity-dependent turnover of PSD-95 and Shank in the PDSs and in homeostatic synaptic scaling.

UPS-dependent protein degradation has emerged as an important theme underlying synaptic plasticity4,16,39,40. A distinguished characteristic of activity-dependent turnover of PSD proteins is that groups of synaptic proteins are co-regulated, perhaps via control of master organizing proteins in the PSD4. Several characteristics of GKAP fit well for an organizer role within the PSD. First, GKAP is an indispensable central linker for the assembly of PSD-95–GKAP–Shank complexes at synapses4, and PSD-95, GKAP and Shank are mutually dependent on each other for stable accumulation at synapses12,41. Indeed, activity-dependent turnover of PSD-95 and Shank was dependent on the turnover of GKAP. Second, GKAP is one of the direct substrates of the UPS (Fig. 4 and ref. 4). Third, and most importantly, the synaptic accumulation of GKAP is controlled bidirectionally by synaptic activity. The level of activity is decoded by different Ca2+ channels. Ca2+ influx through the NMDA receptor triggers polyubiquitination and degradation of GKAP. In contrast, Ca2+ influx through L-VDCC is required for the synaptic accumulation of GKAP during inactivity. However, it is unlikely that GKAP turnover controls all PSD protein remodeling processes. A group of PSD proteins showed opposite changes to GKAP in response to altered activity, for example, NR2A and CaMKIIδ. After GKAP turnover, synaptic scaling requires additional players such as Plk2 to target other synaptic protein complexes for activity-dependent degradation by the UPS33,36.

The opposing functions of CaMKIIα and CaMKIIβ in the bidirectional synaptic scaling are well documented22–24, but the specific regulatory targets of these enzymes have remained unclear. Our finding indicates that GKAP is the critical substrate of these CaMKII isoforms for the activity-dependent control of synaptic strength. How does the activation of CaMKIIα versus CaMKIIβ exert different effects on GKAP turnover? Activity modulates synaptic levels of CaMKIIα and CaMKIIβ, rendering CaMKIIα as a dominant species during overexcitation but leaving CaMKIIβ as a major kinase during low-level activity21–24. Therefore, we expect that Ca2+ influx through NMDA receptors during high activity preferentially acts through CaMKIIα, which translocates to the PSD during high activity, and recruits proteasomes to dendritic spines16. In contrast, Ca2+ influx through L-VDCCs during low-level activity (activated by spontaneous release of glutamate and miniature EPSPs)42 preferentially stimulates CaMKIIβ, which has higher sensitivity to Ca2+ and CaM43, is associated with actin concentrated at the base of dendritic spine heads44, where L-VDCCs protein are also localized45.

What are the underlying molecular mechanisms for GKAP depletion and accumulation at synapses? Our results suggest a removal mechanism in which CaMKIIα phosphorylation in the N-terminal repeat region of GKAP disrupts GKAP interaction with PSD-95 and promotes UPS-dependent degradation (Supplementary Fig. 9a). Although dissociation from PSD-95 requires phosphorylation of Ser54 and Ser201, Ser54 phosphorylation was sufficient for polyubiquitination of GKAP. Notably, the S54D mutant did not show Bic-induced reduction. This is probably due to the overexpression-induced dominant-negative effect of inhibiting polyubiquitination of the S54D mutant itself by saturating specific E3 ubiquitin ligase. GKAP is likely transported away from synapses before degradation by proteasomes, as preventing proteasome activity did not protect synaptic GKAP but rather produced large aggregates of GKAP in the soma. However, this view is different from the previous report proposing in situ degradation of GKAP at synapses4. In addition, that report showed a completely opposite activity-dependent regulation of PSD-95 (ref. 4). At present the basis of these discrepancies is unclear, but differences in the density of hippocampal neurons and the concentration of proteasome inhibitors applied to culture might have contributed to the discrepancies. It is also unclear from our data whether ubiquitination of GKAP occurs at or near synapses. Additional studies are necessary to address these questions.

For the accumulation mechanism for GKAP (Supplementary Fig. 9b), we propose that β-CaMKII phosphorylation of Ser340 and...
Ser384 in the DLC-binding domain of GKAP promotes the dissociation of GKAP from MVA-DLC2 motor protein complexes that transport GKAP to the base of the PSDs, and then the ‘unloaded’ GKAP incorporates into the PSDs. For this, CaMKIIβ association with the actin cytoskeleton was critical, presumably because it provides spatially favored position to regulate the interaction. A similar regulatory role of CaMKIIβ has been shown for MVA-mediated transport of GluR1 to synapses46 and for Kif17-mediated transport of Mint1-NMDAR complex in spines47.

