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Neuron Specific Rab4 Effector GRASP-1 Coordinates Membrane Specialization and Maturation of Recycling Endosomes

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Abstract

The endosomal pathway in neuronal dendrites is essential for membrane receptor trafficking and proper synaptic function and plasticity. However, the molecular mechanisms that organize specific endocytic trafficking routes are poorly understood. Here, we identify GRIP-associated protein-1 (GRASP-1) as a neuron-specific effector of Rab4 and key component of the molecular machinery that coordinates recycling endosome maturation in dendrites. We show that GRASP-1 is necessary for AMPA receptor recycling, maintenance of spine morphology, and synaptic plasticity. At the molecular level, GRASP-1 segregates Rab4 from EEA1/Neep21/Rab5-positive early endosomal membranes and coordinates the coupling to Rab11-labelled recycling endosomes by interacting with the endosomal SNARE syntaxin 13. We propose that GRASP-1 connects early and late recycling endosomal compartments by forming a molecular bridge between Rab-specific membrane domains and the endosomal SNARE machinery. The data uncover a new mechanism to achieve specificity and directionality in neuronal membrane receptor trafficking.

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Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; APV, 2-amino-5-phosphonopentanoic acid; β-gal, β-galactosidase; DIV, days in vitro; EPSC, excitatory postsynaptic current; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP-TfR, GFP-tagged transferrin receptor; GluR, glutamate receptor; GRASP-1, GRIP-associated protein-1; GRIP, glutamate receptor interacting protein; GRP, guanine nucleotide releasing protein; GST, glutathione S-transferase; JNK, Jun-N-terminal kinase; LTD, long-term depression; LTP, long-term potentiation; NB, neurobasal; NEEP21, neuronal endosome enriched protein of 21 kDa; NMDAR, n-methyl-D-aspartic acid receptor; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PSD, post synaptic density; shRNA, short hairpin RNA; TBS, Tris-Buffered Saline; TIRFM, Total Internal Reflection Fluorescence microscopy.

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Introduction

In order to receive, process, and transmit information, neurons need substantially regulated mechanisms to locally redistribute membranes and proteins to synaptic sites. Multiple lines of evidence suggest that the endosomal pathway plays a crucial role in synaptic function and plasticity. At excitatory synapses, the postsynaptic membrane composition is subject to continuous and activity-dependent endocytic cycling of postsynaptic molecules. Based on uptake of extracellular gold particles, visualization of clathrin assembly in living neurons and pre-embedding immunogold electron microscopy, it was shown that endosomal compartments are present in the dendritic shaft and spines and that endocytosis occurs at specialized endocytic zones lateral to the postsynaptic density (PSD) [1]. Using live-cell imaging and serial section electron microscopy, it was demonstrated that recycling endosomes are required for the growth and maintenance of dendritic spines [2]. Membrane recruitment from recycling endosomes is a common mechanism that cells employ to expand the plasma membrane and targets proteins in a polarized manner in such distinct processes as cytokinesis, cell-cell adhesion, phagocytosis, and cell fate determination [3,4].

Author Summary

Neurons communicate with each other through specialized structures called synapses, and proper synapse function is fundamental for information processing and memory storage. The endosomal membrane trafficking pathway is crucial for the structure and function of synapses; however, the components of the neuronal endosomal transport machinery are poorly characterized. In this paper, we report that a protein called GRASP-1 is required for neurotransmitter receptor recycling through endosomes and back to the cell surface, as well as for the normal morphology of dendritic spines—the projections that form synapses—and for synaptic plasticity. We show that GRASP-1 coordinates coupling between early and later steps of the endocytic recycling pathway by binding to Rab4, a regulator of early endosomes, and to another endosomal protein found later in the pathway called syntaxin 13—a so-called SNARE protein involved in membrane fusion. GRASP-1 binds Rab4 with its N terminus and syntaxin 13 with its C terminus, suggesting that these interactions could structurally and functionally link early endosomes to those later in the recycling pathway. We propose a model in which GRASP-1 forms a molecular bridge between different endosomal membranes and the SNARE fusion machinery. Our study thus provides new mechanistic information about endosome function in neurons and highlights GRASP-1 as a key molecule that controls membrane receptor sorting and recycling during synaptic plasticity.

Perhaps the strongest evidence for the importance of endocytic recycling in synaptic function originates from the analysis of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (AMPAR) trafficking [5–8]. AMPARs are the major excitatory neurotransmitter receptors in the brain, and redistribution of AMPARs in and out of the synapse has emerged as an important mechanism for information storage in the brain [6,8]. Increased delivery of AMPARs to the postsynaptic membrane leads to long-term potentiation (LTP), whereas net removal of AMPARs by internalization from the surface through endocytosis seems to underlie long-term depression (LTD) [5–8]. Like any other internalized membrane protein, endocytosed AMPARs undergo endosomal sorting; they can be degraded in lysosomes or recycled back to the surface membrane [9–11]. A popular model holds that the recycling endosomes provides the local intracellular pool of glutamate receptors for LTP [12]. Neuron-enriched endosomal protein of 21 kD (Neep21) and its interacting protein syntaxin 13 are endosomal proteins implicated in regulating AMPAR trafficking during synaptic plasticity [13]. However, it remains unclear how endocytic receptor sorting and recycling is organized and coordinated in neuronal dendrites.

Multiple proteins identified as regulators of endosomal traffic in non-neuronal cells are also important in neuronal endosomes [3,14–16]. Dendritic spines contain the basic components of the endocytic machinery, postsynaptic receptor endocytosis occurs through a dynamin-dependent pathway, and Rab GTPases and their effectors regulate endosomal traffic [17–19]. The classic endosomal Rab proteins, Rab5, Rab4, and Rab11, have all been implicated in endosomal receptor and membrane trafficking in dendrites [12,19–23]. Rab5 controls transport to early endosomes (also called sorting endosomes), whereas Rab4 and Rab11 are involved in the regulation of endosomal recycling back to the plasma membrane [24]. The endosomal pathway can be considered as a mosaic of discrete but overlapping domains that are generated and controlled by Rab proteins and their interacting effector protein networks. The communication and transport between sequentially organized Rab domains is thought to be mediated via proteins that are "shared" by both domains. Bivalent effectors, such as Rabenosyn-5 and Rabaptin-5, have been found that connect proximal Rab5 and distal Rab4 domains on early endosomes [25,26]. However, how Rab4 and Rab11 recycling endosomal domains are coupled is poorly understood.

To gain a better mechanistic understanding of endosome recycling in neurons, we searched for neuronal interacting partners of Rab4 [27]. Using a pull-down and mass spectrometry approach, we identified GRASP-1 as a neuron-specific effector of Rab4 and key component of endocytic recycling in dendrites. GRASP-1 was originally found to interact with glutamate receptor interacting protein (GRIP) and shown to be involved in regulating AMPAR distribution [28]. We show that GRASP-1 is necessary for AMPAR recycling and synaptic plasticity, essential for maintenance of spine morphology and important for endosomal trafficking. GRASP-1 segregates Rab4 from EEA1/Neep21/Rab5-positive early endosomal membranes and coordinates the coupling to Rab11-labelled recycling endosomes via the interaction with t-SNARE syntaxin 13. These results describe a molecular mechanism for regulating recycling endocytosis by GRASP-1.

