GRIP1 is required for homeostatic regulation of AMPAR trafficking

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Homeostatic plasticity is a negative feedback mechanism that stabilizes neurons during periods of perturbed activity. The best-studied form of homeostatic plasticity in the central nervous system is the scaling of excitatory synapses. Postsynaptic AMPA-type glutamate receptors (AMPARs) can be inserted into synapses to compensate for neuronal inactivity or removed to compensate for hyperactivity. However, the molecular mechanisms underlying the homeostatic regulation of AMPARs remain elusive. Here, we show that the expression of GRIP1, a multi-PDZ (postsynaptic density 95/discs large/zona occludens) domain AMPAR-binding protein, is bidirectionally altered by neuronal activity. Furthermore, we observe a subcellular redistribution of GRIP1 and a change in the binding of GRIP1 to GluA2 during synaptic scaling. Using a combination of biochemical, genetic, and electrophysiological methods, we find that loss of GRIP1 blocks the accumulation of surface AMPARs and the scaling up of synaptic strength that occur in response to chronic activity blockade. Collectively, our data point to an essential role of GRIP1-mediated AMPAR trafficking during inactivity-induced synaptic scaling.

synaptic scaling | postsynaptic density | glutamate receptor | PDZ domain

Associative, or Hebbian, synaptic plasticity is widely thought to underlie information storage (1). However, changes in synaptic strength through these learning processes are inherently destabilizing for neuronal networks. Homeostatic scaling is one elegant mechanism proposed to counterbalance the destabilizing forces of associative plasticity and maintain neuronal activity at a set point (2, 3). Homeostatic scaling was first identified in cultured neocortical neurons, where a prolonged increase in neuronal activity globally scaled down excitatory synaptic responses while a chronic blockade of activity scaled up the responses (4, 5).

AMPA-type glutamate receptors (AMPARs) are the principle postsynaptic ionotropic glutamate receptors that mediate fast excitatory synaptic transmission in the central nervous system. AMPARs are tetrameric assemblies of highly homologous subunits encoded by four different genes, GluA1–4 (5, 6). Perturbing network activity induces compensatory changes in surface AMPARs to restore neuronal firing rates (5). Accordingly, the mechanisms of homeostatic regulation of AMPARs are currently under intense scrutiny. AMPAR trafficking is highly dynamic and regulated by binding partners and posttranslational modifications (5, 7) including phosphorylation (8, 9). AMPAR trafficking, binding, and modification are highly subunit-specific. It has been shown that the GluA2 subunit, but not GluA1, is required for inactivity-induced scaling, and the C-terminal domain is crucial (10). Many molecules known to regulate AMPAR trafficking, including AKAP5, Arc, TNFα, β3 integrins, PSD-95, and PICK1, are all involved in or required for synaptic scaling in neuronal cultures (11–16). However, a clear picture of molecular mechanisms underlying scaling still remains largely unknown.

Glutamate receptor interacting protein 1 (GRIP1) and its homolog GRIP2 are major scaffolding proteins for GluA2/3 subunit containing AMPARs. GRIP1 and 2 each contain seven postsynaptic density 95/discs large/zona occludens (PDZ) domains and interact directly with GluA2/3 C-terminal domains through their fourth and fifth PDZ domains (17, 18). The interaction between GRIP1 and GluA2/3 regulates surface expression, membrane trafficking, and synaptic targeting of AMPARs, and the regulation of this interaction is critical for neuronal development and several forms of synaptic plasticity (7, 19–23). Loss of GRIP1/2 slows activity-dependent AMPAR recycling (24) and blocks cerebellar long-term depression (LTD) expression (20). Though the PDZ domains of GRIP1 and GRIP2 are highly conserved, some regions between these PDZ domains are variable. For example, the KIF-5 binding region of GRIP1 (between PDZ6 and PDZ7) is poorly homologous with GRIP2 (25). This divergence suggests that GRIP1 and GRIP2 are not entirely redundant, such that GRIP1 harbors unique abilities to regulate AMPAR trafficking through the interactions with motor proteins. Indeed, GRIP1 plays a primary role in Hebbian plasticity like LTD, given that GRIP1 can completely rescue the LTD deficits in the GRIP1/2 double knockout background, whereas GRIP2 can only partially rescue (20). In stark contrast to the well-known function of GRIP1/2 in Hebbian plasticity, the role of GRIP1/2 in synaptic scaling has not been examined.

Here, we tested whether GRIP1 participates in the homeostatic regulation of AMPARs. We found that GRIP1 expression is bidirectionally altered during synaptic scaling. Activity also changes the subcellular distribution of GRIP1 and its association with GluA2. Using a combination of biochemical, genetic, and electrophysiological methods, we observed that inactivity-induced synaptic scaling up is blocked in GRIP1 knockout neurons due to impaired trafficking of AMPARs, whereas scaling down during elevated activity is intact. Overall, these findings reveal an essential role of GRIP1 in inactivity-induced synaptic scaling by regulating synaptic targeting of AMPARs.