It is remarkable that deletion of R1 in GKAP was sufficient to block the bidirectional activity-dependent turnover of GKAP, which is different from S54A mutation that blocked only Bic-induced removal from synapses. One major difference between these two mutants is the CaMKII-dependent dissociation from PSD-95. The S54A mutant has Ser201, which allows CaMKIIα to prevent GKAP interaction with PSD-95 by phosphorylation, as suggested by the impaired PSD-95 interaction of the S54D,S201D mutant (Fig. 4e,f). In contrast, unlike wild-type GKAP, ΔR1 mutant retained ΔR1–PSD-95 co-clusters even in the presence of constitutively active CaMKIIα in COS cells (Supplementary Fig. 10). Thus, it is likely that deletion of the R1 likely induces changes in the overall conformation of the GKAP repeat region, so that the additional CaMKIIα is masked, rendering GKAP resistant to CaMKIIα regulation of its metabolic of synapses and synaptic scaling but also an essential scaffolding function for synapses, the data suggest that GKAP RNAi led to a loss of synapses. This result supports the notion that the dissociation of GKAP from PSD-95 is required for the accumulation or recruitment of GKAP. Additional studies are required to clarify how deletion of R1 prevents GKAP accumulation by inactivity. Furthermore, the CaMKII isoform-specific phosphorylation of GKAP at different sites needs to be tested by additional biochemical experiments.

GKAP RNAi not only prevented synaptic scaling measured by changes in mEPSC amplitude (Fig. 8e) but, unlike GKAP turnover mutant AR1, also had an additional effect of reducing mEPSC frequency. As mEPSC frequency is mostly determined by the number of synapses, the data suggest that GKAP RNAi led to a loss of synapses. Thus, GKAP is not only important for the activity-dependent remodeling of synapses and synaptic scaling but also an essential scaffolding protein of the PSDs for the maintenance of excitatory synapses.

Bic is a GABA_A receptor antagonist commonly used to induce synaptic scaling and indirectly enhances overall excitatory activity in neurons by reducing inhibitory inputs to excitatory neurons. Synaptic scaling is thought to occur in all synapses2, as cumulative histograms showed a shift of entire mEPSC amplitude distribution toward smaller values after Bic treatment (Fig. 8c). Consistent with this idea, cumulative distribution of GKAP cluster intensities revealed a similar shift after Bic treatment (Supplementary Fig. 11c), indicating that the Bic treatment reduced the amount of GKAP from most synapses (if not all). Therefore, Bic has a global effect and, unlike synapse-specific Hebbian-type plasticity, synaptic scaling affects all synapses.

Proteasome inhibitor treatments did not induce apoptosis of neurons (Supplementary Fig. 12), indicating the specificity of proteasome activity for GKAP degradation. GKAP has been shown to be polyubiquitinated by TRIM3 ubiquitin ligase48, which raises a possibility that TRIM3 may be involved in the CaMKII-mediated GKAP degradation. However, we did not observe a specific association of TRIM3 with the pSer54 peptide or the GKAP S54D mutant by pull-down assays (data not shown), indicating that TRIM3 is unlikely involved in the CaMKII-dependent degradation of GKAP described here. CDK5 is another protein kinase involved in the GKAP degradation induced by soluble amyloid β49. However, this also likely represents an independent GKAP regulatory mechanism, as the CDK5 phosphorylation sites in GKAP are different from the CaMKIIα sites, and notably, CaMKII inhibitors did not prevent GKAP degradation by soluble amyloid β49.

Finally, recent studies on knockout mice lacking SAPAP-3, a GKAP family member highly expressed in the striatum, showed unexpected behavioral abnormalities similar to obsessive-compulsive disorder50. Our findings of the critical role of GKAP in synapse remodeling and homeostatic plasticity offer potentially new ways to think about the pathophysiology of this condition.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

S.M.S., N.Z.G., J.H. and S.H.L. conducted all experiments and analyzed the data. N.Z.G., D.T.S.P., M.S. and S.H.L. contributed to designing experiments and interpretation of the data. S.H.L. and M.S. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Expression constructs and shRNA plasmids. HA-GKAP construct was generated by subcloning the EcoRI fragment of GKAP-encoding cDNA comprising the entire coding region into pGWI-HA. GKAP ΔRI was generated by deleting SmaI–NcoI from the HA-GKAP–encoding sequence cloned in pGWI. For electrophysiological experiments, GKAP or GKAP ΔRI were subcloned into pIRE2-EGFP (Invitrogen). CaMKII RNAi constructs were based on pSuper plasmid as described before. MVA RNAi and GKAP RNAi constructs were prepared by cloning the following nucleotide sequences (not including linker and loop sequences) into pSuper: GTAGAACGTCTTCAGCTAATA (MVA), CAGTGGCAGCTACATCAAA (GKAP #1) and GGGCAGCTACATCAAAAGCCGA (GKAP #3). GST-GKAP fusion protein expression vectors contained a flexible linker (SGGSGGASGGGGS) between GST-encoding and GKAP-encoding cDNA sequence to avoid steric hindrance that might hamper CaMKII phosphorylation of Ser54 of GKAP. CaMKII-ΔRI–AD construct was prepared by inserting sequence encoding Glu319 to Val388 of CaMKIIβ into α-CaMKIIβ residues between Lys322 and Lys323.

