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Differential roles of GRIP1a and GRIP1b in AMPA receptor trafficking

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Abstract

Regulated trafficking controls AMPA receptor (AMPAR) number at the postsynaptic membrane to modify the efficiency of synaptic transmission. The PDZ proteins GRIP1 and the related ABP-L/GRIP2 bind AMPAR subunit GluA2, and have been proposed to play a role in AMPAR trafficking associated with Long Term Depression (LTD) of synaptic transmission. Both GRIP1 and ABP-L/GRIP2 exist in different splice isoforms, including alternative 18 amino acid domains at the extreme N-terminus, which determine whether the protein can be palmitoylated. The implications of this differential splicing for AMPAR trafficking is unknown. Here, we use surface biotinylation and quantitative Western blotting to show that the N-terminal splice variants GRIP1a and GRIP1b have differential effects in NMDA-induced AMPAR internalization in cultured hippocampal neurons. GRIP1a inhibits, but GRIP1b enhances this trafficking event. We further demonstrate that GRIP1a and GRIP1b have dramatically different subcellular distributions in cultured neurons and exhibit NMDA-dependent colocalisation with early endosomes. We propose that GRIP1 palmitoylation modulates NMDA-induced AMPAR internalisation by differential regulation of the early endosomal system.

Keywords

Synaptic plasticity; LTD; Palmitoylation; Glutamate; PDZ domain

AMPA receptors mediate most fast synaptic excitation in the brain and their activity-dependent trafficking is a major determinant of synaptic strength. AMPAR expression at the synaptic plasma membrane is regulated by endocytosis, exocytosis, recycling and lateral diffusion events that contribute to reduced (Long Term Depression, LTD) or increased synaptic strength (Long Term Potentiation, LTP) [5,3,21,17]. Multiple protein interactions with AMPAR subunits mediate these trafficking events [19,11]. Among these the ABP/GRIP family of proteins are multi-PDZ domain proteins that interact with the extreme C-terminus of AMPAR subunits GluA2 and GluA3 [8,23]. GRIP1 and ABP (also known as GRIP2) are expressed from two separate genes and differential RNA splicing gives rise to multiple isoforms of each of these proteins [26,9].

ABP/GRIP proteins are be involved in anchoring AMPARs either at the synapse [18] or at an intracellular location [6,2]. One possible explanation for these distinct subcellular sites of action is that both GRIP1 and ABP exist in two different N-terminal splice forms, only one of which contains a consensus sequence for the posttranslational modification palmitoylation [27,7]. Conjugation of the fatty acid palmitate facilitates substrate protein

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association with specific cellular membrane compartments [14]. GRIP1b and pABP-L splice variants can be palmitoylated, whereas GRIP1a and ABP-L cannot.

Members of the ABP/GRIP family of proteins have been implicated in hippocampal [6], and more recently in cerebellar LTD [25]. Their precise function in AMPAR trafficking remains unclear, although it has been reported that GRIP1 interacts with the endosomal protein NEEP21, and regulates the recycling of internalised AMPARs back to the plasma membrane [24]. ABP/GRIP may also play a role in stabilising the pool of AMPARs internalised during LTD [6]. However, the function in AMPAR trafficking and synaptic plasticity of specific GRIP1 N-terminal splice variants that govern palmitoylation has not been explored.

Here, we investigated the specific roles of GRIP1a and GRIP1b in the regulation of AMPAR surface expression and NMDA-induced AMPAR trafficking. We show that overexpression of GRIP1b enhances, and GRIP1a inhibits NMDA-induced AMPAR internalisation, most likely via a re-organisation of the endosomal system by GRIP1. Our results demonstrate a previously unsuspected divergence in the roles of different GRIP1 isoforms in AMPAR trafficking, and also suggest important differences between GRIP1 and the closely related ABP-L.

Every attempt was made to minimise distress and suffering in animals used to produce neuronal cultures and all procedures were in compliance with the Animal [Scientific Procedures] Act 1986. Hippocampal and cortical primary neuronal cultures were prepared from E18 Wistar rats as previously described [15]. For imaging studies, neurons were plated on glass coverslips at a density of 75,000 per 24 mm coverslip. For biotinylation assays, neurons were plated at a density of 1×10^6 per 60 mm dish. Neurons were maintained in Neurobasal medium with B27, Glutamine and Pen/Strep for 14–21 days before use. Sindbis viruses were prepared according to the Sindbis Expression System manual (Invitrogen). Infections were carried out 16–20 h before experimentation.

