NMDA receptor-dependent activation of the small GTPase Rab5 drives the removal of synaptic AMPA receptors during hippocampal LTD

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ABSTRACT

The activity-dependent removal of AMPA receptors from synapses underlies long-term depression in hippocampal excitatory synapses. In this study we have investigated the role of the small GTPase Rab5 during this process. We propose that Rab5 is a critical link between the signaling cascades triggered by LTD induction, and the machinery that executes the activity-dependent removal of AMPA receptors. We have found that Rab5 activation drives the specific internalization of synaptic AMPA receptors in a clathrin-dependent manner, and that this activity is required for LTD. Interestingly, Rab5 does not participate in the constitutive cycling of AMPA receptors. Rab5 is able to remove both GluR1 and GluR2 AMPA receptor subunits, leading to GluR1 dephosphorylation. Importantly, NMDA receptor-dependent LTD induction produces a rapid and transient increase of active (GTP-bound) Rab5. We propose a model in which synaptic activity leads to Rab5 activation, which in turn drives the removal of AMPA receptors from synapses.

INTRODUCTION

Synaptic connections between neurons in the hippocampus and other brain regions are continuously remodeled in response to neuronal activity. This process, known as synaptic plasticity, is widely thought as the cellular correlate for cognitive functions, such as learning and memory (Bliss and Collingridge, 1993). In particular, short bursts of synaptic activity can lead to long-lasting changes in synaptic efficacy, known as long-term depression (LTD) and long-term potentiation (LTP). Recent studies have concluded that the regulated movement of AMPA-type glutamate receptors (AMPARs) in and out of synapses is an important factor contributing to the changes in synaptic strength that occur during these types of activity-dependent synaptic plasticity. This topic has been the focus of several recent reviews (Barry and Ziff, 2002; Carroll et al., 2001; Malinow and Malenka, 2002; Sheng and Kim, 2002).

AMPARs are hetero-tetramers composed of different combinations of GluR1, GluR2, GluR3, and GluR4 subunits (Hollmann and Heinemann, 1994). The trafficking of AMPARs at synapses is regulated by the subunits' cytoplasmic carboxyl terminus (Hayashi et al., 2000; Passafaro et al., 2001; Shi et al., 2001). GluR2/GluR3-containing receptors continuously cycle in and out of synapses in an activity-independent manner that requires NSF (Luscher et al., 1999; Nishimune et al., 1998; Song et al., 1998) and Hsp90 (Gerges et al., 2004b) function. In contrast, GluR1/GluR2-containing receptors are incorporated into synapses during synaptic plasticity leading to long-term synaptic potentiation (LTP). These two types of trafficking for receptor delivery have been termed constitutive and regulated pathways, respectively (Malinow et al., 2000). Conversely, activity-dependent removal of AMPARs

leads to long-term depression (LTD) in these synapses (Carroll et al., 2001). However, the role of subunit composition during this regulated removal is still unclear.

Although these studies have shown that the regulation of AMPAR function is a critical component for synaptic plasticity, the molecular and cellular mechanisms that govern AMPAR trafficking at synapses are far from clear. In particular, it remains to be elucidated how the signaling cascades that are triggered upon plasticity induction result in AMPAR transport in and out of synapses. The regulated removal of AMPARs during LTD has been proposed to be triggered by activation of different intracellular signaling cascades in different cell types, such as PKC in cerebellum (Xia et al., 2000) and hippocampus (Chung et al., 2000), PKA in dopamine neurons of the ventral tegmental area (Gutlerner et al., 2002), and mitogen-activated protein kinase 38 (p38 MAPK) in hippocampus (Huang et al., 2004; Zhu et al., 2002). In addition, protein phosphatases play a critical role in LTD (Mulkey et al., 1994; Mulkey et al., 1993; Zeng et al., 2001) and AMPAR endocytosis (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000) in the hippocampus. From a mechanistic point of view, regulated AMPAR removal is mediated by clathrin-dependent endocytosis (Carroll et al., 1999a; Man et al., 2000; Wang and Linden, 2000) through the interaction between the GluR2 subunit of the AMPAR and the clathrin adaptor protein AP2 (Lee et al., 2002). However, it is not yet understood how the signaling cascades that mediate these forms of LTD lead to the specific clathrin-mediated internalization of AMPARs from synapses. We believe this gap in our understanding is due to the scarce available information on the cellular machinery that controls the membrane transport and sorting of AMPARs during plasticity.

The members of the Rab family of small GTPases are key mediators of intracellular membrane sorting in eukaryotic cells (Pfeffer, 2001; Zerial and McBride, 2001), and they

have been implicated in the pathology of multiple human diseases (Seabra et al., 2002), including X-linked mental retardation (D'Adamo et al., 1998). One member of the family, Rab5, has been implicated in protein transport from plasma membrane to early endosomes during clathrin-dependent endocytosis in a variety of cellular systems (Mohrmann and van der Sluijs, 1999), including hippocampal neurons (de Hoop et al., 1994; Kanaani et al., 2004). Rab5 activity has also been linked to actin remodeling and cell motility (Lanzetti et al., 2004; Spaargaren and Bos, 1999). However, to our knowledge, the role of Rab5 in synaptic function has not yet been studied.

In this study, we explore the possibility that Rab5 is a critical link between the signaling cascades triggered by LTD induction and the regulated removal of AMPARs from synapses. We have found that Rab5 removes AMPARs from excitatory CA1 synapses and that this removal is both necessary and sufficient for LTD. Importantly we also show that active Rab5 (GTP-bound) is rapidly and transiently up-regulated after NMDA receptor-dependent LTD induction. In contrast, Rab5 is not involved in the constitutive cycling of AMPARs, thus the internalization pathways for the activity-dependent removal and continuous cycling of AMPARs operate via distinct molecular mechanisms. Therefore, we conclude that Rab5 is an activity-regulated component of the endocytic machinery that mediates AMPAR removal from synapses during LTD.

RESULTS

Localization of Rab5 at CA1 hippocampal synapses

Previous work has shown that Rab5 is present in axonal and dendritic compartments in hippocampal neurons (de Hoop et al., 1994) and in synaptic vesicle preparations from brain homogenates (Fischer von Mollard et al., 1994). However, its ultrastructural localization with respect to synaptic sites has never been evaluated. We used post-imbedding immunogold electron microscopy (see Experimental Procedures) to determine the synaptic location of endogenous Rab5 in hippocampal CA1 neurons. Most synaptic Rab5 immunolabeling was found in the postsynaptic terminal (≈75%; Fig. 1A, arrows; Fig. 1B, "POST"). Furthermore, we found that postsynaptic Rab5 labeling was predominantly located outside of the postsynaptic density (PSD) on lateral extrasynaptic membranes (Fig. 1B, "Memb."), roughly 100-300 nm away from the edge of the PSD (Fig. 1C). This localization fits well with "endocytic hotspots" previously described lateral to the PSD (Blanpied et al., 2002; Petralia et al., 2003; Racz et al., 2004).

To examine the postsynaptic function of Rab5 in hippocampal CA1 neurons, we used the Sindbis virus expression system to overexpress Rab5-GFP constructs in organotypic slices of rat hippocampus. Rab5-GFP was well expressed in this system, as assayed by Western blot analysis (Fig. 1D). We have used this system to express both wild type (wt) and dominant negative (dn) forms of Rab5-GFP. As shown in Fig. 1E, most endogenous Rab5 is associated to membrane compartments, due to the covalent addition of geranylgeranyl groups to two cysteines at the Rab5 C-terminus (Seabra, 1998). Recombinant Rab5(wt) is also membrane-bound, although to a lower extent than the endogenous protein. This is probably

due to the overexpression levels, which may saturate the endogenous geranylgeranyl transferases. In contrast, Rab5(dn) was predominantly cytosolic, in agreement with the association of GDP-bound Rab5 to Rab GDI in the cytosol (Ullrich et al., 1994). Confocal imaging of infected CA1 neurons showed ubiquitous distribution of Rab5(wt)-GFP, including distal dendrites and spines (Fig. 1F) (a similar expression was found for Rab5(dn)-GFP; not shown). In addition, the punctate distribution displayed by Rab5-GFP in cell body and proximal dendrites closely resembles the pattern recently described for endocytic zones using a clathrin-GFP fusion protein (Blanpied et al., 2002).

Rab5 overexpression specifically depresses AMPA receptor-mediated synaptic responses

As a first step to evaluate the role of Rab5 in excitatory synaptic function, we performed simultaneous double whole-cell electrophysiological recordings from nearby pairs of infected and control, non-infected, CA1 neurons. Excitatory postsynaptic currents (EPSCs) were evoked by stimulation of the afferent Schaffer collateral fibers. Neurons overexpressing wild-type Rab5-GFP, Rab5(wt), displayed a significantly reduced AMPAR current ($p=0.02$; Fig. 2A), but no change in the NMDA EPSC, relative to control (Fig. 2B). This effect was also reflected by a marked reduction in the AMPA/NMDA ratio ($p=0.05$; Fig. 2C). These results suggest that Rab5 is sufficient to trigger AMPAR removal from synapses, and that this effect, at least at excitatory CA1 synapses, is specific for AMPA *versus* NMDA receptors. In contrast, neurons overexpressing a dominant negative Rab5 construct, Rab5(dn), exhibited no change in the AMPA or NMDA responses, or in the AMPA/NMDA ratio (Fig. 2D-F). Similarly, overexpression of another wild-type Rab protein, Rab8, or

dominant negative forms of Rab4 and Rab11 did not alter AMPA or NMDA responses either (Gerges et al., 2004a). These results verify that the depression of AMPAR responses observed with Rab5(wt) is not due to virus infection or non-specific sequestration of regulatory proteins, like GTP/GDP exchange factors (GEFs) or GDP dissociation inhibitors (GDIs). In addition, the absence of enhancement of AMPAR responses in neurons expressing Rab5(dn) suggests that Rab5 is not involved in the continuous cycling of AMPARs (see also Fig. 6). These results were confirmed using miniature current recordings (see Supplementary Information).

Rab5 removes AMPA receptors from the surface of dendritic spines

Rab5 has been shown to mediate endocytosis of a variety of proteins from the plasma membrane (for review see (Zerial and McBride, 2001)). We first determined that Rab5 overexpression leads to the internalization of AMPARs from the neuronal surface, using a surface crosslinking assay (see Supplementary Information). Then, in order to morphologically evaluate the subcellular localization of AMPAR internalization mediated by Rab5, we carried out a quantitative surface immunostaining assay in hippocampal slices. We used biolistic gene delivery to co-express a GFP-tagged GluR2 AMPAR subunit and Rab5(wt) tagged with a red fluorescence protein (Rab5(wt)-RFP, see Experimental Procedures) (the functionality of Rab5(wt)-RFP was confirmed electrophysiologically; see below in description for Fig. 6). The surface distribution of the recombinant receptor was assessed by immunostaining with an anti-GFP antibody coupled to an infrared fluorophore (Cy5) in non-permeabilized conditions (the GFP tag is placed at the extracellular, N-terminus, of the receptor). Therefore, this experimental design allows us to monitor the total

amount of receptor (GFP channel), the fraction exposed to the surface (Cy5 channel) and the presence of the co-expressed Rab5(wt) (RFP channel) (see Fig. 3A as an example).