Hippocampal culture, transfection, antibodies and immunocytochemistry. Dissociated hippocampal neuron culture was prepared as described previously and grown in neurobasal medium supplemented with B27. All animal protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Neurons were transfected at 14–15 DIV with Lipofectamine 2000 (Invitrogen). Neurons were fixed with cold methanol (−20 °C) for staining of PSD-95, Shank and GKAP. Alternatively, they were incubated first in 2% formaldehyde, 4% sucrose and 1× PBS for 2 min followed by cold methanol for 10 min, for staining with β-Gal (or GFP) and CaMKII, GKAP or PSD-95. For surface staining of GluA neurons, neurons were fixed in 4% formaldehyde for 6 min, which is suboptimal for GKAP staining. GKAP antibodies used in the immunofluorescence staining, rabbit anti–GKAP (1:100) or mouse anti–pan SAPAP (NeuroMab, N127/31, 1:250), recognize all GKAP isoforms (data not shown). Other primary antibodies and their dilution used for immunocytochemistry were mouse anti–β-Gal (Promega, Z3781, 1:1,000), rabbit anti–β-Gal (Abcam, ab290, 1:5,000), mouse anti-CaMKII (Zymed, CBr-2, 1:250), mouse anti–CaMKII (Zymed, CBF-1, 1:250), mouse anti–PSD-95 (Chemicon, K28/43, 1:500), rabbit anti–ps-PS95 PSD-95 (Abcam, ab166495, 1:300), mouse anti–PSD-93 (NeuroMab, N18/30, 1:100), rabbit anti-Shank (3856 antibody), mouse anti-Bassoon (Stressgen, VAM-PS003, 1:200), mouse anti-synaptophysin (Sigma, SVP-38, 1:1,000), rabbit anti–HA (Santa Cruz, T-11, 1:100), mouse anti–HA (Roche, 12CAS, 1:400), mouse anti-my (Santa Cruz, 9E10, 1:100), rabbit anti–Arc (Santa Cruz Biotechnology, C-7, 1:100), rabbit anti–SNK (Santa Cruz Biotechnology, sc-25421, 1:100), rabbit anti–GluR1 (Oncogene, PC246, 5 μg/ml) and mouse anti–GluR2 (Chemicon, MAB397, 5 μg/ml). Bound primary antibodies were visualized by Alexa Fluor 488–conjugated (1:250) or Cy3-conjugated (1:500) secondary antibodies. Anti–pS54 antibody was prepared by immunizing rabbits with KLH–conjugated peptide (CGMRMSGP5YKKA) and purified by affinity chromatography using the antigen peptide after passing through non-phospho peptide column. Rabbit anti–phospho–CaMKII antibody (Phospho solutions, p1005-286) and mouse anti-ubiquitin, Lys48-specific antibody (Millipore, Apu2) were used for western blotting at 1:1,000 and 1:2,000 dilutions, respectively.

Image acquisition and analyses. Images were captured by using Nikon C1 plus laser scanning confocal microscope. Acquired images (z-series stacks) were first converted to projection images (with maximal projection option) and analyzed using Metamorph software (Molecular Devices). To measure puncta number per given length of dendrites, per image, 5 dendritic segments (~15–30 μm in length each) were selected from transfected and neighboring nontransfected neurons, respectively. After applying threshold, only puncta larger than 3 pixels were counted, and their pixel area and total and average intensity were also measured. For quantification of HA-GKAP staining, clusters formed inside dendritic shafts were excluded from quantification to avoid erroneous inclusion of nonsynaptic clusters. All data collected were transferred to Microsoft Excel for computation and statistical significance analyses. Image acquisition and analyses were done in a double-blinded manner to eliminate experimenter bias.

Statistical analysis. All values represent means ± s.e.m., unless otherwise indicated. All transfaction experiments were done in triplicate. Statistical significance for pair was analyzed by the Student’s t-test (unpaired, two-tailed, assuming unequal variance), unless otherwise indicated. ANOVA with Tukey’s post-hoc test were used for group comparisons. Cumulative plot data were analyzed by Kolmogrov–Smirnov test. P < 0.05 was considered significant.