Results

GRASP-1 Is a Rab4-GTP-Binding Protein

To identify Rab4-interacting proteins, we performed glutathione S-transferase (GST) pull-down assays with pig brain extracts using GTPγS-loaded GST-Rab4 affinity columns and analyzed the isolated proteins by mass spectrometry (Figure 1A). Among the proteins that were highly enriched in the GST-Rab4-GTPγS pull-downs but were not detected by mass spectrometry in the pull-down assays using GST-Rab4-GDP or GST alone, we found known binding partners of Rab4, such as the bivalent Rab effectors Rabaptin-5 and Rabenosyn-5 (Table 1) [25,29]. The most significant novel hit was GRASP-1, which was originally identified as a GRIP/AMPAR interacting protein. GRASP-1 has been shown to regulate AMPAR targeting and Jun-N-terminal kinase (JNK) signaling [28,30]. The association between GRASP-1 and Rab4 was confirmed by immunoblotting with an antibody against GRASP-1 (Figure 1B). Binding of GRASP-1 to Rab4 was direct and specific since GRASP-1 associates with GST-Rab4 but not with the other tested Rab proteins, such as Rab3, Rab5, and Rab11 (Figure 1C). Some weaker binding was detected with Rab7 in this assay. Immunoprecipitation experiments from COS-7 cells co-expressing myc-GRASP-1 and Flag-Rab4 or Flag-Rab5 further confirmed the interaction of GRASP-1 with Rab4 (Figure 1D). Fluorescence microscopic analysis of Hela cells transfected with myc-GRASP-1 and GFP-Rab4 showed that the distribution of GRASP-1 fully coincided with GFP-Rab4 (Figure 1E). Analysis of the endosomal compartment in the same cells, as visualized by internalized Alexa594-Transferrin (Tf-594), indicated that GRASP-1 localizes to the Rab4-positive domain of the early endosomal recycling system. These immunofluorescence data are in line with the reported endosomal localization of GRASP-1 in Hep-2 cells, detected with an autoimmune GRASP-1 serum from a patient with recurrent infections and a presumed immune deficiency [31].

GRASP-1 has an extensive propensity to form coiled-coils and contains a caspase-3 cleavage site, a PDZ-like GRIP binding domain, and a central glutamate-rich stretch (Figure 1F). To define the minimal Rab4 binding domain on GRASP-1, we generated a series of myc-GRASP-1 truncations (Figure 1F). GST-Rab4

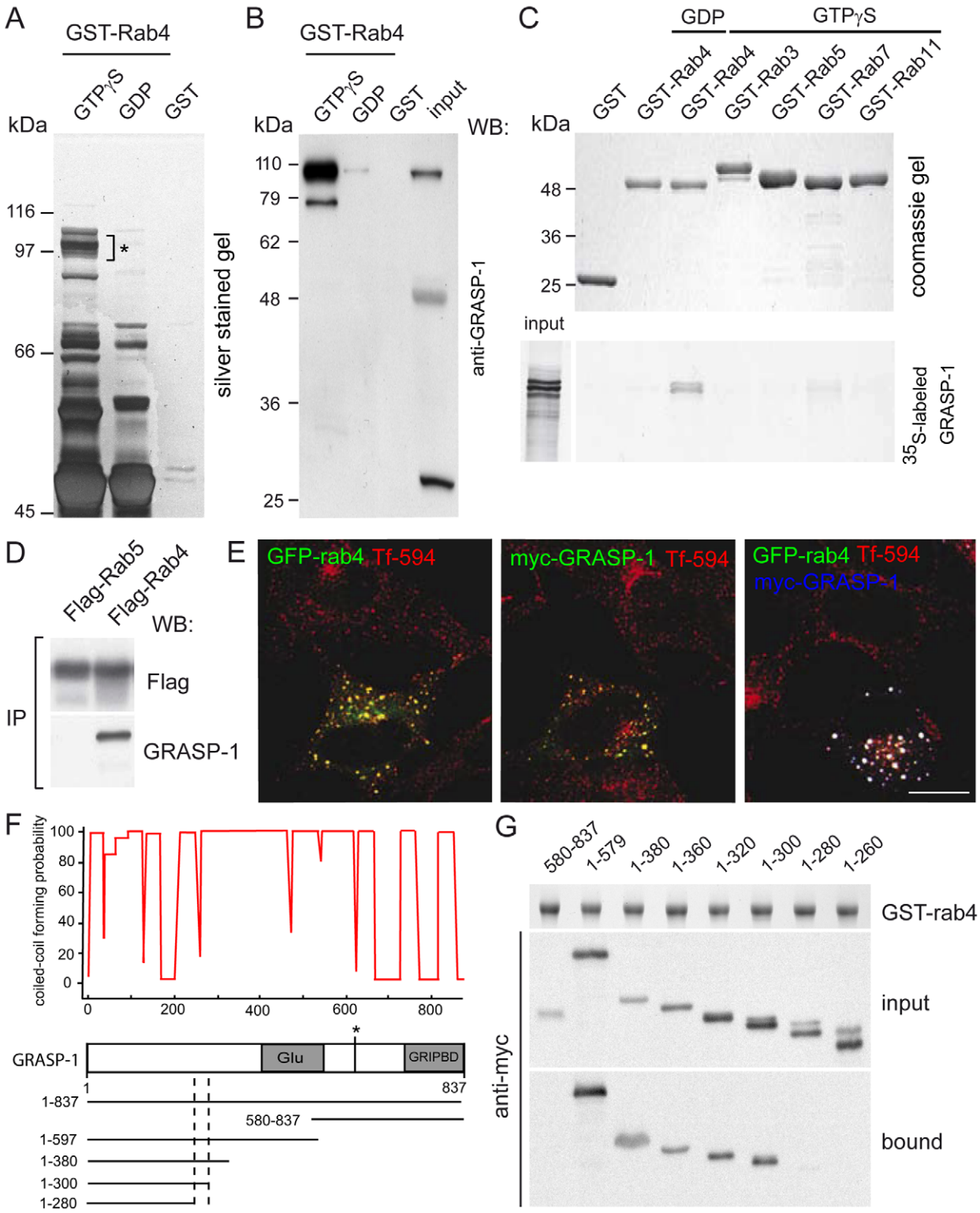

Figure 1. GRASP-1 is a Rab4GTP-binding protein. (A) Silver stained gel showing isolation of GSTRab4-GTPγS binding proteins from brain cytosol. Asterisk denotes band from which GRASP-1 was identified. (B) Western blot of samples from (A) probed with GRASP-1 antibody. (C) Binding assay of ^{35}S-labeled GRASP and GSTRab4-GTPγS, or GSTRab4-GDP, and other GTPγS charged GST-Rab proteins. (D) FLAG-tagged Rabs were co-expressed with myc-GRASP-1 in COS-7 cells. Anti-FLAG immunoprecipitates (IP) were analyzed by Western blot with myc antibody. (E) Hela cells were transfected with GFP-Rab4, myc-GRASP-1, or both. Prior to fixation, cells were incubated for 60 min with Alexa594-labeled Tf at 37°C. Bar is 10 μm. (F) Coiled-coil prediction and domain architecture of GRASP-1. Glu, glutamic acid rich domain; asterisk, caspase-3 cleavage site; GRIPBD, GRIP1 binding domain. (G) Binding domain analysis using lysates of COS-7 cells expressing myc-tagged GRASP-1 truncations and GTPγS-charged GST-Rab4.
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Table 1. Binding partners of GST-Rab4-GTP in pig brain extracts identified by mass spectrometry.