To assess local AMPAR endocytosis from synaptic sites, we quantified GFP and Cy5 fluorescence intensities across spine heads and adjacent dendritic shafts using line plots (Gerges et al., 2004a). Surface ratios are then calculated for spine and dendrites by dividing Cy5 and GFP fluorescence peaks after background subtraction (see Fig. 3B). When GluR2-GFP was expressed alone, the surface ratio at spines was significantly higher than at the adjacent dendritic shaft (Fig. 3C, left). In contrast, co-expression of Rab5(wt) with GluR2-GFP strongly reduced surface ratio at spines, which became lower than the surface ratio at dendrites (Fig. 3C, middle). This is also reflected in the fraction of spines that had lower surface ratios than their adjacent dendrites: 30% in the absence of Rab5(wt) *versus* 70% in its presence (cumulative probability values for spine/dendrite=1; Fig. 3C, right, dashed lines). Therefore, these data indicate that Rab5 removes AMPARs locally from the spine plasma membrane, where synapses are located. To note, these results do not imply that Rab5 internalizes AMPARs exclusively from spines; however, they do show that there is a preferential removal from the spine surface, as compared to the adjacent dendritic plasma membrane. Interestingly, Rab5(wt) did not decrease the total amount of receptor (GFP channel) in the spine as compared to the dendrite (Fig. 3D). This result suggests that Rab5 does not transport AMPARs from the spine into the dendritic shaft, and supports the interpretation that Rab5 is involved in a local trafficking step from the plasma membrane of the postsynaptic terminal into an intracellular compartment inside the spine.

Rab5 mediates long-term synaptic depression (LTD)

To examine whether Rab5 activity is involved in the removal of synaptic AMPARs that accompanies long-term depression, we induced LTD according to a pairing protocol (see Experimental Procedures) in neurons expressing Rab5(dn), Rab5(wt) and non-infected, control neurons. These experiments were carried out blind with respect to the protein being expressed. Non-infected neurons displayed a statistically significant LTD, relative to the unpaired pathway that did not receive LTD stimuli ($p<0.0001$, Fig. 4). In contrast, neurons expressing Rab5(dn) failed to exhibit any long-lasting depression of AMPAR-mediated transmission (Fig. 4). This result indicates that activation of Rab5 is necessary for LTD at CA1 synapses.

We have shown that Rab5 overexpression leads to depression of synaptic AMPA responses (Fig. 2A and Supplementary Fig. 1). Therefore, we examined whether increasing Rab5 levels could occlude LTD. Indeed, LTD-inducing stimuli on neurons overexpressing Rab5(wt) failed to elicit any long-term depression of AMPAR responses (Fig. 4). This result indicates that expression of Rab5(wt) mimics and occludes LTD, and suggests that Rab5 removes the same population of receptors that is affected by LTD. These results indicate that Rab5 activity is both necessary and sufficient to elicit long-term depression at hippocampal CA1 synapses.

Rapid and transient up-regulation of Rab5-GTP upon LTD induction

Previous studies have shown that Rab5 association with Rab-GDI can be regulated by p38 MAPK (Cavalli et al., 2001; Huang et al., 2004). This scenario implies that p38 MAPK

is potentially able to modulate Rab5 function. Therefore we tested whether LTD induction triggers the p38 MAPK cascade and leads to Rab5 activation. These experiments were carried out using a "chemical LTD" protocol (Lee et al., 1998) to maximize the number of synapses undergoing plasticity. As shown in Fig. 5A, a brief application of 20 μM NMDA produces a long-lasting depression of field excitatory responses recorded from acute hippocampal slices (see Experimental Procedures). This form of LTD is dependent on NMDA receptor activation, since it is prevented by preincubation with the NMDA receptor antagonist AP5 (Fig. 5B) (see also (Lee et al., 1998)).

In order to monitor the levels of active (GTP-bound) Rab5 during LTD induction, we designed a GST "pull-down" assay using the C-terminus (last 74 amino acids) of the Rab5 effector protein, rabaptin-5, which specifically binds Rab5-GTP (Vitale et al., 1998). To evaluate the specificity of the GST fusion protein, we preloaded hippocampal extracts with a non-hydrolysable GTP analog or with GDP, followed by incubation with beads containing either GST alone or GST fused to the Rab5-binding domain (GST-R5BD) (see Experimental Procedures). As shown in Fig. 5C (left panels), GST-R5BD specifically binds to Rab5-GTP, with only a weak binding to Rab5-GDP. Also, the low amount of Rab5 pulled down directly from hippocampal extracts (without nucleotide preloading) indicates that the levels of Rab5-GTP in basal conditions in the hippocampus are very low. As a control, GST alone did not bind detectable amounts of Rab5 (Fig. 5C, right panels).

We then examined the time-course of Rab5 activation during NMDA receptor-dependent LTD. We found that the amount of active, Rab5-GTP is rapidly and transiently up-regulated during LTD induction. Specifically, the amount of Rab5-GTP doubled during the NMDA treatment, relative to untreated, control slices (Fig. 5D, E, t= 2.5 min). This

increase was blocked by AP5, and therefore was dependent on NMDA receptor activation (Fig. 5D, E, "+AP5"). Interestingly, Rab5-GTP levels rapidly declined by the end of the NMDA treatment and during wash out, and returned to basal levels at the end of the time course (Fig. 5E, t=5-20 min). In contrast, phophorylation of p38 MAPK slowly increases during LTD induction and is maintained for at least 15 minutes after NMDA treatment (Fig. 5D, E–inset). These results indicate that LTD induction leads to p38 MAPK phosphorylation (as previously observed (Bolshakov et al., 2000)) and, importantly, to the activation of Rab5. This is, to our knowledge, the first reported evidence for an activity-dependent up-regulation of the endocytic machinery responsible for the removal of AMPARs during LTD. On the other hand, the differing time courses for p38 MAPK phosphorylation and Rab5-GTP formation do not necessarily support a role for the p38 MAPK cascade in Rab5 activation.

Rab5 is not involved in the constitutive synaptic cycling of AMPA receptors

The constitutive cycling of AMPARs into synapses depends on a direct interaction between GluR2 and NSF (N-ethylmaleimide sensitive factor). When this interaction is prevented by intracellular perfusion of a peptide containing the NSF-binding sequence of GluR2 (pep2m/G10), AMPAR-mediated responses rapidly decline, as the receptors continue to be internalized, but failed to be reinserted into synapses (Luscher et al., 1999; Nishimune et al., 1998; Song et al., 1998). Therefore, this peptide provides a valuable tool to monitor the constitutive, activity-independent endocytosis of AMPARs, and the possible role of Rab5 in this process.

We carried out whole-cell recordings on uninfected, Rab5(wt)- and Rab5(dn)-expressing CA1 neurons while they were loaded intracellularly with the pep2m peptide. As

expected, control cells showed a fast "run down" of synaptic transmission, reflecting the constitutive internalization of AMPARs ($p<0.05$; Fig. 6A, C). The decline of AMPAR responses produced by the peptide was essentially identical in neurons overexpressing Rab5(dn) (Fig. 6A, C), indicating that Rab5 activity is not required for the continuous endocytosis of AMPARs during their constitutive cycling. Cells expressing Rab5(wt) also displayed "run down" of AMPA transmission ($p=0.02$ relative to baseline), which was even more pronounced than that of Rab5(dn) or uninfected cells. This result is compatible with Rab5 removing both cycling and non-cycling populations of AMPARs, although with some preference for non-cycling receptors. As a control, similar recordings using a peptide (pep4c) corresponding to the homologous GluR4c sequence which does not bind NSF (Nishimune et al., 1998) did not produce "run down" in either uninfected or Rab5(wt)-expressing neurons (Fig. 6B, C).

Rab5 drives the removal of both GluR1 and GluR2 receptors

It has been demonstrated that the population of AMPARs that undergoes constitutive cycling is composed of GluR2/GluR3 subunits (Passafaro et al., 2001; Shi et al., 2001). In contrast, it remains unclear what population of receptors is internalized during LTD. Since Rab5 seems to exclusively mediate the regulated endocytosis of AMPARs, we tested what population of receptors is susceptible to Rab5-driven internalization. We evaluated the presence of recombinant AMPARs at synapses using an electrophysiological tag. In this assay, an increase in the rectification index of synaptic transmission (defined here as the ratio between AMPAR currents at holding potentials of -60 mV and +40 mV) indicates that homomeric recombinant AMPARs have been incorporated into synapses (Hayashi et al.,

2000) (see Experimental Procedures). We used the gene gun transfection system to express recombinant AMPARs alone or with Rab5(wt). As previously reported (Shi et al., 2001), neurons transfected with GluR2(R607Q) exhibited a statistically significant rectification, relative to control, non-transfected neurons ($p=0.001$; Fig. 6D). In contrast, simultaneous transfection of GluR2(R607Q) with Rab5(wt) blocked this rectification ($p=0.003$, relative to GluR2(R607Q) alone; Fig. 6D). Similar rectification values were obtained by co-expressing Rab5(wt)-GFP (2.1±0.2, n=10) or Rab5(wt)-RFP (2.2±0.2, n=8). This result suggests that Rab5 removes GluR2 from synapses.

Homomeric GluR1 receptors can be delivered into synapses by coexpression with a constitutively active CaMKII (tCaMKII), which mimics long-term potentiation (Hayashi et al., 2000). As previously reported, simultaneous transfection of GluR1 and tCaMKII resulted in an increase in rectification, compared to controls ($p=0.008$; Fig. 6E). To examine whether Rab5(wt) could also remove recombinant GluR1, we transfected neurons with Rab5(wt), GluR1, and tCaMKII. The addition of Rab5(wt) abolished the observed rectification due to synaptic delivery of GluR1 ($p=0.01$, relative to GluR1 plus tCaMKII; Fig. 6E). These results suggest that Rab5(wt) is capable of removing both GluR1- and GluR2-containing AMPARs from CA1 synapses, in agreement with our previous data (Fig. 6A-C) monitoring internalization of cycling and non-cycling endogenous receptors.

Rab5-driven internalization of AMPA receptors is mediated by clathrin

It has been previously shown that the C-terminus of GluR2 interacts with the clathrin adaptor protein, AP2, and that this interaction is necessary for LTD in CA1 neurons (Lee et al., 2002). To further explore the role of Rab5 in this process, we tested whether Rab5 would

internalize a mutant GluR2 receptor that does not bind AP2: GluR2(R845A) (Lee et al., 2002). As expected, the double mutant GluR2(R607Q, R845A) formed homomeric receptors that were detectable at synapses via an increase in rectification, relative to controls ($p=0.001$; Fig. 6F). Interestingly co-expression of GluR2(R607Q, R845A) and Rab5(wt) did not block, and indeed enhanced, the increase in rectification as compared with GluR2(R607Q, R845A) alone ($p=0.046$; Fig. 6F). This result indicates that the Rab5-mediated removal of AMPARs from synapses requires the interaction with the clathrin-dependent endocytic machinery. Incidentally, the enhancement of rectification observed by coexpressing Rab5(wt) and GluR2(R607Q, R845A) is consistent with Rab5 removing endogenous (non-rectifying) receptors, while leaving unaffected the mutated recombinant receptors. Also, this result verifies that Rab5 overexpression does not interfere with receptor insertion at synapses or with other cellular processes not related to clathrin-dependent endocytosis.

Rab5-mediated removal of AMPA receptors results in GluR1 dephosphorylation

Previous studies have shown that LTD in CA1 neurons is associated with dephosphorylation of the GluR1 AMPAR subunit (Lee et al., 2000; Lee et al., 1998). To examine the role of Rab5-driven endocytosis on GluR1 phosphorylation, we prepared homogenates of microdissected CA1 regions from Rab5(wt) and control uninfected slice cultures and assayed the phosphorylation levels of S845 and S831 of GluR1. Extracts from Rab5(wt)-overexpressing slices showed a significant decrease in phosphorylation of GluR1 S845 and S831 relative to controls ($p=0.008$ and $p=0.04$, respectively; Fig. 7A, C). These results indicate that Rab5-mediated internalization of AMPARs results in GluR1 dephosphorylation, and therefore, they suggest that GluR1 dephosphorylation occurs

downstream of AMPAR removal from synapses. As controls, neither PKA activity nor phosphorylation of αCaMKII at T286 were affected by Rab5(wt) overexpression (Fig. 7A-C).