In situ and in vitro phosphorylation. In situ phosphorylation reaction was performed by incubating purified PSD I fraction (30 μg) at 37 °C for 5 min in the 100 μl reaction mixture of 20 mM HEPES (pH 7.5), 10 mM Mg-acetate, 1 mM CaCl2, 5 μM CaM, 0.5 μM [γ-32P]ATP, 10 mM DTT, 0.1% Triton X-100, 1 μM microcystin-LR and 1× protease inhibitor cocktail (Roche). Ca2+ dependence was examined by substituting 1 mM EGTA for CaCl2 and CaM in the reaction mixture. The reaction was immediately stopped by adding EDTA to final 25 mM and putting on ice. Phosphorylation of specific proteins was examined by subsequent immunoprecipitation with respective antibodies under denaturing condition. Briefly, 1/10 volume of 2% SDS (final 0.2% concentration) was added to the reaction mixture and boiled for 3 min. After centrifugation at 15,000g for 10 min at 4 °C, supernatant was mixed with equal volume of 2× RIPA (20 mM Tris-Cl, pH7.4, 2, 4 mM EDTA, 300 mM NaCl, 2% Triton X-100, 1% sodium deoxycholate and 1 mM βSA). Aliquots were mixed with 5 μg of indicated antibodies and mixed overnight at 4 °C, followed by incubation with protein-A sepharose beads (20 μl) for additional 2 h. After washing the beads with 1× RIPA 3 times, immunoprecipitated proteins were eluted by boiling in SDS sample buffer, followed by subsequent SDS-PAGE and autoradiography.

Coimmunoprecipitation. Coimmunoprecipitation of GKAP and PSD-95 or DLC was done as described before. Briefly, HA-tagged GKAP constructs were transfected with either myc-tagged PSD-95 or myc-tagged DLC2 in COS cells using Lipofectamine (Invitrogen). Forty-eight hours after transfection, collected cells were lysed in buffer A (50 mM Tris-Cl, pH7.4, 75 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 2 mM DTT, 1% SDS and protease inhibitor cocktail (Roche)). After mixing by pipetting up and down briefly, the lysate was quickly quenched in a 4× volume of buffer B (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 2 mM DTT, 1% Triton X-100 and protease inhibitor cocktail). After removing particulate materials by centrifugation at 16,000g for 30 min at 20 °C, we used the supernatant for immunoprecipitation using myc-agarose beads (Santa Cruz). For brain tissues, GFP fluorescence and morphological inspection. Whole-cell patch recordings were performed by voltage-clamping neurons at −70 mV in bath solution (in mM, 4 Mg-ATP, 0.3 Na2-GTP, 0.2 EGTA and 10 HEPES, adjusted to pH 7.2 and 290 mOsm). mEPSCs were acquired through a MultiClamp 700B amplifier (Molecular Devices), filtered at 2 kHz, digitized at 10 kHz, using the ‘gap-free’ protocol. mEPSCs were detected and analyzed with MiniAnalyses software (Synaptosoft) by setting amplitude threshold to 5 pA (usually VMS × 3 values were lower than 4), further filtered by selecting mini events of 10–90% rise time < 3 ms. Cumulative probability plots were generated by combining mini events from all recorded neurons.

Electrophysiology and miniature excitatory postsynaptic current analysis. Hippocampal neurons, plated at the density of 150,000 cells/cover slip, were transfected at 10 DIV with pIRE2-EGFP plasmid encoding either wild-type GKAP or ΔRI. After 1 d after transfection, neurons were treated by either TTX (2 μM) or Bic (40 μM) for 48 h before recording. Transfected pyramidal neurons were identified by GFP fluorescence and morphological inspection. Whole-cell patch recordings were performed by voltage-clamping neurons at −70 mV in bath solution (in mM, 119 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 30 glucose and 10 HEPES, pH 7.4, 300 mOsm) containing TTX (1 μM) and Bic (20 μM), continuously perfused at the rate of ~0.5 ml/min. Internal solution was composed of 140 K-gluconate, 5 mM MgCl2, 4 ATP; 0.3 Na2-GTP, 0.2 EGTA and 10 HEPEs, adjusted to pH 7.2 and 290 mOsm. mEPSCs were acquired through a MultiClamp 700B amplifier (Molecular Devices), filtered at 2 kHz, digitized at 10 kHz, using the ‘gap-free’ protocol. mEPSCs were detected and analyzed with MiniAnalyses software (Synaptosoft) by setting amplitude threshold to 5 pA (usually VMS × 3 values were lower than 4), further filtered by selecting mini events of 10–90% rise time < 3 ms. Cumulative probability plots were generated by combining mini events from all recorded neurons.

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