Low density hippocampal cultures infected with Sindbis viruses were fixed with 4% paraformaldehyde, quenched with 0.1 M glycine and permeabilised for 10 min with 0.1% triton, 5% BSA in PBS [15]. For NMDA-application experiments, cultures were treated with 25 μM NMDA for 3 min at 37 °C, followed by 12 min after drug washout. Neurons were then stained with primary antibodies against flag (Sigma), EEA1 (Transduction Laboratories), or LAMP1 (StressGen) for 1 h, followed by Cy2 or Cy3 conjugated secondary antibodies. Visualisation of oxerexpressed proteins was performed on a Leica TCS-NT confocal laser scanning microscope.

High density cortical neurons were pre-incubated with 2 μM TTX for 1 h. For AMPA-induced trafficking, 100 μM AMPA was applied for 10 min at 37 °C. For NMDA-induced trafficking, 25 μM NMDA was applied for 3 min at 37 °C, followed by 12 min after drug washout. Following drug application, neurons were cooled to 4 °C, washed in ice cold PBS and incubated with 0.3 mg/ml Sulfo-NHS-SS-biotin for 10 min with gentle shaking. Cells were then washed in PBS and twice in PBS + 1% BSA for 5 min, followed by three further washes in PBS. Cells were lysed in lysis buffer (150 mM NaCl, 20 mM HEPES pH 7.5, 1% TX-100, 0.2% SDS) containing protease inhibitors. Lysates were then incubated with streptavidin-agarose beads for 2 h at 4 °C. Following 4 washes in lysis buffer, bound protein was detected by western blotting using anti-GluA2 (Chemicon) and anti-mouse IgG (Pierce).

Western blots were developed using ECL reagents and the results from at least five identical independent experiments were analysed using Image J. For biotinylation experiments, a ratio of values for bands representing surface verses those for total GluA2 was determined for

each condition. Error bars are standard errors, and *t*-tests were carried out to determine significant differences.

Since differential palmitoylation has been suggested to regulate protein association with specific cellular membrane compartments [14], we analysed the subcellular distribution of GRIP1a and GRIP1b in cultured hippocampal neurons. All available GRIP1 antibodies recognise both GRIP1a and GRIP1b, therefore to distinguish between the isoforms we expressed fluorescent protein tagged constructs (YFP-GRIP1a and GFP-GRIP1b) in dissociated hippocampal cultures using Sindbis virus. Confocal imaging of GFP/YFP fluorescence revealed that GRIP1a has a strongly punctate distribution and is present throughout the soma and primary dendrites. In contrast, GRIP1b has a mainly reticular distribution, but is also prominent in the soma and primary dendrites (Fig. 1). To our surprise, no fluorescence from either GRIP1 isoform was evident in dendritic spines. These data suggest that palmitoylation alone is not sufficient to determine the subcellular localisation of ABP/GRIP proteins.

To investigate the function of specific isoforms of GRIP in AMPAR trafficking, we overexpressed palmitoylatable or non-palmitoylatable forms of GRIP1 in dissociated hippocampal neurons and analysed the surface expression of GluA2 using membrane impermeant biotinylation assays. Surface GluA2 was unaffected by overexpression of GRIP1a or GRIP1b under basal (TTX treated) conditions (Fig. 2). AMPAR internalisation can be induced by bath application of either AMPA or NMDA [15,4,1]. Fig. 2A shows that application of 100 μM AMPA for 10 min resulted in a 60% reduction in the proportion of GluA2 expressed on the cell surface in control GFP-expressing cells. Overexpression of either GRIP1a or GRIP1b had no significant effect on AMPA-induced AMPAR internalisation (Fig. 2A) suggesting that GRIP1 is not involved in constitutive or agonist-induced AMPAR trafficking in hippocampal neurons.