Several recent studies have shown that GluR2 phosphorylation at S880 promotes receptor internalization and accompanies LTD (Chung et al., 2000; Kim et al., 2001; Xia et al., 2000). To examine the role of GluR2 phosphorylation during Rab5-driven receptor internalization, we assayed the phosphorylation levels of S880 GluR2 in slices overexpressing Rab5(wt), as described above. No significant change in phosphorylation of GluR2 S880 was observed upon Rab5 overexpression (Fig. 7A, C). These results suggest that phosphorylation of GluR2 S880 during LTD occurs upstream of or independent from Rab5 mediated removal of AMPARs.

DISCUSSION

In this study we have identified a small GTPase of the Rab family, Rab5, as a critical link between the signaling cascades that mediate LTD and the clathrin-dependent endocytic machinery that executes AMPAR removal from synapses. This is based on four main lines of evidence. First, Rab5 function is necessary for synaptically evoked LTD, and Rab5 overexpression is sufficient to drive AMPAR removal from synapses, occluding further LTD expression. Second, Rab5 is activated during LTD induction, downstream from NMDAR opening. Third, Rab5 is located at the right place to mediate AMPAR synaptic removal, that is, at "endocytic hotspots" lateral from the PSD that have been shown to associate with AMPAR endocytosis (Petralia et al., 2003). And fourth, Rab5 preferentially removes AMPARs from the spine plasma membrane, where synapses are located.

These results allow us to propose a minimal model that accounts for the specific removal of AMPARs during NMDAR-dependent LTD. This model is schematized in Fig. 8. NMDAR opening would trigger several signal transduction cascades, which may include activation of p38 MAPK (Zhu et al., 2002). These signaling pathways would lead to activation of Rab5 at the plasma membrane, either through p38 MAPK phosphorylation of Rab-GDI and extraction of Rab5 from endosomal membranes (Cavalli et al., 2001; Huang et al., 2004) or through other unknown mechanisms. Once activated, Rab5 would trigger endocytosis by facilitating the formation of clathrin-coated endocytic pits and sorting of membrane proteins into endosomes (Bucci et al., 1992; McLauchlan et al., 1998).

The role of p38 MAPK in this pathway is intriguing, given its ability to promote Rab-GDI-Rab5 interaction (Cavalli et al., 2001; Huang et al., 2004) and its reported involvement

in AMPAR removal during LTD (Huang et al., 2004; Zhu et al., 2002). However, not all experimental evidence fits this scenario. For instance, the kinetics of Rab5 activation and p38 MAPK phosphorylation upon NMDA receptor activation are not correlated, to the point that maximal p38 MAPK phosphorylation is reached when Rab5-GTP levels are back to baseline. In addition, activation of the p38 MAPK pathway by Rap1 has been shown to remove exclusively the GluR2/GluR3 population of AMPARs (Zhu et al., 2002), whereas Rab5 activation internalizes both GluR1/GluR2 and GluR2/GluR3 populations (see below). Finally, the role of p38 MAPK in the different forms of hippocampal LTD is still controversial. The Rap1-p38 MAPK pathway has been proposed to mediate NMDA receptor-dependent LTD in CA1 synapses (Zhu et al., 2002). However, most investigations have linked p38 MAPK with hippocampal LTD induced by metabotropic glutamate receptors (mGluRs) (Bolshakov et al., 2000; Huang et al., 2004; Rush et al., 2002). Nevertheless, by experimentally showing that NMDAR activation leads to the up-regulation of Rab5-GTP and that Rab5 mediates LTD, we demonstrate that the endocytic machinery is not a passive mediator of AMPAR removal, but a regulated component in the signaling cascades that underlie synaptic plasticity.

This model clarifies several controversial aspects of AMPAR synaptic removal and LTD. For instance, both AMPARs (Carroll et al., 1999a) and NMDARs (Lavezzari et al., 2003) are internalized through clathrin-dependent endocytosis. Induction of LTD in hippocampal slices can lead to a marked reduction of both AMPA and NMDA receptor-mediated responses (Gean and Lin, 1993; Montgomery and Madison, 2002; Selig et al., 1995). Nonetheless, there are also multiple evidences for LTD-like manipulations that lead to the specific internalization of AMPARs without altering NMDARs (Carroll et al., 1999b;

Ehlers, 2000; Kim and Lisman, 2001; Luscher et al., 1999; Man et al., 2000). One possible explanation is that LTD induction leads to dissociation of the anchoring mechanisms that keep AMPARs at synapses, making them more susceptible for clathrin-mediated internalization. In fact, it has been shown that the presence of GluR2 at synapses is modulated by its phosphorylation-dependent association with the scaffolding molecules GRIP/ABP and PICK1 (Daw et al., 2000; Matsuda et al., 2000; Perez et al., 2001; Seidenman et al., 2003; Xia et al., 2000). However, this cannot be the sole mechanism mediating LTD, since GluR2 (Jia et al., 1996) and GluR2/3 (Meng et al., 2003) knockout animals still display LTD in the hippocampus. In addition, we show that cell-wide activation of the endocytic machinery through Rab5 overexpression, which bypasses the triggering of signaling mechanisms, still leads to the specific removal of AMPARs over NMDARs at excitatory CA1 synapses. Therefore, in the absence of LTD induction, AMPARs seem to be more amenable for clathrin-mediated internalization. The simplest scenario to explain these results implies that AMPARs are intrinsically loose components of the synaptic membrane, and that they may diffuse constantly between synaptic and local extrasynaptic membranes, where clathrin-dependent endocytosis may take place (Fig. 8, upper left). This is supported by multiple biochemical studies showing that AMPARs are not strongly associated with the postsynaptic density (Lee et al., 2001; Sans et al., 2001), and by live imaging experiments where AMPARs displayed local lateral diffusion from the place of synaptic contact (Groc et al., 2004; Tardin et al., 2003). According to this interpretation, AMPARs would be constantly traveling in and out of lateral “endocytic hotspots”, but their internalization would only be executed when the endocytic machinery is activated, through Rab5-GTP formation, upon LTD induction (Fig. 8, right and lower left). This scenario fits well with the recently

reported rapid internalization of extrasynaptic AMPARs after NMDAR activation (Ashby et al., 2004).

The subunit composition of the AMPARs that undergo activity-dependent removal is another mechanistic aspect of LTD that is still debated. Regulated and constitutive pathways for AMPAR synaptic delivery act on different receptor populations according to their subunit composition (Malinow et al., 2000; Passafaro et al., 2001; Shi et al., 2001). Similarly, subunit-specific rules have been recently described for the redistribution of AMPARs after internalization (Lee et al., 2004). In contrast, the results that we present here do not support subunit specificity for the regulated endocytosis of AMPARs. In particular, we show that Rab5 is able to efficiently remove both recombinant GluR1 and GluR2 homomeric receptors, as well as cycling (GluR2/GluR3) and non-cycling (GluR1/GluR2) populations of endogenous AMPARs. According to the model shown in Fig. 8, we would expect that both GluR1/GluR2 and GluR2/GluR3 receptors diffuse locally between synaptic and extrasynaptic membranes. Then, once the Rab5-dependent endocytic machinery is activated upon LTD induction, receptors would be internalized irrespective of their subunit composition. This interpretation is consistent with the combined observations that GluR2 homomers are removed from synapses during LTD (Seidenman et al., 2003), and that the GluR2/3 knockout, which presumably only contains GluR1 homomers at synapses, still displays LTD in the hippocampus (Meng et al., 2003). An indiscriminate removal of AMPARs during LTD is also supported by the fact that GluR1, GluR2 and GluR3 cytoplasmic C-termini bind the clathrin adaptor AP2 (Lee et al., 2002). This interaction, at least in the case of GluR2, is necessary for LTD (Lee et al., 2002). It is worth mentioning again that the Rap1-p38 MAPK pathway has been shown to internalize exclusively the

cycling (GluR2/GluR3) population of AMPARs (Zhu et al., 2002). However, multiple studies indicate that activation of NMDA receptors leads to the internalization of both GluR1- and GluR2-containing receptors (Beattie et al., 2000; Ehlers, 2000; Lee et al., 2004; Lin et al., 2000). Still, subunit-specific internalization pathways have been previously described, such as the GluR2-specific endocytosis induced by insulin in hippocampal neurons (Lin et al., 2000; Man et al., 2000), a process that depends on tyrosine phosphorylation of the GluR2 C-terminus (Ahmadian et al., 2004). Also, cerebellar LTD specifically requires PKC phosphorylation of GluR2 at Ser880 (Chung et al., 2003). These observations suggest that additional, subunit-specific mechanisms may exist to control the synaptic removal of AMPARs under different stimuli or in different brain regions.

Our results also provide new mechanistic information about the distinct endocytic machinery that acts on the regulated and constitutive internalization of AMPARs. We have shown that blocking Rab5 function with a dominant negative did not have any effect on the constitutive endocytosis of AMPARs, although the same construct completely blocked LTD expression. Additionally, it has been reported that the interaction between AMPARs and the clathrin adaptor AP2 is required for LTD, but not for constitutive internalization (Lee et al., 2002). This fits well with our observation that Rab5-driven removal of GluR2 requires an intact AP2 binding site in the AMPAR subunit. Rab5 is known to participate in clathrin-dependent endocytosis (Bucci et al., 1992), and it is not required for clathrin-independent endocytosis (Sabharanjak et al., 2002). Therefore, these results support the notion that Rab5 is involved in a clathrin-mediated endocytic process that operates only for the regulated, activity-dependent removal of AMPARs from synapses.

Finally, our results also provide information about the relation between AMPAR phosphorylation and LTD. It is well established that GluR1 dephosphorylation correlates with LTD induction (Kameyama et al., 1998; Lee et al., 2000; Lee et al., 1998). We show that Rab5 overexpression leads to GluR1 dephosphorylation in the absence of LTD-inducing stimuli. These results would then suggest that GluR1 dephosphorylation is a consequence, rather than a cause, of AMPAR removal from synapses driven by Rab5 (see model in Fig. 8). However, it has been reported that phosphatase activity (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Mulkey et al., 1994; Mulkey et al., 1993; Zeng et al., 2001) and GluR1 dephosphorylation (Lee et al., 2003) are required for LTD, hence, suggesting that dephosphorylation is upstream from AMPAR removal. A simple scenario to reconcile these results would suggest that GluR1 dephosphorylation occurs after removal from synapses, but this dephosphorylation would then be required to prevent receptor reinsertion. According to this interpretation, blocking GluR1 dephosphorylation would produce short-term depression, as receptors fail to be retained away from synapses. Indeed, this is consistent with the original observation that phosphatase activity is needed for LTD maintenance (Mulkey et al., 1993). Furthermore, a retention mechanism that keeps unphosphorylated receptors away from synapses has been proposed for GluR4-containing AMPARs (Esteban et al., 2003). One advantage of this model is that it would explain how a transient activation of the endocytic machinery results in long-lasting receptor removal. In addition, we have found that Rab5-driven internalization of GluR2 does not alter its phosphorylation at S880. However, as discussed above, there have been multiple reports linking GluR2 phosphorylation to dissociation of AMPARs from synaptic scaffolding molecules and internalization. Once again, it seems that there are GluR2-specific events that may operate independently from the

regulatory cascade that we have described here, and that would provide additional levels of control to the regulated removal of AMPARs during LTD.

In conclusion, our results shed light into the complex array of events regulating the activity-dependent removal of synaptic AMPARs during LTD and, for the first time, provide a direct link between the signaling cascades triggered by synaptic activity and the intracellular machinery that executes AMPAR trafficking.