Identified Protein	MW (kDa)	Pept. Total	NCBI GI Number	References
Rabaptin-5	99.7	68	1050523	[29]
GRASP-1	96.3	9	16758652	[28]
Rabenosyn-5	89.5	3	58037445	[25]

The table shows proteins identified with a significant Mascot score in GST-Rab4-GTP pull-downs from pig brain extracts. The list is corrected for background proteins, which were identified in a control GST-Rab4-GDP and GST pull-down. For each identified protein, the list is filtered for duplicates and shows only the hits with identified peptides.

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pull-down assays with COS-7 cell extracts expressing GRASP-1 mutants showed that the N-terminal domain of GRASP-1 binds to Rab4 and that the coiled-coil region between amino acid 280–300 is required for this interaction (Figure 1G). However, full-length GRASP-1 lacking amino acid 280–300 partially retained Rab4 binding (unpublished data). These data argue for an important role of the N-terminal coiled-coil region in Rab4 binding but show that other regions might also be involved.

It has been reported that GRASP-1 may serve as a guanine nucleotide exchange factor (GEF) for H-ras [28]. We tested whether GRASP-1 might be a GEF for Rab4 by analyzing recombinant GRASP-1(1–594) in a GEF assay using fluorescent mantGDP. GRASP-1 did not act as GEF for Rab4 (Figure 2A,B). However, unlike the positive control cdc25, GRASP-1 also did not exhibit noticeable GEF activity towards H-ras (Figure 2A). Full-length GRASP-1 also failed to increase GTP-loading of H-Ras in vivo as measured in pull-down assays with the recombinant ras binding domain of Raf-1. The bona fide GEF Ras-GRP markedly increased the amount of H-Ras in the GTP state (Figure 2C), which was further enhanced through its membrane recruitment via a phorbol myristate acetate (PMA)-controlled pathway [32]. In line with these results, careful sequence analysis of GRASP-1 did not reveal significant homology to any known rasGEF. Together these data suggest that GRASP-1 is not a rasGEF but a Rab4 effector.

GRASP-1 Localizes to a Sub-Domain of Rab4-Positive Early Recycling Endosomes in Neurons

We examined GRASP-1 expression in mouse tissues and cell lines and showed by Western blot that GRASP-1 is highly expressed throughout the central nervous system, including cortex, cerebellum, midbrain, and spinal cord, and in primary cultured

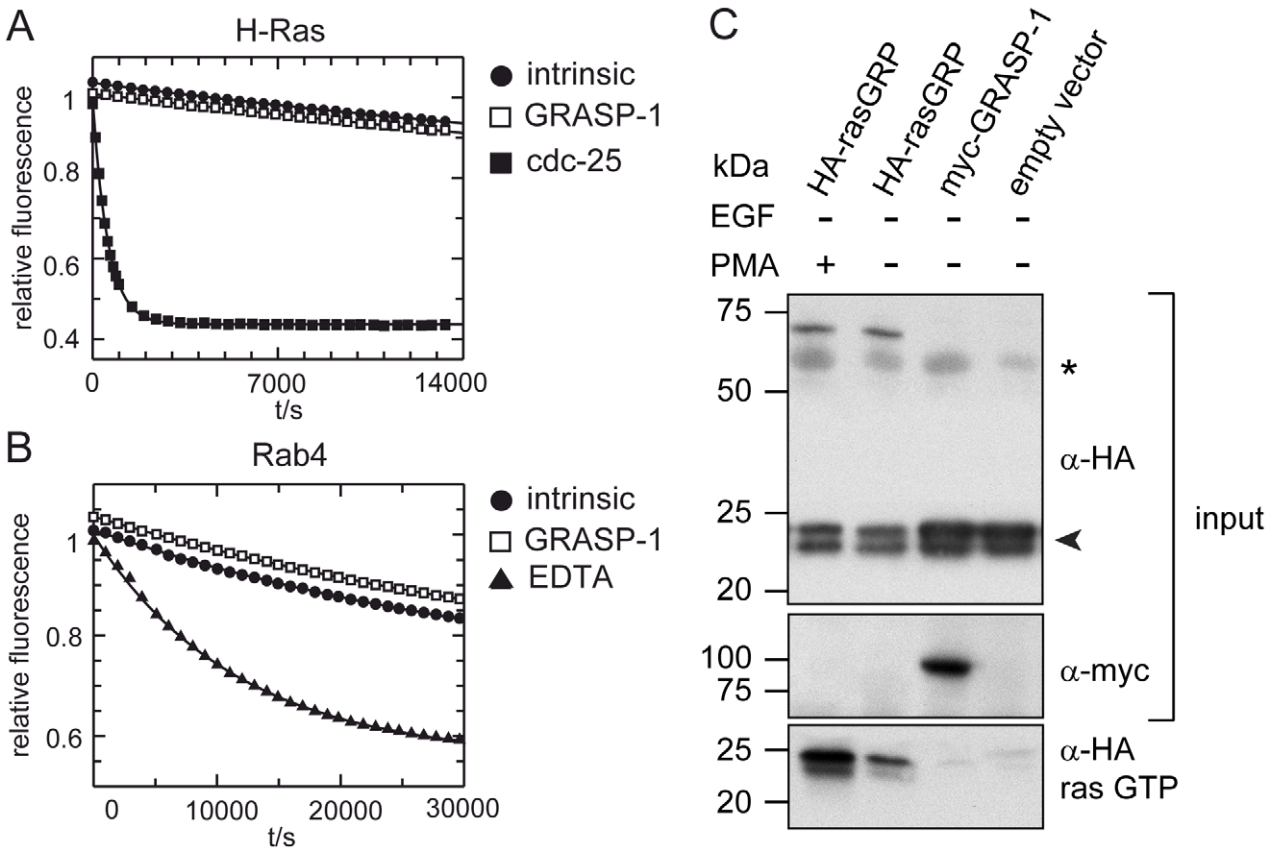

Figure 2. GRASP-1 does not have GEF activity on H-ras and Rab4. (A–B) 0.2 μM H-ras or Rab4 loaded with fluorescent mantGDP was incubated with an excess of GDP at 25°C, in the absence or in the presence of 10 μM GRASP-1(1–594), 0.2 μM cdc-25, or 10 μM EDTA. Dissociation of mGDP was monitored by measuring the decrease in relative fluorescence that accompanies release of mGDP from the GTPase. (C) COS-7 cells were transfected with HA-Hras in combination with indicated constructs and treated with or without PMA. Ras-GTP was isolated on GSH beads containing the ras binding domain of the ras effector raf and analyzed by Western blot with HA antibody. Note that full-length GRASP-1 did not increase rasGTP level above non-transfected control. Asterisk and arrowhead in HA Western blot of input material denote a background band and the position of HA-ras, respectively.