Results

GRIP1 Expression Is Bidirectionally Regulated by Activity. We first examined GRIP1 protein levels during synaptic scaling. Cultured rat cortical neurons were treated with bicuculline (to induce

Significance

Homeostatic plasticity constrains neuronal networks, allowing the brain to maintain a dynamic equilibrium. Loss of homeostatic plasticity disrupts normal brain function and has been implicated in autism spectrum disorder. Much of homeostatic regulation centers on synapses. In particular, excitatory AMPA-type glutamate receptors (AMPARs) are moved in and out of synapses to recalibrate neuronal activity. AMPARs can be inserted at the surface to compensate for inactivity or removed to compensate for hyperactivity. We found that GRIP1, an AMPAR-binding protein implicated in autism, regulates the homeostatic shuttling of AMPARs between cytoplasmic and synaptic pools. Restoring GRIP1 regulation may therefore prove therapeutically useful in autism.

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hyperactivity) or TTX (to induce inactivity) for 48 h, which has been shown to induce homeostatic scaling down or scaling up, respectively (4, 26). We found that bicuculline treatment significantly increased, whereas TTX treatment significantly decreased, total GRIP1 protein levels (Fig. 1 *A* and *B*), suggesting that GRIP1 protein levels may be important in controlling synaptic strength during homeostatic scaling.

To test whether GRIP1/2 are involved in the scaling process, we used GRIP1 and GRIP2 knockout mice. GRIP1 conventional knockout mice have an early embryonic lethal phenotype, but GRIP2 knockout mice are viable (27, 28). We therefore used GRIP1 conditional knockout mice (GRIP1$^{-/-}$), GRIP2 conventional knockout mice (GRIP2$^{-/-}$), and double-knockout GRIP1 conditional plus GRIP2 conventional mice (GRIP1/2$^{-/-}$). To achieve conditional knockout of GRIP1 in cultured neurons, lentiviruses expressing Cre recombinase (EGFP-IRES-Cre) were introduced. As previously described (20), 7–10 d after EGFP-IRES-Cre virus infection, GRIP1 protein could no longer be detected (Fig. 1 *C* and *D*). Therefore, we routinely infected cortical neurons with lentiviruses at day in vitro (DIV) 3 and performed experiments 8–10 d later. Complete loss of GRIP1 was confirmed by Western blot for each experiment. In both WT and GRIP2 knockout mouse neurons, GRIP1 was significantly increased or decreased after 48 h of bicuculline or TTX treatments, respectively (Fig. 1 *D* and *E*). These data show that GRIP1 expression is bidirectionally regulated during synaptic scaling in both WT and GRIP2 knockout neurons.

Loss of GRIP1 or GRIP2 Decreases Surface AMPARs. We first examined the consequences of GRIP deletion on AMPAR surface or total expression. Using a surface biotinylation assay, we found a significant reduction in surface GluA1, GluA2, and GluA3 AMPAR subunits in all GRIP knockout neurons (GRIP1 conditional knockout, GRIP2 conventional knockout, and GRIP1/GRIP2 double-knockout neurons; Fig. 2 *A–D*). However, total AMPAR subunit expression was comparable to WT neurons (Fig. 2 *E–H*). We therefore concluded that the decrease of surface AMPARs in GRIP knockout neurons is due to impaired receptor trafficking rather than protein synthesis or degradation defects.

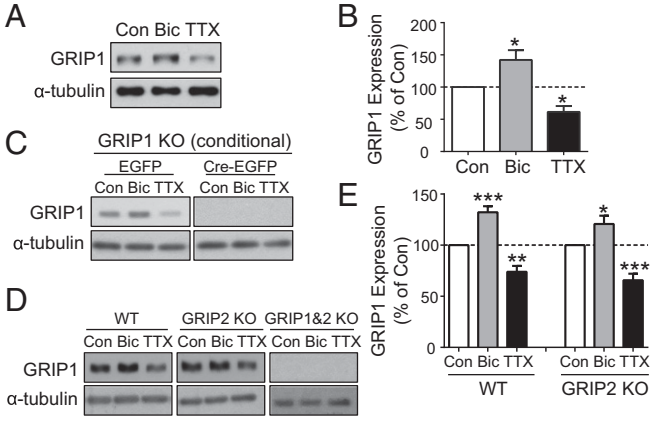

Fig. 1. Synaptic scaling bidirectionally regulates GRIP1 protein level. (*A*) Rat cortical neurons (DIV11–13) were treated with control solution (Con), bicuculline (Bic; 20 μM), or TTX (1 μM) for 48 h, followed by Western blot. (*B*) Quantification of GRIP1 protein levels after normalizing to α-tubulin. Data represent mean ± SEM of band intensities normalized to control values (ANOVA, $*P < 0.05$; $n = 9$). (*C* and *D*) Western blot analysis of GRIP1 in WT and GRIP knockout mouse cortical neurons (DIV11–13) treated with Con/Bic/TTX for 48 h. (*E*) Quantification of GRIP1 protein levels after normalizing to α-tubulin. Data represent mean ± SEM of band intensities normalized to control values (ANOVA, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 9–10$).