EXPERIMENTAL PROCEDURES

Molecular Biology. The GFP-tagged AMPAR subunit constructs (GluR1–GFP and GluR2 R607Q–GFP), and the truncated CaMKII construct were made as previously described (Shi et al., 2001). Human Rab5a cDNA was generously provided by Dr. Ronald Holz (University of Michigan Medical School). Other details of the constructs used in this work are described in Supplementary Information.

Biochemistry. Hippocampal extracts were prepared in homogenization buffer containing 10 mM HEPES, 0.5 M NaCl, 10 mM NaF, 1 μM Microcystin LR, 10 mM EDTA, 0.1 mM PMSF, 1% Triton X-100 and a protease inhibitor cocktail (2 μg/ml) containing chemostatin, leupeptin, antipain and pepstatin. See Supplementary Information for a list of the antibodies used.

Membrane fractionation. Hippocampal extracts were prepared as described above but in homogenization buffer without Triton X-100. Cytosolic and membrane fractions were separated by centrifugation (13,000 rpm) for 15 min at 4 ºC. Membrane fraction (pellet) was then resuspended in homogenization buffer with 1 % Triton X-100.

GST "Pull-down". The C-terminus of rabaptin-5 (last 74 amino acids), which was previously shown to bind specifically Rab5-GTP, was fused downstream from GST in the pGEX-2T vector. GST fusion proteins were expressed in BL21/DE3 bacteria and loaded onto glutathione beads (Amersham). "Pull-down" controls were carried out with 0.1 mM non-hydrolyzable GTP analog (GMP-PNP; Sigma) or 1 mM GDP (Sigma). Hippocampal extracts were prepared in the homogenization buffer described above, with the following changes: 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 5% glycerol. Extracts from each condition

were incubated with GST beads for 1.5 hours at 4°C, followed by three washes in homogeneization buffer.

Imaging. Confocal and electron microscopy experiments are described in Supplementary Information.

Electrophysiology. Simultaneous double whole-cell recordings were obtained from nearby pairs of infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. Synaptic responses were evoked with two bipolar stimulating electrodes placed over Schaffer collateral fibers between 300 μm and 500 μm from the recorded cells. The responses obtained from the two stimulating electrodes were averaged for each cell and counted as an "n" of 1. Therefore, "n" equals the number of cells in all electrophysiology experiments. Composition of perfusion and internal solutions and experimental details for the different electrophysiological experiments are described in Supplementary Information.

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FIGURE LEGENDS

Figure 1. Localization of endogenous Rab5 at postsynaptic terminals and expression of Rab5-GFP in hippocampal slices. (**A**) Ultrastructural localization of endogenous Rab5 in CA1 neurons of rat hippocampus. Rab5 immunogold particles (arrows) were often found in the pery-synaptic membrane. Asterisks indicate presynaptic terminal. Scale bar is 100 nm. (**B**) Quantification of immunogold labeling of endogenous Rab5 in presynaptic terminals ("PRE"), intracellular compartments underneath the postsynaptic membrane ("Intra."), postsynaptic density ("PSD") and plasma membrane lateral to the PSD ("Memb."). For this quantification and the one shown in C, only gold particles within 600 nm of the synapse were included. n=339: total number of gold particles. (**C**) Frequency distribution of membrane immunogold labeling (compartments "PSD" plus "Memb." in B) according to its distance to the edge of the PSD. Empty bars (negative distances) represent gold particles within the PSD. Filled bars (positive distances) represent gold particles lateral from the PSD. n=211: total number of membrane-bound gold particles. (**D**) Western blot analysis of the expression of recombinant Rab5-GFP in organotypic hippocampal slice cultures using the Sindbis virus method. Non-infected slices are included as control. (**E**) Distribution of endogenous and recombinant Rab5 proteins in cytosol *versus* membranes. Membrane fractionation was carried out as described in Experimental Procedures from slices expressing Rab5(wt)-GFP, Rab5(dn)-GFP or uninfected slices. Percentages of protein present in the membrane fraction were as follows. Endogenous Rab5: 91%; Rab5(wt)-GFP: 55%; Rab5(dn)-GFP: 30%. (**F**) Representative confocal images showing the distribution of recombinant Rab5-GFP in soma, distal dendrites and spines of CA1 pyramidal neurons.

Figure 2. Rab5 overexpression selectively depresses AMPA receptor-mediated synaptic transmission. (**A**) Average evoked AMPAR-mediated response recorded at -60 mV simultaneously from nearby pairs of CA1 non-infected, control neurons and Rab5(wt)-infected neurons (p=0.02; n=7). (**B**) Simultaneous recordings of evoked NMDAR-mediated responses recorded at +40 mV from Rab5(wt) infected and non-infected neurons (p=0.7; n=8). (**C**) Ratio of AMPAR versus NMDAR responses from Rab5(wt) and control neurons (p=0.05; control, n=13; Rab5(wt), n=12). (**D**) Comparison of evoked AMPAR-mediated responses from Rab5(dn)-infected and control neurons (n=11). (**E**) Comparisons of evoked NMDAR-mediated responses from Rab5(dn)-infected and control neurons (n=9), as in B. (**F**) Ratio of AMPAR versus NMDAR responses from Rab5(dn) and control neurons (control: n=11; Rab5(dn): n=11).

Figure 3. Rab5 removes AMPARs locally from the spine plasma membrane. (**A**) Representative example of a neuron transfected with GluR2-GFP and Rab5(wt)-RFP and imaged with confocal microscopy. Left: GFP fluorescence signal showing total GluR2 receptor distribution. Middle: surface GluR2-GFP receptors assayed with an anti-GFP antibody coupled to Cy5 under non-permeabilized conditions. Right: RFP fluorescence from the same neuron showing expression of the co-transfected Rab5(wt)-RFP (neurons transfected only with GluR2-GFP do not show any RFP signal above background; not shown). Scale bar: 20 μm. (**B**) Representative line plot analysis of total (GFP signal; top) and surface (Cy5 signal; bottom) receptors across a spine and the adjacent dendritic shaft. Values for surface and total receptors were taken from the fluorescence intensity peaks after background subtraction (dashed line). Scale bar: 2 μm. (**C**) Surface ratio from pairs of spines

and dendrites in GluR2 transfected neurons (left) or in GluR2 and Rab5(wt) cotransfected neurons (middle). Surface ratios for spines and dendrites were calculated by dividing the corresponding background subtracted Cy5 and GFP fluorescence intensities. Plotted values are normalized by the mean surface ratio at dendrites. Spines showed a significantly higher surface ratio than dendrites in GluR2 transfected cells ($p<0.001$; n=63 spine-dendrite pairs from five different cells). The converse was observed for GluR2 plus Rab5(wt) cotransfected cells ($p=0.002$; n=64 spine-dendrite pairs from five cells). Cumulative probability distributions (right) of spine/dendrite ratios show a significant difference between the surface ratio distributions of GluR2 and GluR2 plus Rab5(wt) transfected neurons. For comparison, dashed lines indicate cumulative probability values for spine/dendrite=1 (equal surface distribution in spine and dendrite). (**D**) Total receptor amount (GFP signal) in spines and dendrites are calculated in GluR2 transfected neurons (left) and in GluR2 plus Rab5(wt) cotransfected neurons (middle). Values are normalized by the mean GFP fluorescence in dendrites. Cumulative probability plots (right) indicate that Rab5(wt) does not significantly change the total receptor distribution in spines versus dendrites.

Figure 4. Rab5(dn) blocks and Rab5(wt) mimics LTD in CA1 neurons. (**A**) LTD of AMPAR-mediated responses induced in neurons expressing Rab5(wt), Rab5(dn), or control, non-infected CA1 neurons. LTD was induced by pairing low-frequency stimulation (1 Hz) with moderate depolarization (-40 mV). Recordings were carried out with two stimulating electrodes place on Schaffer collaterals. One pathway did not receive low-frequency stimulation (unpaired pathway). Inset, sample traces from baseline (thin lines) and 30 minutes after LTD induction (thick lines). (**B**) Average AMPAR-mediated responses

collected between 40 and 50 minutes of the time course shown in A (30 minutes after LTD induction), and normalized to the baseline. Synaptic AMPA responses were significantly depressed in control neurons, relative to the unpaired pathway ($p<0.001$; n=11). Responses from Rab5(wt) (n=9) and Rab5(dn)-infected neurons (n=10) after LTD induction were not statistically different from baseline responses.

Figure 5. Transient increase in Rab5-GTP upon NMDA-induced chemical LTD. (**A**) Bath-application of 20 μM NMDA for 5 min produces a significant long-lasting depression of field excitatory postsynaptic potentials (fEPSP) in acute hippocampal slices (50% depression with respect to the baseline; $p=0.04$, n=4). Responses were evoked by stimulating Schaffer collaterals, and recorded from CA1 stratum radiatum. (**B**) Bath application of 100 μM AP5 10 min before adding NMDA completely block long-term depression (n=4). (**C**) Western blot analysis of Rab5 "pulled-down" with GST fused to the Rab5-GTP binding domain (GST-R5BD; left) or with GST alone (GST; right) from hippocampal extracts. Extracts were untreated, preloaded with non-hydrolyzable GTP or with GDP, as indicated. Relative Rab5 signal obtained with GTP and GDP incubations indicates that binding is mostly specific for Rab5-GTP. (**D**) Representative example of the levels of Rab5-GTP, total Rab5, phospho-p38 MAPK, and total p38 MAPK obtained after 2.5 min incubation with NMDA during a chemical LTD experiment. This treatment leads to a marked increase in the amount of Rab5-GTP (left), which is blocked in slices preincubated with AP5 (right). (**E**) Time course of Rab5-GTP formation and p38 MAPK phosphorylation (inset) during a chemical LTD experiment. Hippocampal slices were perfused with 20 μM NMDA for 5 min, and then transferred to regular perfusion solution. Extracts were quickly prepared at different

time points and incubated with GST-R5BD. Time points of 2.5 min (n=4) and 5 min (n=4) represent slices taken during or immediately after the NMDA incubation, respectively. Time points of 10 min (n=3) and 20 min (n=2) were taken during wash out of NMDA. Separate point at 2.5 min (+AP5) represents slices preincubated with AP5 and then treated for 2.5 min with NMDA (n=2). The amount of Rab5-GTP (bound to GST-R5BD) was normalized by the total amount of Rab5 in the extracts. Average ± standard error of the mean is plotted. Each experimental point (n=1) is the result of a pull-down assay carried out with extracts from 35 to 45 slices. Therefore, each experimental point can be considered the average value obtained from about 40 slices. Total and phosphorylated p38 MAPK were assayed from the non-bound fraction after GST pull-down.