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hippocampal neurons but is absent in non-neuronal tissues and cell types with the exception of neuroendocrine insulinoma cells (Figure 3A). These results are consistent with previous immunoblot and immunohistochemistry analyses [28], indicating that GRASP-1 is expressed in neurons throughout the CNS, with highest expression levels in the hippocampus. Double labeling confocal immunofluorescence on mouse brain and spinal cord sections showed that GRASP-1 immunoreactivity was associated with punctate structures throughout the somato-dendritic compartment of neurons (Figure S1 and unpublished data). These punctate structures generally were immunoreactive for Rab4, although various GRASP-1 positive structures did not label for Rab4 and vise versa (Figure S1).

Immunofluorescence labeling in mature hippocampal neurons (>days in vitro 17; DIV 17) revealed that endogenous GRASP-1, although present in axons, is predominantly localized within the somatodendritic compartment, as evidenced by its labeling pattern and the codistribution with the dendritic marker MAP2 (Figure 3B). GRASP-1 is associated with punctate structures that occasionally extend beyond the dendritic shaft (arrowheads in Figure 3C), overlap with the synaptic markers PSD-95 (arrowheads in Figure 3D) and Bassoon (arrowheads in Figure 3E), and localize within the dendritic spines visualized in β-galactosidase (β-gal) filled neurons (unpublished data). In line with the immunohistochemistry data (Figure S1) [28], colocalization of endogenous Rab4 and GRASP-1 is observed in primary hippocampal neurons (Figure 3F). Immunoelectron microscopy showed that endogenous GRASP-1 and Rab4 localize on an extensive tubular network that appeared to emanate from endosomes with a morphology that is characteristic of recycling tubules (Figure 4A). The ability of GRASP-1 to associate with Rab4 positive endosomes was further confirmed by simultaneous dual color live imaging of mRFP-GRASP-1 and GFP-Rab4: GRASP-1 was observed on mobile Rab4-positive vesicles and tubular structures which dock and fuse with larger GRASP-1/Rab4 endosomal domains (Figure 3H; Videos S1 and S2). Overexpression of GFP-Rab4 in hippocampal neurons increased the size of the endosomal structures where GRASP-1 and Rab4 coincide (Figure 3G). Close inspection of these structures revealed that endogenous GRASP-1 localizes to a sub-domain of the large Rab4-positive endosome (Figure 3G, inset), suggesting that GRASP-1 might regulate a particular step in the endosomal recycling pathway. To test whether endosomal GRASP-1 localization depends on Rab4 activity, neurons were transfected with dominant negative Rab4 (Rab4S22N). Expression of Rab4S22N redistributed GRASP-1 away from punctate endosomes, while other endosomal proteins were unaffected (Figure S2). Although it is likely that Rab4S22N inhibits membrane localization of its effector GRASP-1, we cannot exclude that overall levels of GRASP1 are also affected by Rab4S22N. Together these data indicate that GRASP-1 is selectively expressed in neurons, where it is partially localized to Rab4-positive endosomes in dendrites and present in spines near postsynaptic structures.

GRASP-1 Is Required for Dendritic Spine Morphology

To explore the function of GRASP-1, we used RNA interference to knock down expression of GRASP-1 in mature hippocampal neurons. We found two independent GRASP-1-shRNA sequences (#2 and #5) that specifically inhibited expression of GRASP-1 in hippocampal neurons (Figure S3). GRASP-1 antibodies detected more than ~80% reduction of GRASP-1 staining intensity in the cell body as well as in dendrites in GRASP-1-shRNA transfected neurons (Figure S3B), while other antibody staining, such as of MAP2, were unaffected (unpublished data). Both GRASP-1-shRNAs constructs produced similar phenotypic effects.

In view of previous observations that inhibition of endosomal recycling by dominant negative forms of Rab4 and Rab11 alters the morphology of dendritic spines [2], we first examined the effect of GRASP-1 knock-down on dendritic spines. In neurons co-expressing GRASP-1-shRNA and β-gal, we observed a marked decrease in the total number of protrusions (Figure 5A). The remaining dendritic protrusions were classified as filopodia-shaped protrusions and mushroom-shaped spines based on the ratio of spine head width to protrusion length. Quantification revealed that knock-down of GRASP-1 decreased the number of mushroom-headed spines (Figure 5B,C). Neurons expressing GRASP-1* (which is resistant to GRASP-1-shRNA#2 knock-down) largely reversed the spine phenotype (Figure 5A–C). A similar spine phenotype was observed by expressing dominant negative forms of Rab11 (Rab11S25N) and Rab4 (Rab4S22N) (Figure 5B,C). We next tested whether GRASP-1 knock-down could inhibit LTP-induced spine growth by glycine stimulation, a protocol used to induce chemical LTP in cultured hippocampal neurons [2]. In control neurons, glycine treatment induced new spine formation and preexisting spine growth, while in the absence of GRASP-1 spine growth is blocked (Figure 5D,E). Together these data indicate that GRASP-1 plays an essential role within the recycling endosomal pathway to maintain dendritic spine morphology and regulate LTP-induced spine growth.

GRASP-1 Regulates Recycling Endosome Distribution

To directly examine the effect of GRASP-1 knock-down on recycling endosomes distribution in spines, we analyzed its localization with GFP-tagged transferrin receptor (GFP-TfR), which is an archetype recycling cargo that at steady state resides in recycling endosomes [2]. As expected GRASP-1 and GFP-TfR showed a strong colocalisation within dendrites (Figure 5F). TfR-GFP-labeled endosomes were present in the dendritic shaft at the base of spines (a), in the spine neck (b), and in the spine head (c) (Figure 5G). In neurons transfected with GRASP-1-shRNA, GFP-TfR-labeled endosomes were abundantly present in the dendritic shaft at the base of spines but were depleted from the spines (Figure 5H). Quantitative analysis revealed that in control neurons ~50% of the spines had TfR-GFP-labeled endosomes in their neck and head (b, c, and b+c), whereas in the absence of GRASP-1 only ~10% of the spines contained recycling endosomes (Figure 5G). These data show that GRASP-1 regulates recycling endosomal localization into dendritic spines and most likely explains the observed GRASP-1 knock-down spine phenotype.

GRASP-1 Regulates AMPAR Recycling

To further explore the functional importance of GRASP-1 in endosomal recycling, we studied the effect of GRASP-1 knock-down on endocytic trafficking of AMPAR. First, we analyzed GRASP-1 colocalization with internalized AMPARs by using the fluorescence-based antibody feeding assay [10]. Live hippocampal neurons expressing extracellular HA-tagged GluR1 or GluR2 subunits were surface labeled with HA antibody, stimulated with AMPA (100 μM, in the presence of 50 μM APV, a selective n-methyl-D-aspartic acid (NMDA) receptor antagonist), fixed, permeabilized, and stained for internalized GluR subunits and endogenous GRASP-1. At 2 min after AMPA stimulation, only ~5% of internalized HA-GluR1 or HA-GluR2 colocalized with GRASP-1 (Figure S4A,B). After 10 min following stimulation, colocalization between internalized GluR subunits with GRASP-1 was increased to ~30% (Figure 6A, Figure S4A,B), which is