Since ABP/GRIP have been implicated in LTD [6] we next used a “chemical LTD” protocol in which NMDA receptors are activated by bath application of NMDA to induce AMPAR internalisation [4,1,10,12]. Application of 25 μM NMDA resulted in a 36% reduction in the proportion of GluA2 expressed on the cell surface in control GFP-expressing cells (Fig. 2B). In cells expressing GRIP1a, NMDA-induced AMPAR internalisation was significantly reduced (11% reduction compared to non-NMDA control), suggesting that the non-palmitoylatable isoform is involved specifically in restricting NMDAR-induced AMPAR endocytosis or, alternatively, promoting recycling of GluA2-containing AMPARs, following NMDAR activation. In contrast, GRIP1b significantly enhanced the NMDA-induced internalisation of surface GluA2 (66% reduction compared to non-NMDA control), suggesting that the palmitoylatable isoform either contributes to endocytosis or reduces receptor recycling. These data demonstrate a specific role for GRIP1 in regulating NMDA-induced trafficking of GluA2-containing AMPARs, and suggest a role for GRIP1 palmitoylation in this process.

It has been reported that GRIP1 plays a role in regulating AMPAR recycling [24]. Therefore, we investigated the possibility that GRIP1 isoforms associate with endosomal compartments. We expressed YFP-GRIP1a and GFP-GRIP1b in hippocampal neurons and assayed their colocalisation with endosomal proteins by immunocytochemistry. Early Endosome Antigen 1 (EEA1) is a marker for early endosomes, and Lysosome Associated Membrane Protein 1 (LAMP1) is a marker for late endosomes and lysosomes. EEA1 and LAMP1 immunostaining both showed a punctate distribution in the neuronal cell body and dendrites, as shown previously [13]. To our surprise, neither GRIP1 isoform showed any colocalisation with either of these endosomal markers (Fig. 3).

Our biotinylation data suggest that GRIP1 influences AMPAR trafficking only when NMDARs are activated so we investigated the possibility that NMDA regulates the association of GRIP1 with endosomal compartments. Consistent with this hypothesis, NMDA application induced an altered distribution of EEA1 resulting in colocalisation with both GRIP1 isoforms (Fig. 4). Since GRIP1a and GRIP1b have very different subcellular distributions (see also Fig. 1), which are unaffected by NMDAR activation (Fig. 4), it is striking that both GRIP1 isoforms can induce an NMDA-dependent colocalisation with the endosomal system. In neurons overexpressing GRIP1b, NMDAR activation results in a radical redistribution of EEA1 immunopositive compartments to colocalise with GRIP1b. Thus, these results raise the possibility that GRIP1 may regulate AMPAR trafficking by redistributing specific components of the endosomal system in a palmitoylation-dependent manner (Fig. 5).

This study identifies differential roles for GRIP1 splice variants in AMPAR trafficking. More specifically, GRIP1b enhances, whereas GRIP1a inhibits NMDA-induced GluA2 internalisation. It has been proposed that ABP/GRIP proteins may be involved in maintaining the pool of AMPARs internalised during LTD [6]. Our data suggest that the palmitoylatable isoform GRIP1b mediates this effect.

Furthermore, GRIP1a and GRIP1b exhibit different subcellular distributions in neurons under basal conditions, neither of which colocalise with early or late endosomes but following NMDAR activation both isoforms colocalise with early endosomes, via redistribution of the endosomal compartments. This NMDA-dependent association of GRIP1 isoforms with early endosomes suggests that these proteins are not exerting their effects at the synapse, but at an endosomal compartment.

It is interesting that GRIP1 overexpression has no effect on surface levels of GluA2 under basal conditions. We demonstrate that GRIP1 associates with early endosomes only after NMDAR activation, so it is likely that the GRIP1-endosome interaction, possibly via NEEP21 [24], is required for appropriate regulation of AMPAR trafficking. An alternative explanation is that the GluA2-GRIP1b interaction may be activity-dependent, and GRIP1b is specifically recruited to AMPARs upon NMDA receptor activation. In this way, GRIP1b, but not GRIP1a, may anchor GluA2 internalised during LTD at an intracellular membrane compartment, preventing its recycling back to the plasma membrane. This mechanistic difference presumably reflects the differential subcellular distribution of GRIP1a and GRIP1b. Although both isoforms colocalise with early endosomes following NMDAR activation, the resulting distribution of EEA1 immunostaining is dramatically different, depending on which isoform is expressed.