Figure 6. Rab5 does not participate in constitutive receptor cycling, and it removes both GluR1 and GluR2 AMPA receptor subunits. (**A**) Time course of AMPAR-mediated responses recorded from CA1 neurons expressing Rab5(wt), Rab5(dn) or control non-infected cells during whole-cell pipette infusion of the GluR2-NSF interfering peptide, pep2m. Responses are normalized to a 2 min baseline from the beginning of the recording. Intracellular loading with pep2m produced a statistically significant depression of AMPA responses after 30 minutes of recording relative to baseline in all cases (uninfected: n=8, p=0.05; Rab5(wt): n=6, p=0.02; Rab5(dn): n=7, p=0.03). (**B**) Similar recordings carried out while infusing a peptide corresponding to the homologous sequence in GluR4c, pep4c, in uninfected and Rab5(wt)-expressing cells. No significant depression relative to baseline was observed (uninfected: n=5; Rab5(wt): n=5). (**C**) Average remaining current from time courses shown in A and B, taken from minutes 25 to 30 and normalized to a 2 min baseline

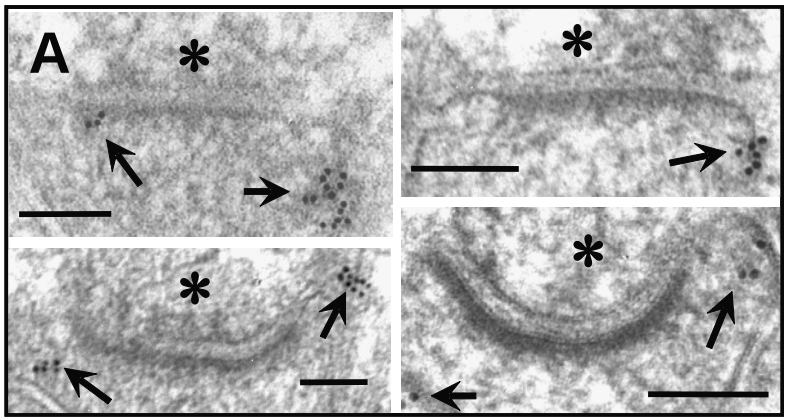
from the beginning of the recording. Inset, sample traces from baseline (thin lines) and 25 to 30 minute average (thick lines). **(D-F)** AMPAR-mediated responses were recorded at -60 mV and +40 mV. The rectification index is calculated as the ratio between the responses at these holding potentials ($I_{-60\ mV}$ / $I_{+40\ mV}$). Delivery of recombinant receptors is accompanied by an increase in rectification index, since homomeric recombinant receptors do not conduct currents at positive potentials (inward rectification). (**D**) Rectification values obtained from control, untransfected neurons or from neurons transfected with GluR2(R607Q), with or without Rab5(wt), as indicated (point mutation R607Q in the GluR2 channel confers inward rectification to these receptors). The increased rectification index obtained with GluR2(R607Q) (n=5) was significantly reduced upon Rab5(wt) coexpression (n=10; p=0.003). (**E**) Rectification values obtained from neurons transfected with GluR1 plus a constitutively active CaMKII (tCaMKII), with or without Rab5(wt) coexpression, as indicated. The increased rectification index obtained with GluR1 plus tCaMKII (n=10) was significantly reduced upon Rab5(wt) coexpression (n=8; p=0.01). (**F**) Increased rectification values from neurons transfected with the rectifying GluR2 mutant that does not bind the clathrin adaptor AP2, GluR2(R607Q, R845A) (n=8) was significantly enhanced by coexpression of Rab5(wt) (n=9; p=0.046).

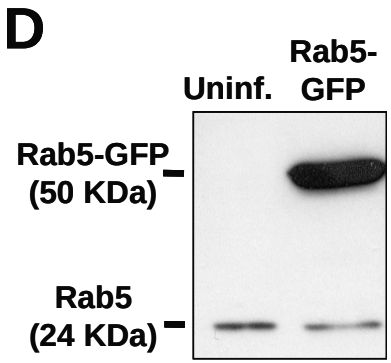
Figure 7. Changes in AMPAR phosphorylation upon Rab5-driven synaptic removal. (**A**) Representative Western blot to evaluate the phosphorylation level of GluR1 and GluR2 AMPAR subunits and αCaMKII in control (non-infected, untreated) slices, Rab5(wt)-overexpressing slices, and non-infected slices treated with forskolin (50 μM) and IBMX (10 μM) for 1 hour to enhance PKA activity. Extracts were prepared from microdissected CA1

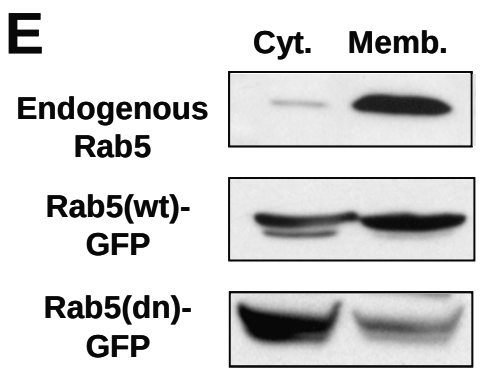
regions. (**B**) Quantification of PKA activity in hippocampal extracts from dissected CA1 regions of non-infected (control) or Rab5(wt) overexpressing slices. PKA kinase activity was assayed with a kit from Stressgen (catalog # EKS-390A) using different amounts of slice extracts, as indicated. Overexpression of Rab5(wt) did not alter intrinsic PKA activity. (**C**) Quantification of several experiments as the ones shown in A and B using non-infected and Rab5(wt) overexpressing slices. Phosphorylation of AMPAR subunits and αCaMKII were normalized to their total amounts assayed with phosphorylation insensitive antibodies. PKA activity was calculated as shown in B, using linear amounts of protein extracts. All values are normalized to those obtained from non-infected slices. Phosphorylation levels of GluR1 S845 and S831 were significantly reduced in Rab5(wt) expressing slices (n=4, p=0.008, and n=4, p=0.04, respectively). Phosphorylation of GluR2 S880 (n=2), αCaMKII T286 (n=3) or PKA activity (n=3) were not significantly altered due to expression of Rab5(wt).

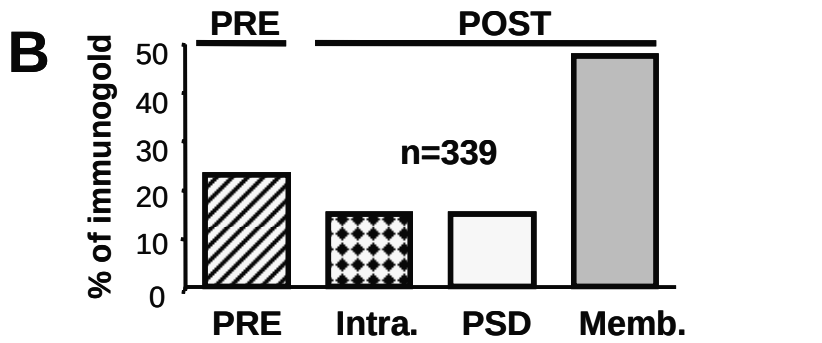
Figure 8. Model for the Rab5-driven removal of AMPA receptors from synapses during LTD. Upper left. Under conditions of basal synaptic transmission, GluR2/GluR3 receptors cycle continuously between the synaptic membrane and an intracellular compartment (RE: recycling endosome). In addition, both GluR1/GluR2 and GluR2/GluR3 receptors diffuse locally between the synaptic membrane and lateral extrasynaptic membrane. Rab5 is mostly in its inactive (GDP-bound) configuration, in early endosomes (EE). **Right**. Upon LTD induction, Ca^{2+} entry through NMDARs leads to activation of Rab-GDI and delivery of Rab5 at the plasma membrane, where it is activated (Rab5-GTP) and stimulates the formation of endocytic pits. AMPARs are recruited through their interaction with the clathrin adaptor AP2, independent from their subunit composition. **Lower left.** Active Rab5 drives these

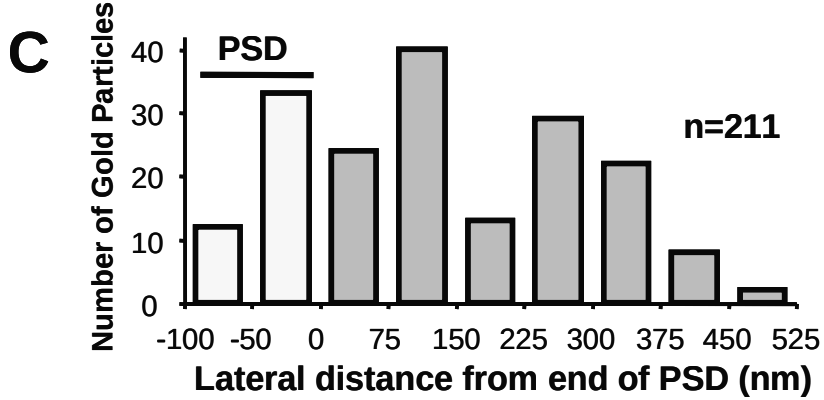
endocytic vesicles (EV) into early endosomes (EE). This process is accompanied by GDP hydrolysis (Rab5 inactivation) and dephosphorylation of GluR1. Dephosphorylated GluR1/GluR2 receptors are kept away from synapses. GluR2/GluR3 receptors might repopulate the pool of constitutively recycling receptors. This process leads to a net reduction in the number of AMPARs at synapses.


A


D
Uninf.
Rab5-GFP
Rab5-GFP (50 KDa)
Rab5 (24 KDa)


E
Cyt.
Memb.
Endogenous Rab5
Rab5(wt)-GFP
Rab5(dn)-GFP


B
PRE
POST
% of immunogold
n=339
PRE
Intra.
PSD
Memb.


C
PSD
Number of Gold Particles
n=211
Lateral distance from end of PSD (nm)