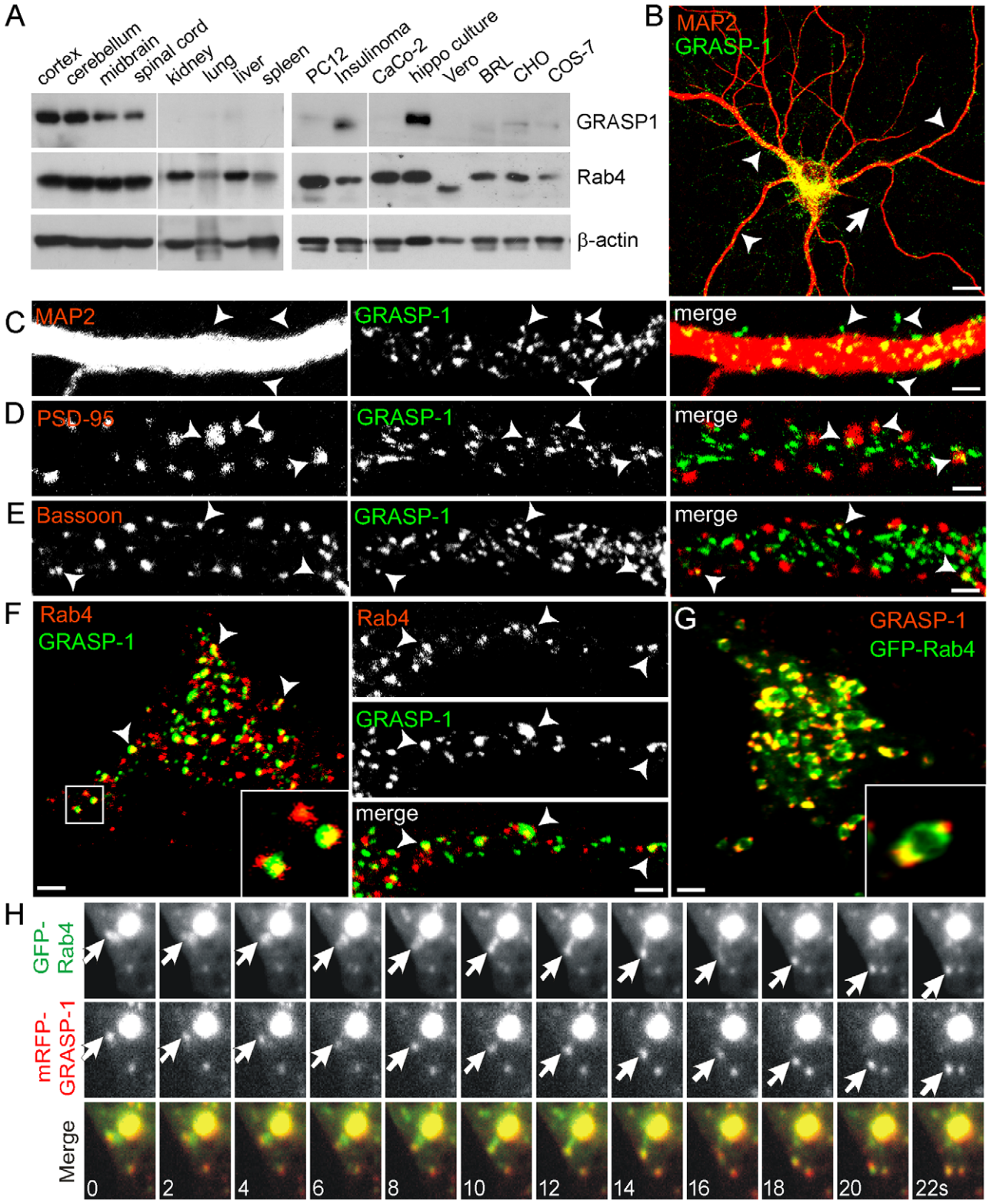

Figure 3. Colocalization of GRASP-1 and Rab4 in hippocampal neurons. (A) Expression pattern of Rab4 and GRASP-1 in mouse tissue and cultured cells visualized on Western blot. (B–F) Representative images of hippocampal neurons double-labeled with antibodies against GRASP-1 and endogenous markers. (B) MAP2 and GRASP-1, arrow denotes axon and arrowheads dendrites. (C) MAP2 and GRASP-1, arrow heads mark GRASP-1 signal beyond the dendritic shaft. (D) PSD-95 and GRASP-1. (E) Bassoon and GRASP-1, arrowheads denote localization of GRASP-1 to synaptic sites. ~15% of the synapses colocalize with GRASP-1, while the "random" colocalization is ~5% as determined by rotating the red channel image. (F) Rab4 and GRASP-1 in the cell body (left) and dendrites (right). Arrowheads denote areas of colocalization, inset show magnified regions. Bar in B is 10 μm; Bar in (C–F) is 1 μm. (G) Image of the cell body of hippocampal neurons transfected at DIV13 with GFP-Rab4 and stained for GRASP-1. Magnified region is shown as inset; note the partial localization of GRASP-1 on the distal domain of GFP-Rab4 endosomes. Bar is 1 μm. (H) Simultaneous imaging of GFP-Rab4 (green) and mRFP-GRASP-1 (red) in transfected hippocampal neurons. Successive frames are shown and time (seconds) is indicated in the merge panel.
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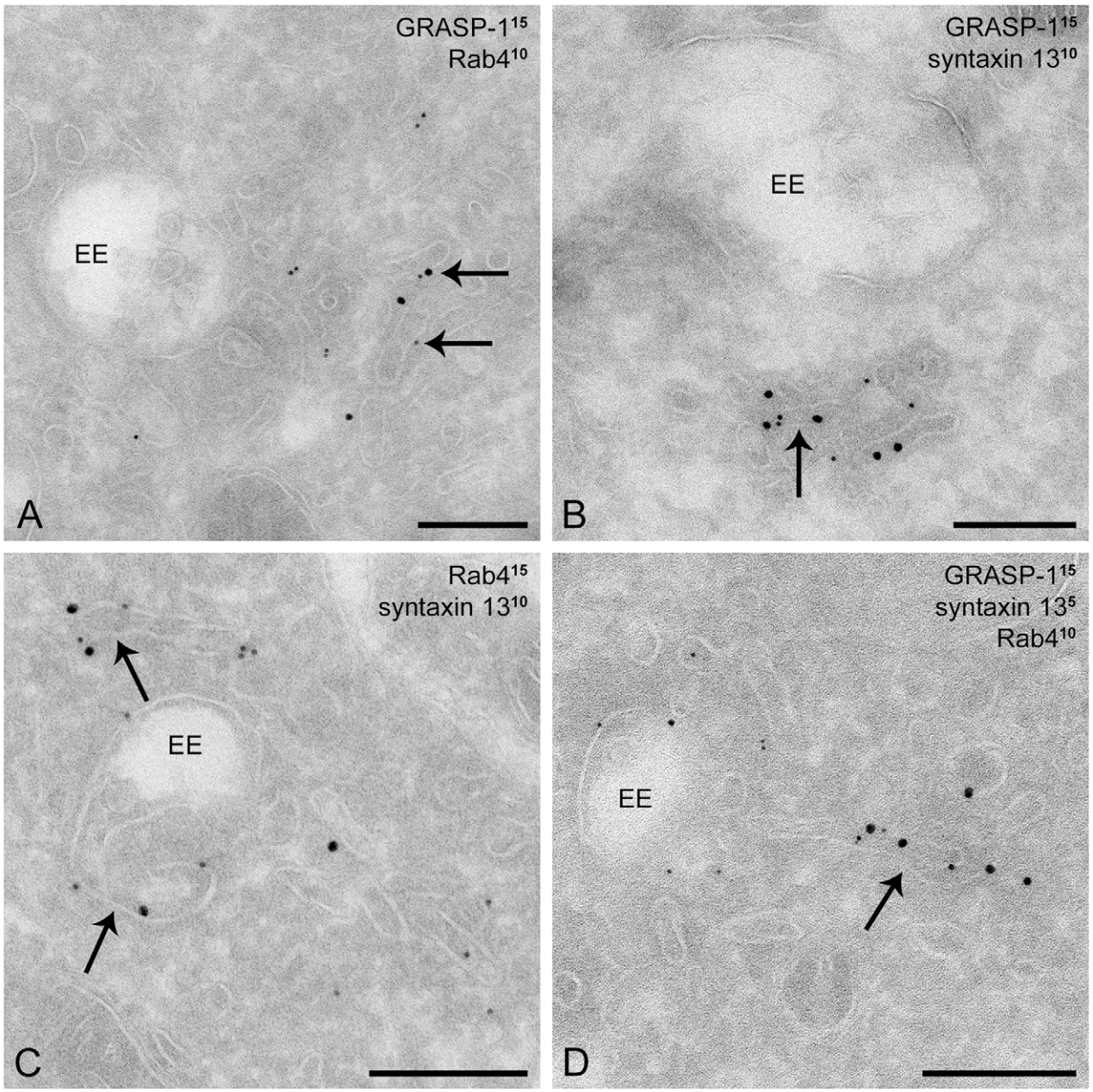