How palmitoylation regulates the subcellular distribution of GRIP1 remains unclear, but it is likely that it promotes the association of GRIP1b with a specific intracellular membrane. In a similar manner, GRIP1a affects AMPAR trafficking only under conditions of NMDAR activation, but in contrast to GRIP1b, the non-palmitoylatable splice variant inhibits the internalisation of GluA2-containing AMPARs. The NMDA-induced colocalisation of GRIP1a with early endosomes again suggests that GRIP1a influences AMPAR trafficking at an intracellular location rather than the plasma membrane. To inhibit overall removal of surface GluA2, GRIP1a presumably acts via promoting receptor sorting from early endosomes to the recycling endosome and back to the plasma membrane (Fig. 5). This is consistent with a previous report demonstrating that unspecified GRIP1 associates with the early endosomal protein NEEP21 to stimulate trafficking to the recycling pathway [24]. Since GRIP1a and GRIP1b are identical apart from the extreme N-terminal 18 amino acids [27], the differences we observe in subcellular localisation and AMPAR trafficking must be attributable to this differential splicing. While it remains possible that additional factors

could be involved, the small size of the splice cassette strongly suggests that the potential for palmitoylation is the predominant characteristic that differs between GRIP1a and GRIP1b.

Recently it has been shown that overexpression of palmitoylatable pABP-L, but not non-palmitoylatable ABP-L, increases surface expression of GluR1 and GluR2, resulting in larger mEP-SCs [16]. Consistent with this observation, ABP-L colocalises with intracellular pools of GluA2 in neurons whereas pABP-L colocalises with surface GluA2 [7]. These data for ABP-L contrast with ours, which show that under basal conditions, neither GRIP1a nor GRIP1b overexpression affects surface AMPARs, suggesting interesting differences between the two related proteins GRIP1 and ABP-L. Furthermore, the subcellular distributions of GRIP1a and GRIP1b in neurons that we report here are different to those of p-ABP-L and ABP-L [7]. pABP-L localises to the plasma membrane and dendritic spines, whereas ABP-L is diffusely distributed in the cytoplasm [7]. In contrast, GRIP1a and GRIP1b appear to be localised to distinct, yet in both cases, intracellular compartments. These differences between GRIP1 and ABP-L indicate that factors in addition to palmitoylation are crucial for regulation of AMPAR trafficking. The PDZ domains of ABP-L and GRIP1 are highly homologous but the linker regions between PDZ domains show very low homology [9]. It is likely, therefore, that these regions contribute to determining the subcellular localisation and the specific membrane compartment with which ABP/GRIP proteins interact.

GRIP1a and GRIP1b appear to behave in a similar manner with respect to endosomal compartments. Neither isoform colocalises with early or late endosomal markers under basal conditions, however following NMDA application, a dramatic redistribution of EEA1 immunostaining results in colocalisation with both overexpressed GRIP1 isoforms. The altered localisation of the endosomal system, especially following NMDAR activation in GRIP1b-overexpressing cells may explain the differential effects of GRIP1a and GRIP1b in AMPAR trafficking. The mechanism that underlies this regulation of the endosomal system by GRIP1 remains to be determined. As discussed above, GRIP1 is known to bind to the early endosomal protein NEEP21 [24]. Furthermore, GRIP1 has been shown to interact with kinesin heavy chain KIF5 [20], and via this interaction targets AMPAR-containing vesicles to dendrites. Since the endosomal system is also regulated by microtubule motors [22], GRIP1 could influence membrane compartments via kinesins.

This work indicates differential functions of the N-terminal splice variants of GRIP1 in AMPAR internalisation, suggesting a role for GRIP1 palmitoylation. It also provides evidence that GRIP1 regulates these trafficking events via the early endosomal system. By comparison with other recent work [16], this work also suggests that palmitoylation of GRIP1 has distinct effects on AMPAR trafficking compared to palmitoylation of ABP-L.