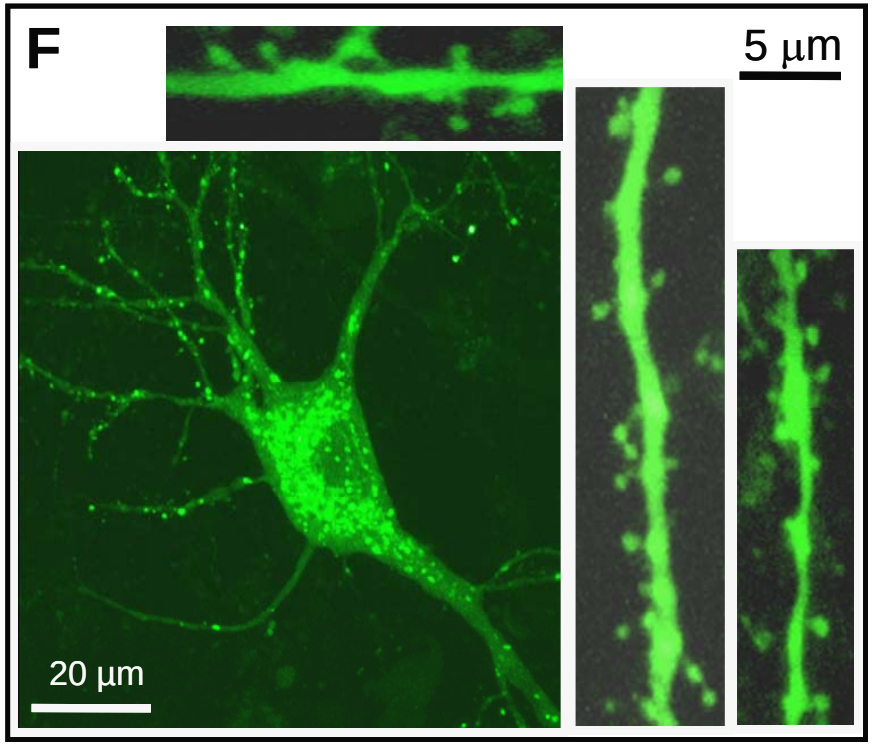

F
5 µm
20 µm

Figure 1

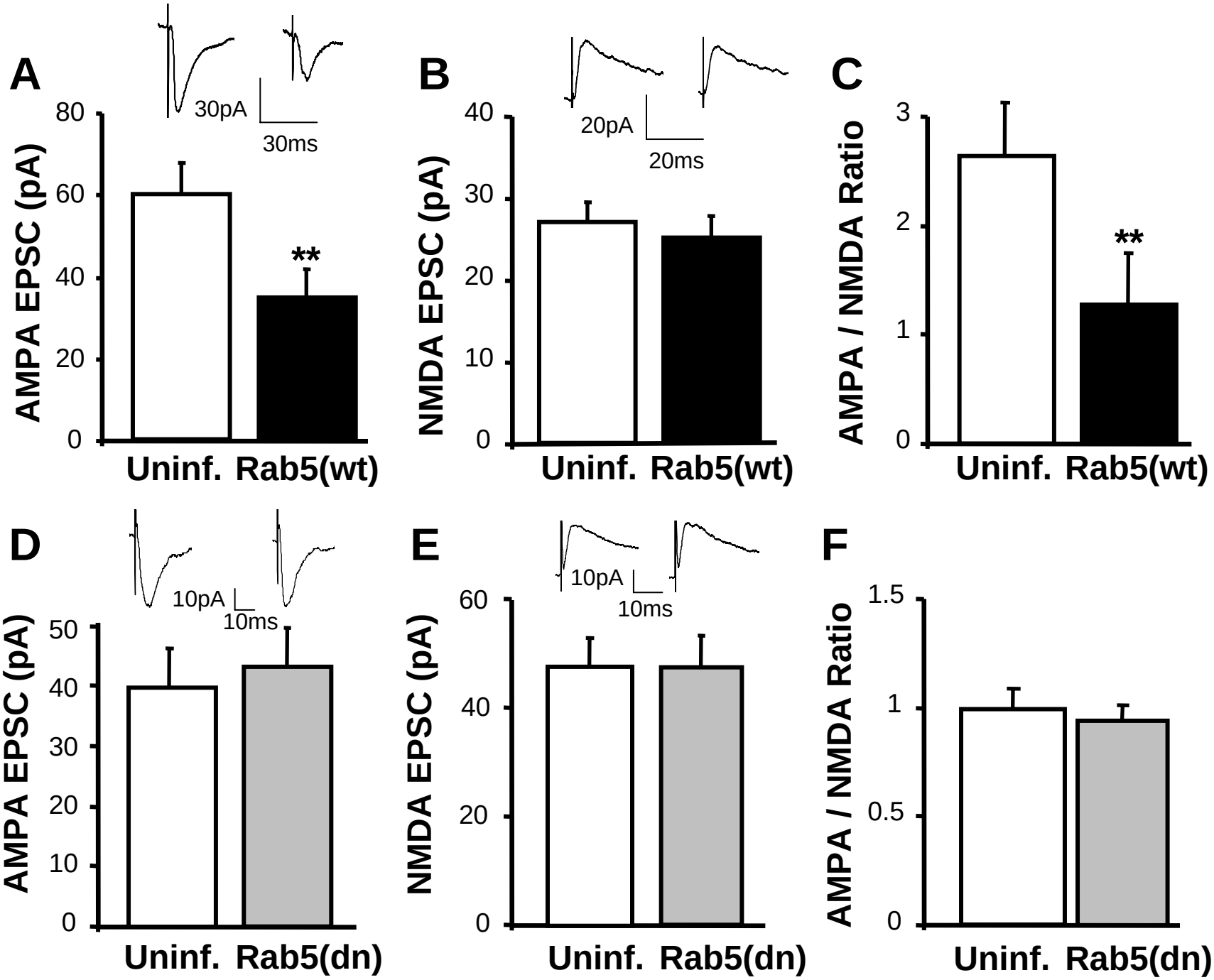

A
30pA
30ms
AMPA EPSC (pA)
80
60
40
20
0
**
Uninf.
Rab5(wt)
B
20pA
20ms
NMDA EPSC (pA)
40
30
20
10
0
Uninf.
Rab5(wt)
C
AMPA / NMDA Ratio
3
2
1
0
**
Uninf.
Rab5(wt)
D
10pA
10ms
AMPA EPSC (pA)
50
40
30
20
10
0
Uninf.
Rab5(dn)
E
10pA
10ms
NMDA EPSC (pA)
60
40
20
0
Uninf.
Rab5(dn)
F
AMPA / NMDA Ratio
1.5
1
0.5
0
Uninf.
Rab5(dn)

Figure 2

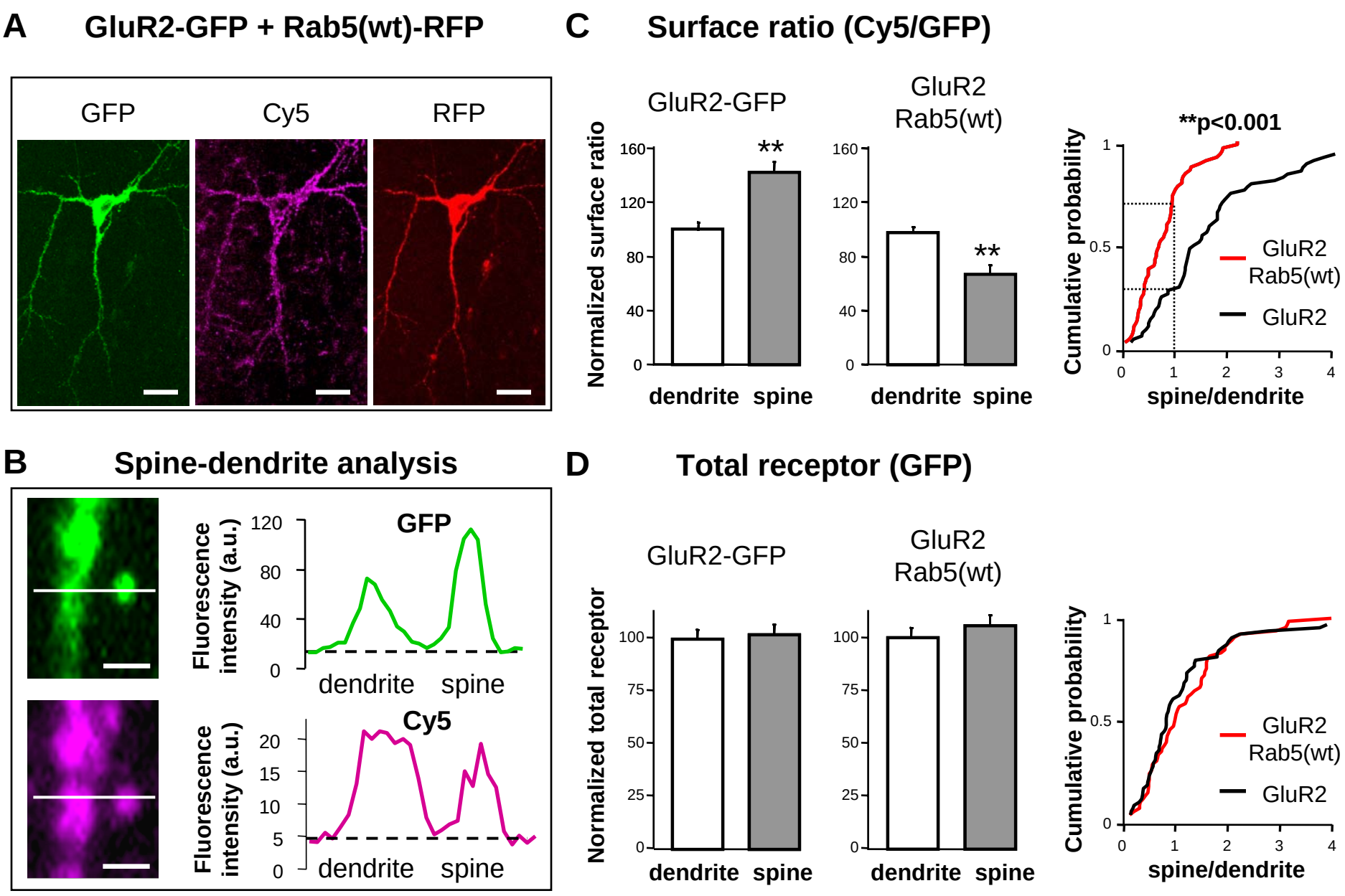

A
GluR2-GFP + Rab5(wt)-RFP
GFP
Cy5
RFP
B
Spine-dendrite analysis
Fluorescence intensity (a.u.)
GFP
dendrite
spine
Cy5
C
Surface ratio (Cy5/GFP)
GluR2-GFP
GluR2 Rab5(wt)
Normalized surface ratio
**
dendrite
spine
**p<0.001
Cumulative probability
spine/dendrite
GluR2 Rab5(wt)
GluR2
D
Total receptor (GFP)
Normalized total receptor

Figure 3

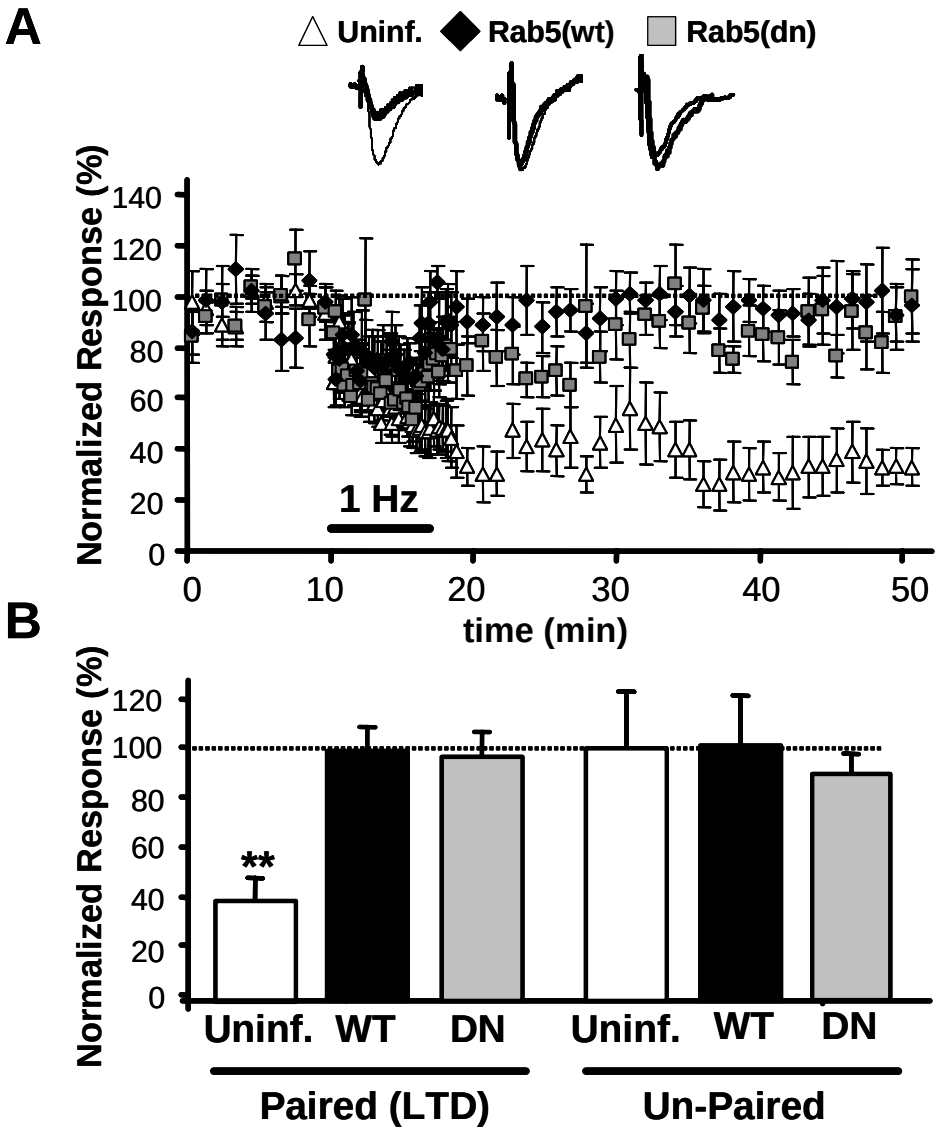

A
Uninf.
Rab5(wt)
Rab5(dn)
Normalized Response (%)
140
120
100
80
60
40
20
0
1 Hz
0
10
20
30
40
50
time (min)
B
Normalized Response (%)
120
100
80
60
40
20
0
**
Uninf.
WT
DN
Uninf.
WT
DN
Paired (LTD)
Un-Paired