Figure 4. Endogenous GRASP-1, Rab4, and syntaxin 13 coincide on recycling endosomal tubules. Immunogold EM of hippocampal neurons labeled with 10 nm protein A gold for Rab4 and with 15 nm protein A gold for GRASP-1 (A), with 10 nm protein A gold for syntaxin 13 and with 15 nm protein A gold for GRASP-1 (B), with 10 nm protein A gold for syntaxin 13 and with 15 nm protein A gold for Rab4 (C), or with 15 nm protein A gold for GRASP-1, with 5 nm protein gold for syntaxin 13, and with 10 nm protein A gold for rab4 (D). Arrow denotes tubular endosomal membrane to which GRASP-1, syntaxin 13, and Rab4 localized. EE indicates early endosomes and scale bar is 100 nm.
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consistent with the kinetics of internalized AMPAR colocalization with Rab4 [9].

Next, we transfected hippocampal neurons either with GFP and control vector or GFP with GRASP-1-shRNA and analyzed internalization and recycling of endogenous AMPAR following AMPA stimulation by immunolabeling for surface GluR1 and GluR2. At steady state, GRASP-1 knock-down neurons showed a modest but significant reduction (~15%) in surface labeling for GluR1 (Figure 6B,D) and GluR2 (Figure 6C,E) compared to controls. After 10 min of stimulation, GluR1 and GluR2 decreased at the neuronal surface in both control and GRASP-1 shRNA expressing neurons, reflecting receptor internalization (Figure 6B,C). At 60 min, reappearance of both GluR1 and GluR2 was strongly impaired (~50%) by GRASP-1 shRNA compared to controls (Figure 6B–E). Consistently, in a protocol where surface HA-GluR2 receptors were stripped away after labeling [33], recycling of HA-GluR2 back to the surface was significantly decreased in neurons expressing GRASP-1-shRNA compared to control neurons (Figure 6F). No difference was observed in the level of intracellular HA-GluR2 after 8 min AMPA stimulation (Figure S4C,D). However, we observed that in GRASP-1 knock-down neurons, more intracellular HA-GluR2 is