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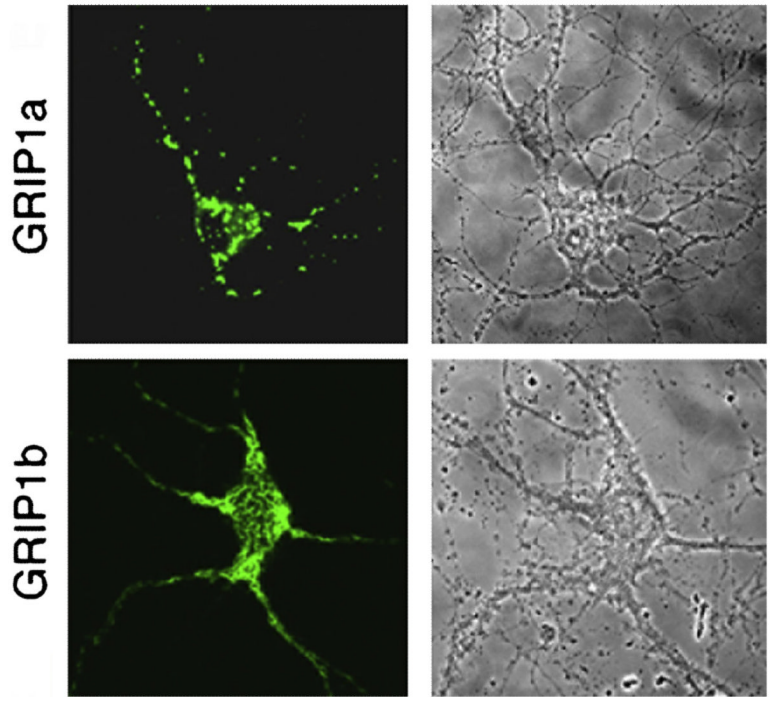

Fig. 1.

Differential subcellular distributions of GRIP1a and GRIP1b isoforms. Dissociated hippocampal neurons (18 DIV) were infected with Sindbis virus expressing GFP-GRIP1a or GFP-GRIP1b. Cells were fixed and imaged by confocal microscopy 24 h later. Left panels show GFP/YFP fluorescence, right panels show transmission images.

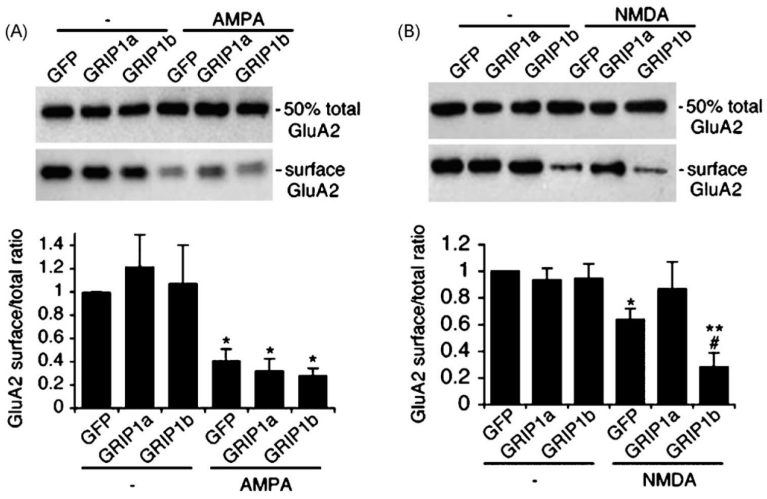

Fig. 2.

Effects of GRIP1a and GRIP1b overexpression on regulated AMPAR internalisation. (A) GRIP1a/GRIP1b overexpression does not affect AMPA-induced internalisation of GluA2-containing AMPARs. Dissociated hippocampal neurons infected with Sindbis virus expressing GFP-GRIP1a or GFP-GRIP1b were pretreated with TTX for 60 min, and stimulated with 100 μM AMPA for 10 min. Top panel shows representative western blots for total and surface GluA2. Graph shows pooled data presented as ratios of surface over total GluA2. $n = 6$,*$p < 0.05$, compared to equivalent condition without AMPA treatment. (B) GRIP1a and GRIP1b have differential effects on NMDA-induced internalisation of GluA2-containing AMPARs. Dissociated hippocampal neurons infected with Sindbis virus expressing GFP-GRIP1a or GFP-GRIP1b were pretreated with TTX for 60 min, and stimulated with 25 μM NMDA for 3 min, with 10 min incubation following drug washout. Top panel shows representative western blots for total and surface GluA2. Graph shows pooled data presented as ratios of surface over total GluA2. $n = 5$, *$p < 0.05$, **$p < 0.01$, compared to equivalent condition without NMDA treatment. #$p < 0.05$, compared to GFP + NMDA condition.