Figure 4

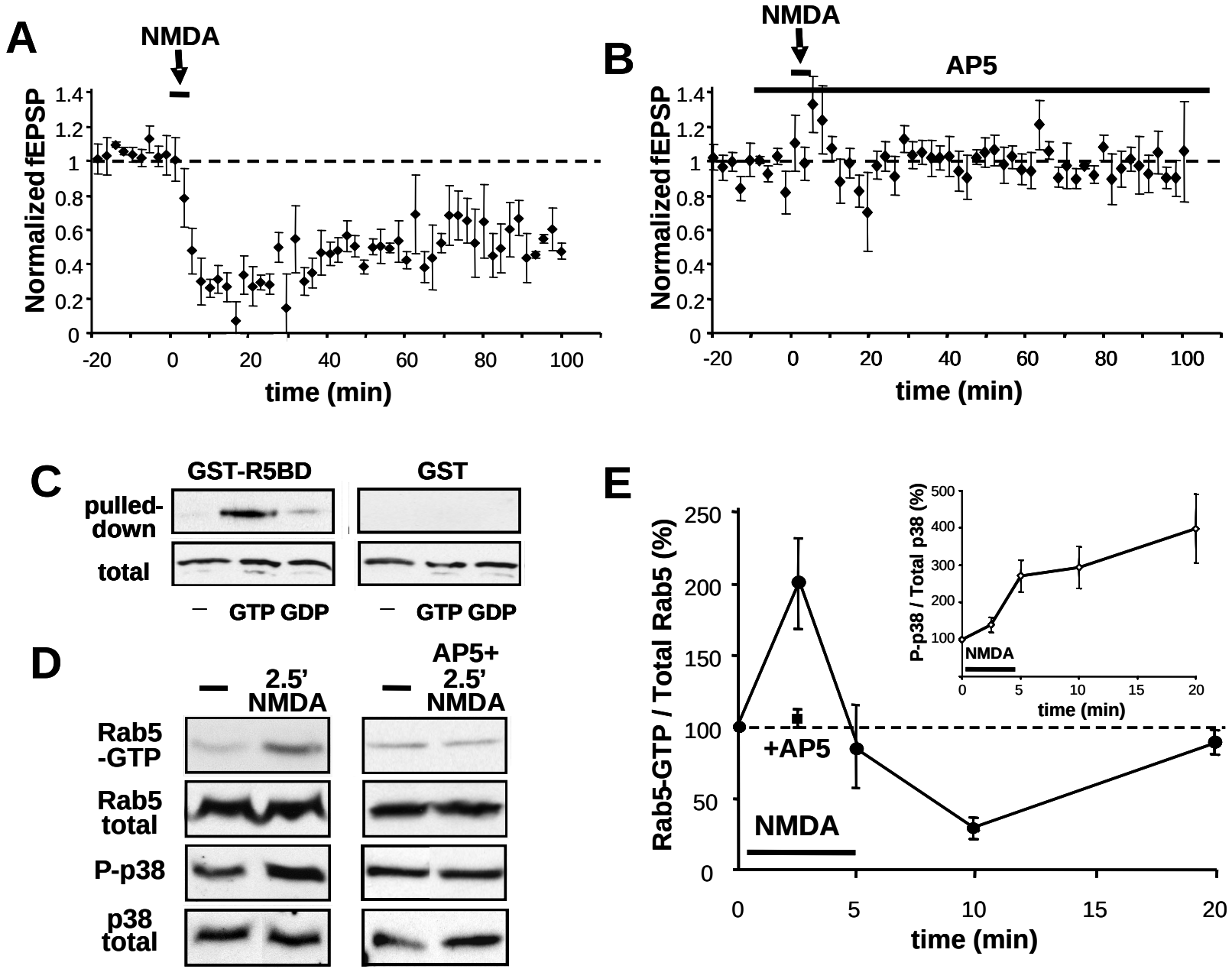

A
NMDA
Normalized fEPSP
time (min)
B
NMDA
AP5
Normalized fEPSP
time (min)
C
GST-R5BD
GST
pulled-down
total
– GTP GDP
– GTP GDP
D
– 2.5' NMDA
– AP5+ 2.5' NMDA
Rab5 -GTP
Rab5 total
P-p38
p38 total
E
Rab5-GTP / Total Rab5 (%)
+AP5
NMDA
time (min)
P-p38 / Total p38 (%)
NMDA
time (min)

Figure 5

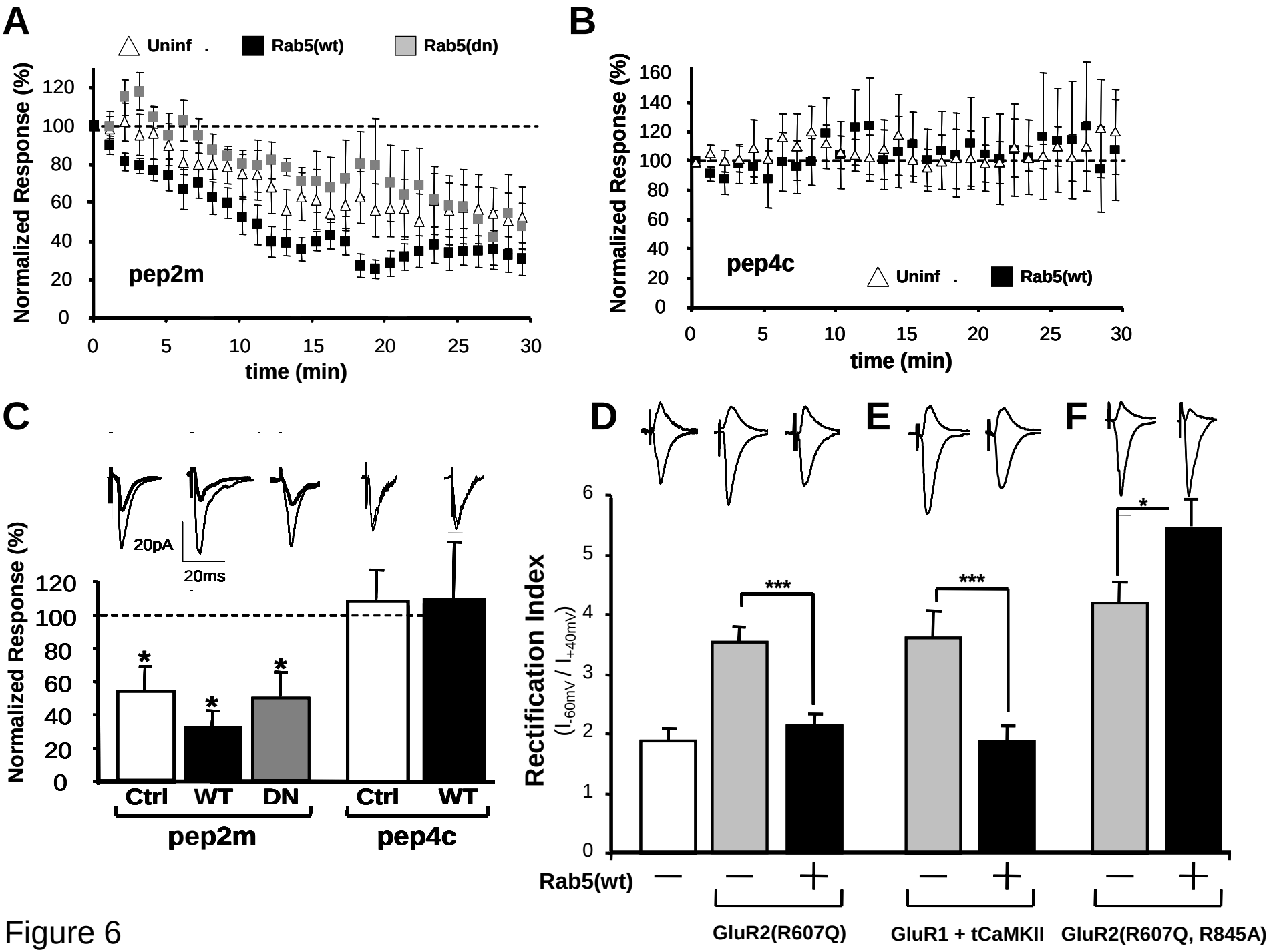

A
Uninf .
Rab5(wt)
Rab5(dn)
Normalized Response (%)
pep2m
time (min)
B
Normalized Response (%)
pep4c
Uninf .
Rab5(wt)
time (min)
C
20pA
20ms
Normalized Response (%)
Ctrl
WT
DN
Ctrl
WT
pep2m
pep4c
D
E
F
Rectification Index
(I-60mV / I+40mV)
***
***
*
Rab5(wt)
GluR2(R607Q)
GluR1 + tCaMKII
GluR2(R607Q, R845A)