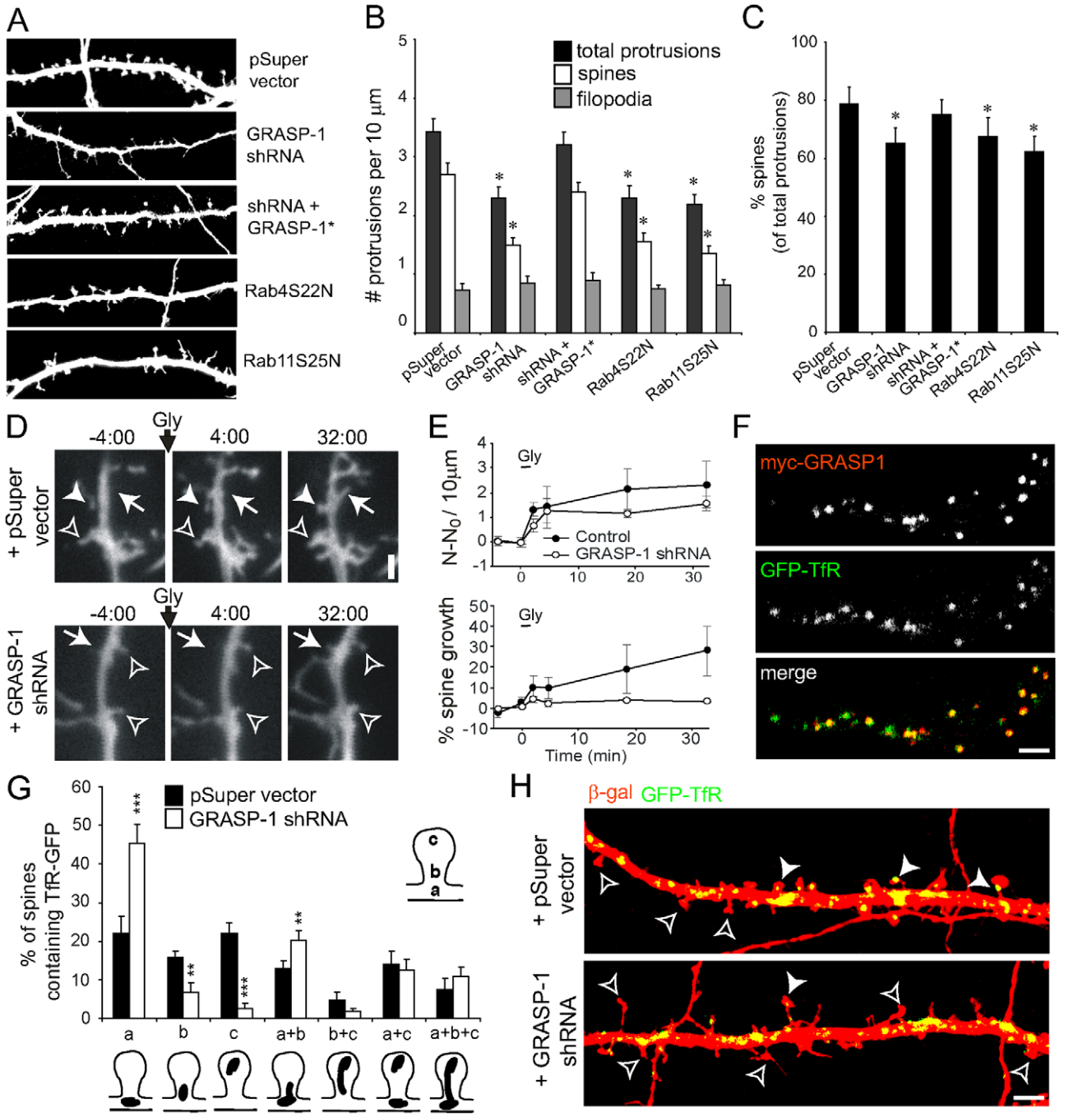

Figure 5. GRASP-1 is required for the maintenance of dendritic spines. (A) Representative high magnification images of dendrites of hippocampal neurons co-transfected at DIV13 for 4 d with β-galactosidase (to mark the dendrites), and either pSuper, pSuper-GRASP-1-shRNA#2, GRASP-1-shRNA#2 and GFP-GRASP-1*, Rab4S22N or Rab11S25N, and labeled with anti-β-galactosidase. (B) Quantification of number of protrusions per 10 μm dendrites in hippocampal neurons transfected as indicated in (A). (C) Percentage of spines of hippocampal neurons transfected as indicated in (A). (D) Neurons expressing GFP (to mark the dendrite), and either pSuper or pSuper-GRASP-1-shRNA#2 were stimulated with glycine (200 mM, 3 min), and then imaged for >30 min after glycine stimulation. Arrows indicated spine formation. Closed and open arrowheads represent spine growth and stable protrusions, respectively. (E) Quantification of protrusion formation (top) and spine growth (bottom) following glycine stimulation. N, number of dendritic protrusions per 10 μm at the indicated time; N_0, average number of dendritic protrusions per 10 μm before application of glycine. Spine growth was probed as the change in sum of spine widths per 10 μm and comprises both addition of new spines and growth of pre-existing spines. Glycine-stimulated spine growth is blocked by GRASP-1-shRNA#2 (bottom). (F) High magnification images of dendrites of hippocampal neurons cotransfected at DIV13 for 4 d with myc-GRASP-1 (red) and GFP-TfR. (G,H) Percentage of spines containing TfR-GFP positive endosomes at the indicated locations. Hippocampal neurons were co-transfected at DIV13 for 4 d with β-galactosidase (to mark dendrites) and GFP-TfR (to mark endosomes) and pSuper control vector or pSuper-GRASP-1-shRNA#2 as shown in (H). Closed and open arrowheads denote protrusions with and without GFP-TfR marked endosomes in the spine head, respectively. Error bars indicate S.E.M. ** $p<0.005$. *** $p<0.0005$. Bar is 1 μm.

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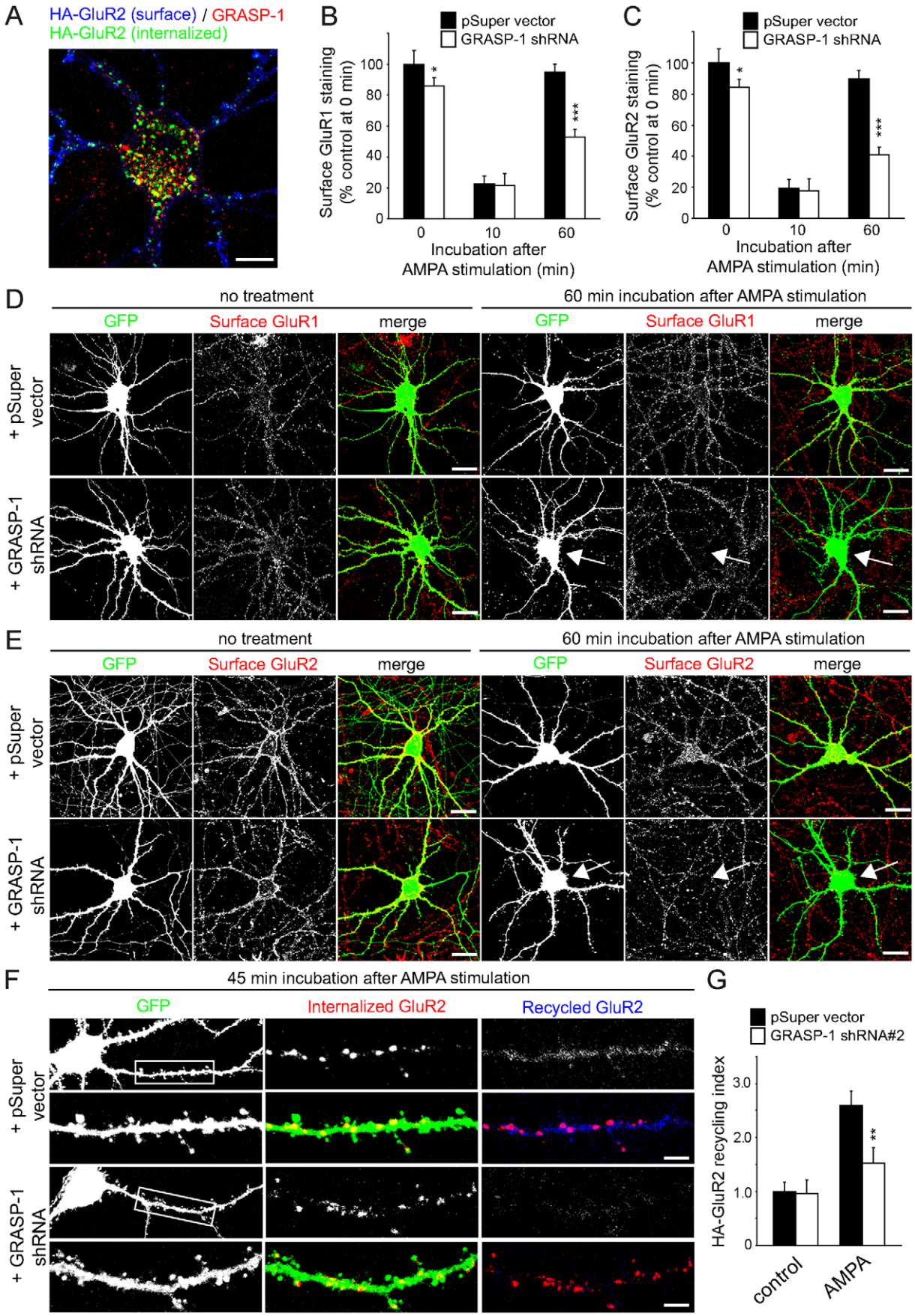
A
HA-GluR2 (surface) / GRASP-1
HA-GluR2 (internalized)
B
pSuper vector
GRASP-1 shRNA
Surface GluR1 staining (% control at 0 min)
Incubation after AMPA stimulation (min)
C
pSuper vector
GRASP-1 shRNA
Surface GluR2 staining (% control at 0 min)
Incubation after AMPA stimulation (min)
D
no treatment
60 min incubation after AMPA stimulation
GFP
Surface GluR1
merge
+ pSuper vector
+ GRASP-1 shRNA
E
no treatment
60 min incubation after AMPA stimulation
GFP
Surface GluR2
merge
+ pSuper vector
+ GRASP-1 shRNA
F
45 min incubation after AMPA stimulation
GFP
Internalized GluR2
Recycled GluR2
+ pSuper vector
+ GRASP-1 shRNA
G
pSuper vector
GRASP-1 shRNA#2
HA-GluR2 recycling index
control
AMPA