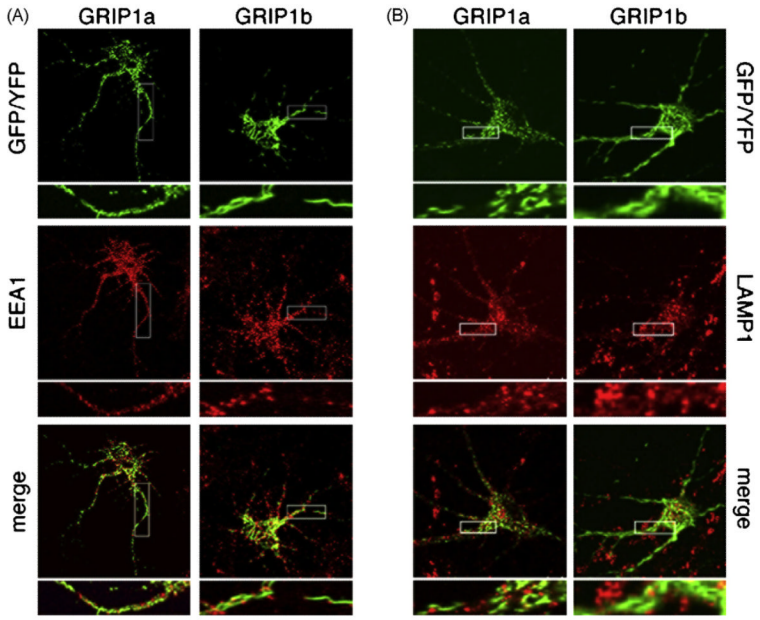

Fig. 3.

GRIP1a and GRIP1b do not colocalise with EEA1 or LAMP1 under basal conditions. Dissociated hippocampal neurons (18 DIV) were infected with Sindbis virus expressing GFP-GRIP1a or GFP-GRIP1b. Cells were fixed, processed for immunostaining using anti-EEA1 or anti-LAMP1 and imaged by confocal microscopy 24 h later.

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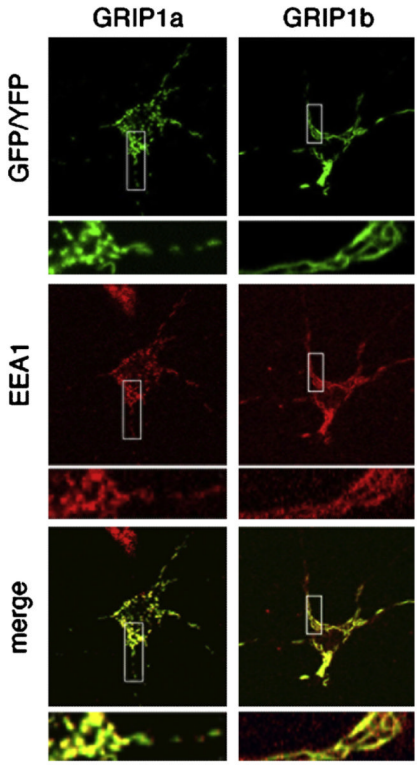

Fig. 4.

Both GRIP1a and GRIP1b colocalise with EEA1 following NMDAR activation. Dissociated hippocampal neurons infected with Sindbis virus expressing GFP-GRIP1a or GFP-GRIP1b were pretreated with TTX for 60 min, and stimulated with 25 μM NMDA for 3 min, with 10 min incubation following drug washout. Cells were fixed, processed for immunostaining using anti-EEA1 and imaged by confocal microscopy.

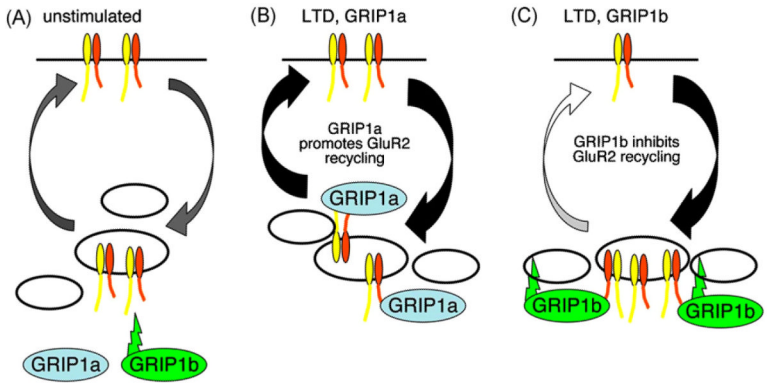

Fig. 5.
Schematic illustrating the roles of GRIP1a and GRIP1b on AMPAR recycling following NMDAR-induced endocytosis.