Figure 6

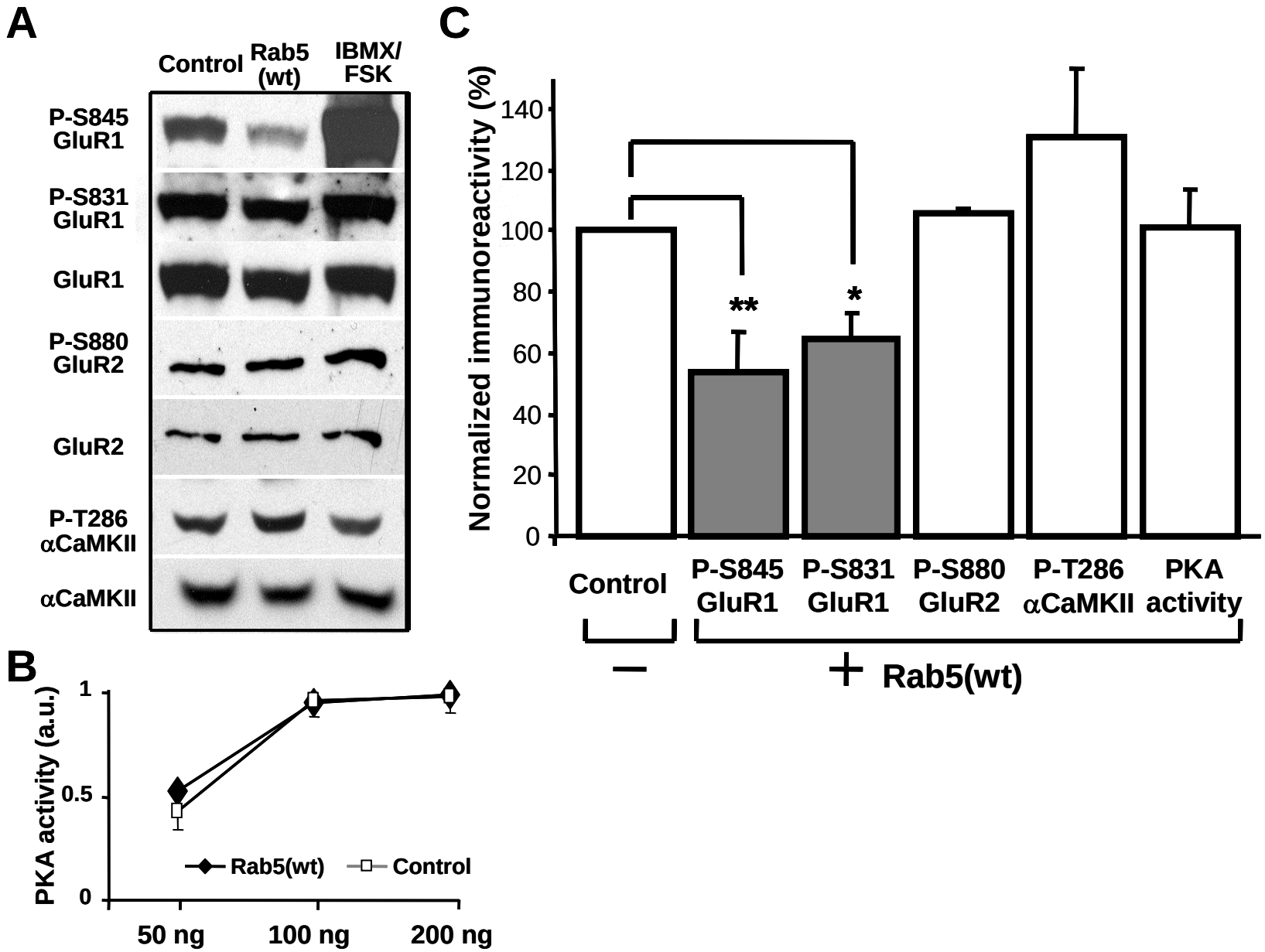

A
Control
Rab5 (wt)
IBMX/ FSK
P-S845 GluR1
P-S831 GluR1
GluR1
P-S880 GluR2
GluR2
P-T286 αCaMKII
αCaMKII
B
PKA activity (a.u.)
1
0.5
0
Rab5(wt)
Control
50 ng
100 ng
200 ng
C
Normalized immunoreactivity (%)
140
120
100
80
60
40
20
0
**
*
Control
P-S845 GluR1
P-S831 GluR1
P-S880 GluR2
P-T286 αCaMKII
PKA activity
–
+ Rab5(wt)

Figure 7

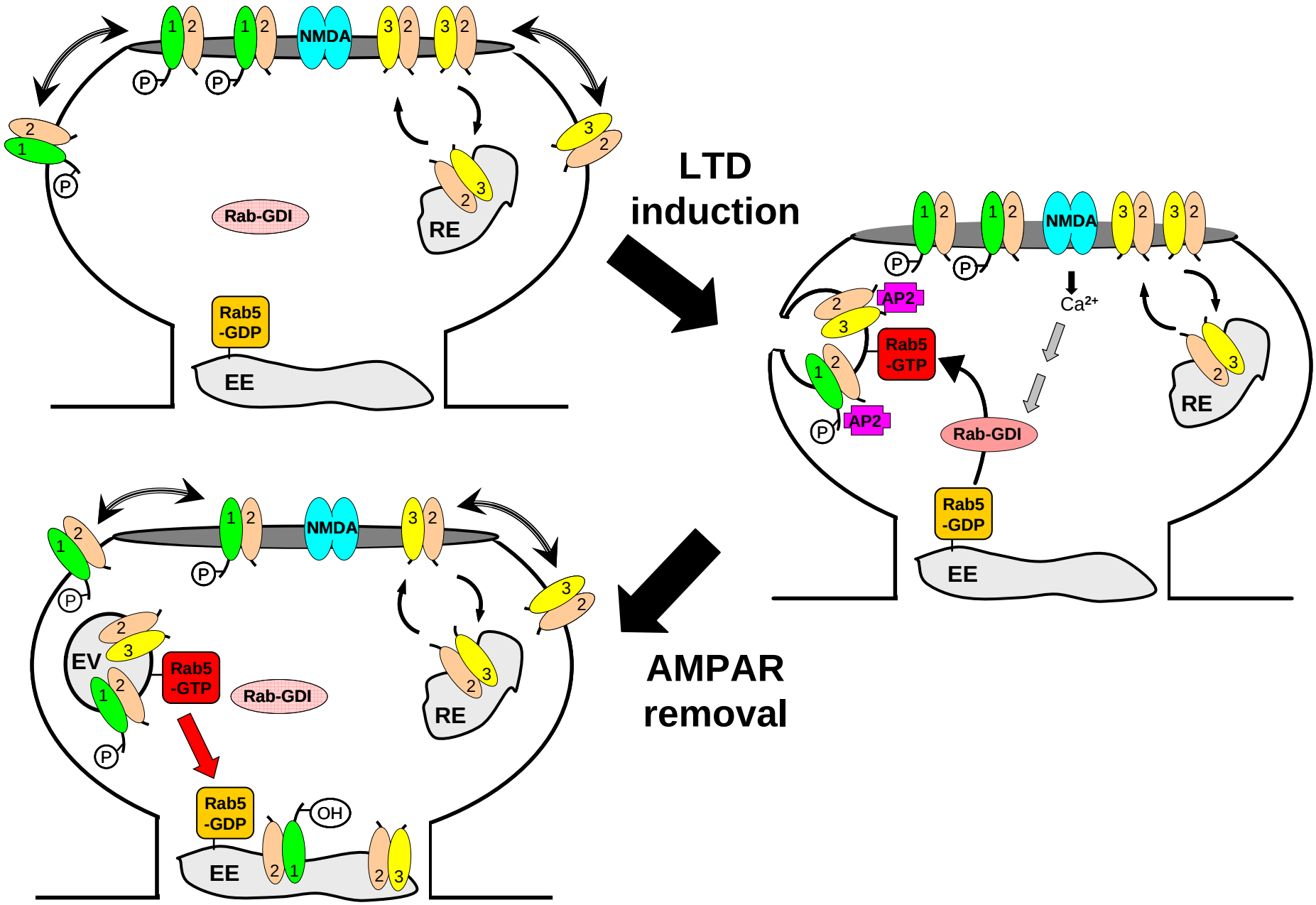
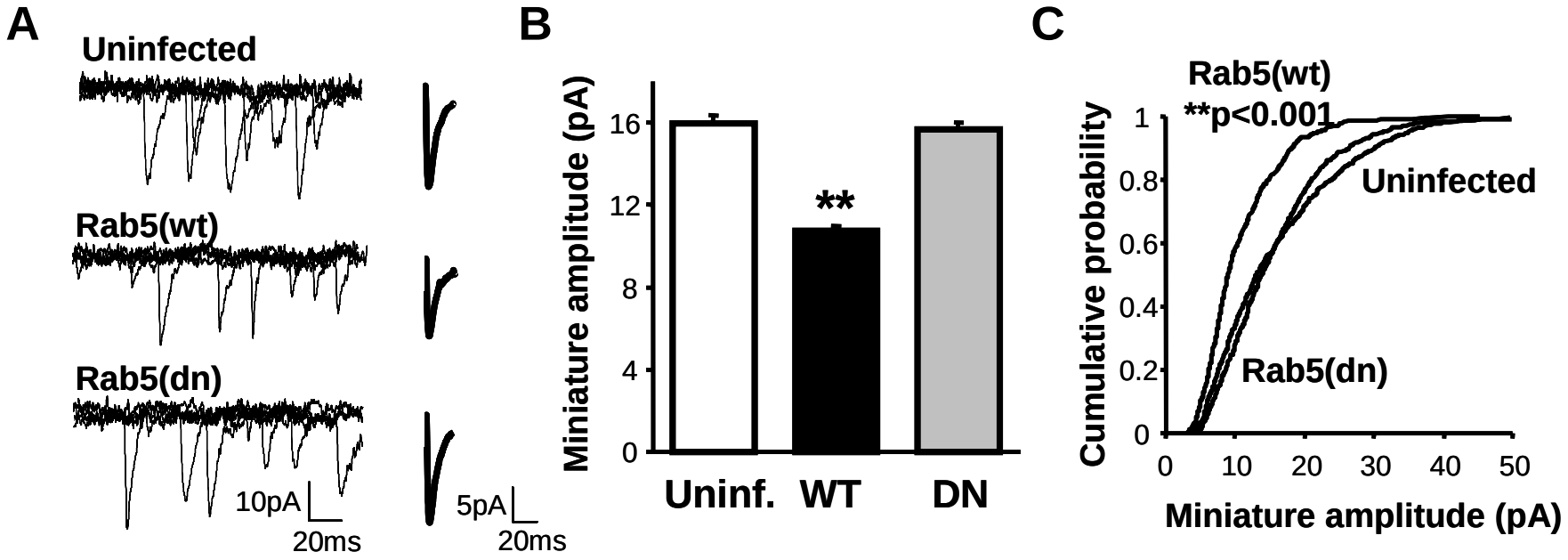
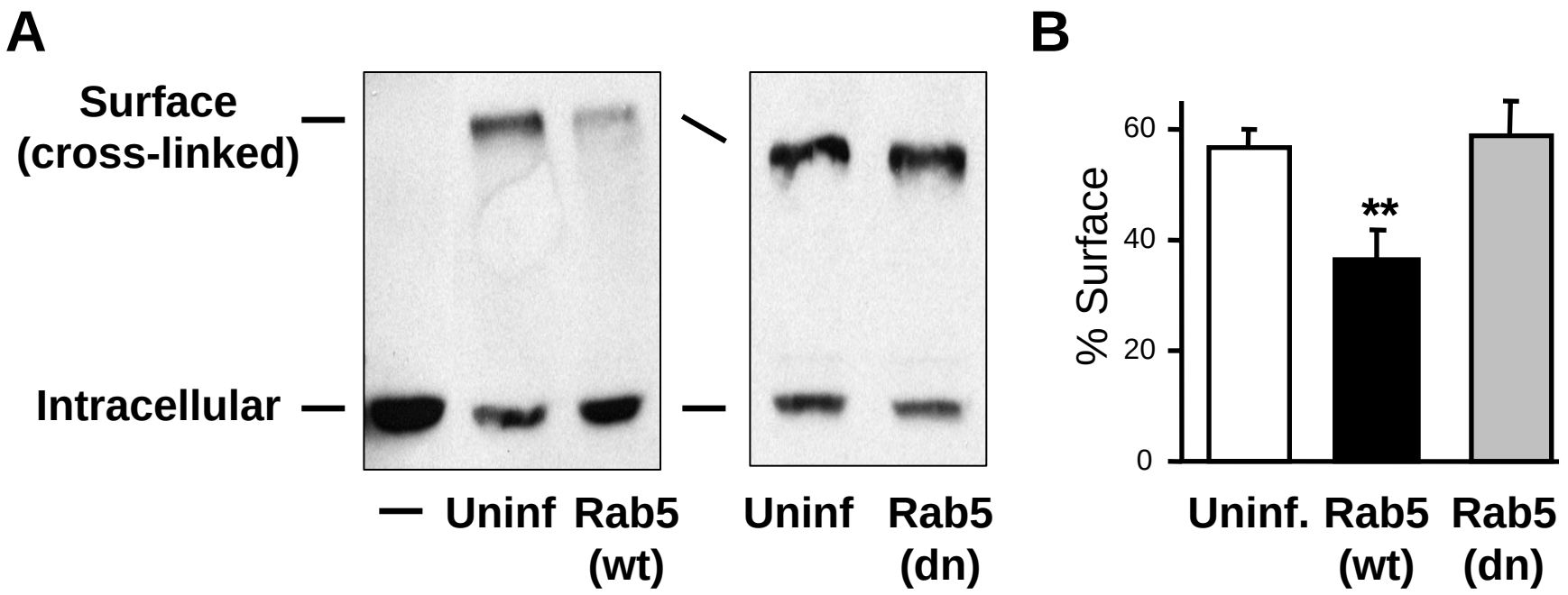

NMDA
Rab-GDI
RE
Rab5
-GDP
EE
LTD
induction
AP2
Ca2+
Rab5
-GTP
AMPAR
removal
EV
OH

Figure 8



Supplementary Figure 1



Supplementary Figure 2