Figure 6. Knock-down of GRASP-1 reduces AMPAR recycling. (A) Representative merge image of surface HA-GluR2 (blue) and internalized HA-GluR2 (green) in soma and dendrites of hippocampal neurons labeled for GRASP-1 (red) after 10 min AMPA stimulation. Bar is 10 μm. (B,C) Quantification of the surface fluorescence intensities of endogenous GluR1 (B) and GluR2 (C) in control pSuper vector or GRASP-1-shRNA#2 transfected neurons. The cells were untreated (0 min) or stimulated with AMPA for indicated times. Histograms show fluorescent intensity of surface GluR subunit staining relative to the intensity of GFP transfected control neurons at basal levels. $n = 20$ cells for each group. (D,E) Representative images of hippocampal neurons stained for endogenous surface GluR1 (D) and GluR2 (E). Hippocampal neurons at DIV13 were cotransfected with GFP and pSuper control vector or GRASP-1-shRNA#2. At DIV17, neurons were fixed (0 min, no treatment) or stimulated for 2 min with 100 μM AMPA in the presence of 50 μM APV and further incubated for a total of 10 or 60 min before fixation. Endogenous surface GluR1 (D) or GluR2 (E) was revealed by immunofluorescence labeling without permeabilization using specific extracellular AMPAR antibodies. Bar is 20 μm. (F) Neurons transfected with GFP, HA-GluR2, and either pSuper control vector or GRASP-1-shRNA#2 were stained live with an anti-HA antibody, stimulated for 2 min with AMPA/APV, acid stripped, and incubated in conditioned media for 45 min. Recycled HA-GluR2 (blue) and internalized HA-GluR2 (red) were sequentially labeled. Bar is 1 μm. (G) Quantification of the ratio of recycled to internalized HA-GluR2 and normalized to unstimulated wild-type control neurons (HA-GluR2 recycling index) as indicated in (F). Error bars indicate S.E.M. * $p<0.05$. ** $p<0.005$. *** $p<0.0005$.
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present in LAMP-1 positive lysosomal compartments after AMPA treatment (Figure S4E,F). These data show that GRASP-1 is important for activity-induced AMPAR recycling.

GRASP-1 Regulates Synaptic Plasticity

Next we examined the role of GRASP-1 in excitatory transmission and LTP and recorded excitatory synaptic responses from CA1 pyramidal neurons in organotypic cultures of hippocampal slices. Simultaneous recordings were obtained from both transfected neurons (identified by cotransfected GFP) and a neighboring untransfected neuron. Both control luciferase-shRNA and GRASP-1-shRNA expressing cells had no effect on basal AMPAR-mediated excitatory postsynaptic currents (EPSCs) (GRASP-1 shRNA#5: 0.93±0.09-fold relative to untransfected cells, luciferase shRNA: 1.21±0.18) and NMDAR-EPSCs (GRASP-1 shRNA#5: 0.86±0.09-fold, luciferase shRNA: 1.03±0.32) (Figure 7A,B). The importance of GRASP-1-mediated AMPAR recycling in slices became more evident by testing for synaptic plasticity. After induction of LTP, cells expressing GRASP-1 shRNA induced comparable levels of potentiation to that of neighboring untransfected cells up to 20 min after the LTP induction protocol. Subsequently, however, the response from GRASP-1 shRNA transfected cells started to fall and eventually returned to the baseline level at 30 min after LTP induction (Figure 7C, untransfected neuron: 1.75±0.18-fold enhancement of EPSC at 29–30 min after LTP induction, transfected neuron: 1.17±0.10). In contrast, control luciferase shRNA transfected, and neighboring untransfected neurons expressed stable LTP lasting for at least 30 min (Figure 7D, untransfected neuron: 2.04±0.16-fold enhancement of EPSC, transfected neuron: 2.45±0.44). These data indicate that GRASP-1 is important for synaptic plasticity and particularly for the phase of LTP after the first 20 min. The results suggest that delivery of AMPAR from recycling endosomes might be important for this later phase of LTP.

GRASP-1 Segregates Rab4 from EEA1/Neep21 Endosomal Membranes

To define more precisely the function of GRASP-1 within the endosomal system, we first examined the localization of exogenous GRASP-1 with respect to early endosomal marker proteins in Hela cells. We found little if any co-distribution with GFP-Rab5 but extensive colocalization with GFP-Rab4 (Figure S5). The same results were obtained in transfected hippocampal neurons, where >80% of Rab4 structures contained GRASP-1 both in dendrites and the cell body, while little overlap was seen with Rab5 (Figures 8A,B, S6). In agreement with this observation, the Rab5 domain marker EEA1 and endogenous GRASP-1 displayed mutually exclusive distributions (Figure 8D), whereas ~40% of EEA1 structures in the cell body and dendrites colocalized with GFP-Rab4 (Figure 8C,E, top row). These results suggested that Rab4 in neurons is interfaced between a proximal EEA1 and distal GRASP-1 endosomal domain.

To determine whether the endosomal domain organization is regulated by GRASP-1, we knocked down the expression of GRASP-1 and then assayed the co-distribution of EEA1 and GFP-Rab4. Hippocampal neurons transfected with GRASP-1-shRNA showed a strong increase in colocalized EEA1 and GFP-Rab4 (~80%) compared to control neurons (~40%) (Figure 8C,E). In contrast, in neurons transfected with myc-GRASP-1, the overlap between EEA1 and GFP-Rab4 was significantly decreased (~20%) (Figure 8C,E). Similar results were obtained in Hela cells, where myc-GRASP-1 strongly reduced colocalization between GFP-Rab4 and EEA1, while the co-distribution of GFP-Rab5 and EEA1 was not affected (Figure S7). To confirm our results we tested the effect of GRASP-1 on the localization of other early endosomal markers, such as Neep21 [13]. Endogenous Neep21 staining strongly coincides with Rab5 and EEA1 (~80%) and to a lesser extent with Rab4 (~40%) (Figure S8 and unpublished data). However in neurons transfected with myc-GRASP-1 the overlap between Neep21 and GFP-Rab4 was significantly reduced (~20%), consistent with the effect on EEA1 distribution (Figure S8D). In contrast, GRASP-1-shRNA enhances Neep21/Rab4 colocalization (Figure S8D). Together these results suggest that GRASP-1 is able to separate Rab4 from EEA1/Neep21 endosomal domains.

GRASP-1 Regulates the Coupling between Rab4 and Rab11 Domains

We next determined GRASP-1 localization with respect to late and recycling endosomal markers in Hela cells (Figure S6) and hippocampal neurons (Figure 9A). We found little GRASP-1 colocalization with the Rab7 endosomal domains, whereas GRASP-1 labeling coincided extensively with Rab11-positive compartments (~70%) (Figures 9A,C, S6). These data strongly suggest that GRASP-1 is localized to distal aspects of the endosomal recycling pathway and might serve to couple Rab4 and Rab11 domains. This observation was confirmed by simultaneous dual color live imaging of mRFP-GRASP-1 and GFP-Rab11: GRASP-1 and Rab11 colocalize on larger endosomal domains, while dynamic Rab11-positive structures segregate into distinct tubular or vesicular structures (Figure 9B; Videos S3 and S4). Most motile Rab11-positive tubules only transiently overlap with GRASP-1-positive endosomes (Videos S3 and S4). Rab4, Rab11, and GRASP-1 largely localized to overlapping regions on these large endosomal structures in the neuronal cell bodies and dendrites (Figure 9E). We further explored a possible role for GRASP-1 in coupling Rab4 and Rab11 domains by determining the Rab4/Rab11 co-distribution when GRASP-1 was knocked down as well as after overexpression of myc-GRASP-1. In