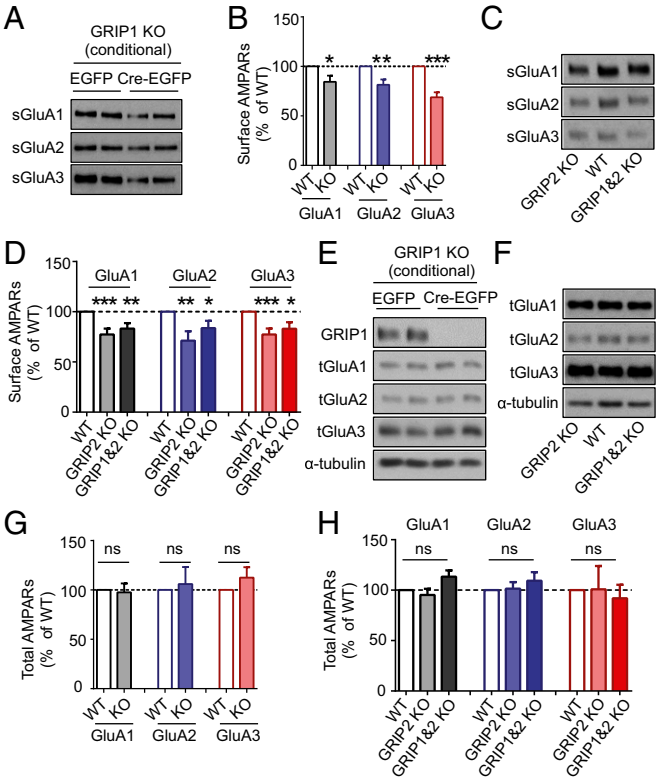

Fig. 2. Loss of GRIP decreases surface AMPAR expression. (*A*, *C*, *E*, and *F*) Cultured cortical neurons from GRIP knockout or WT mice were subjected to surface biotinylation assay. The relative amount of surface and total AMPARs was assessed by Western blot using specific antibodies against GluA1, GluA2, and GluA3. (*B*, *D*, *G*, and *H*) Quantification of the surface (*B* and *D*) and total (*G* and *H*) GluA1, GluA2, and GluA3 after normalizing against α-tubulin. Data represent mean ± SEM of band intensities normalized to values of WT neurons (*t* test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 5–13$).

Inactivity-Induced Synaptic Scaling Is Blocked in GRIP Knockout Neurons. We next directly tested whether GRIP1/2 participate in homeostatic scaling. Scaling was induced pharmacologically as described above, in WT and knockout mouse neurons. As expected, WT neurons exhibited a significant reduction in surface GluA1, GluA2, and GluA3 in response to bicuculline treatment (Fig. 3 *A–D*) (9, 26). Conversely, TTX-induced inactivity caused a significant increase in surface GluA1 and GluA2 with no change in surface GluA3 (Fig. 3 *A–D*). However, TTX treatment failed to generate a compensatory elevation of surface GluA1 or GluA2 in GRIP1 knockout neurons, and even resulted in a small decrease in surface GluA3 (Fig. 3 *A* and *B*). In GRIP1/2 double-knockout neurons, TTX treatment resulted in no change to surface GluA1 levels and a decrease in surface GluA2 and GluA3 compared with untreated controls (Fig. 3 *C* and *D*). To determine whether GRIP1 or 2 predominated in the inactivity-induced synaptic upscaling, we examined scaling in GRIP2 knockout neurons. In the absence of GRIP2, surface GluA1 levels still increased in response to TTX, whereas surface GluA2 showed no change and surface GluA3 was reduced (Fig. 3 *C* and *D*). These data indicate that the homeostatic enrichment of surface glutamate receptors during chronic activity suppression is blocked in GRIP knockout neurons. Moreover, inactivity was able to increase surface GluA1 in GRIP2 knockout neurons but not GRIP1 knockout neurons, suggesting that GRIP1 plays a dominant role in scaling up. In contrast, scaling down of synaptic strength through the application of bicuculline was intact in all neurons, regardless of GRIP expression. No defect of AMPAR

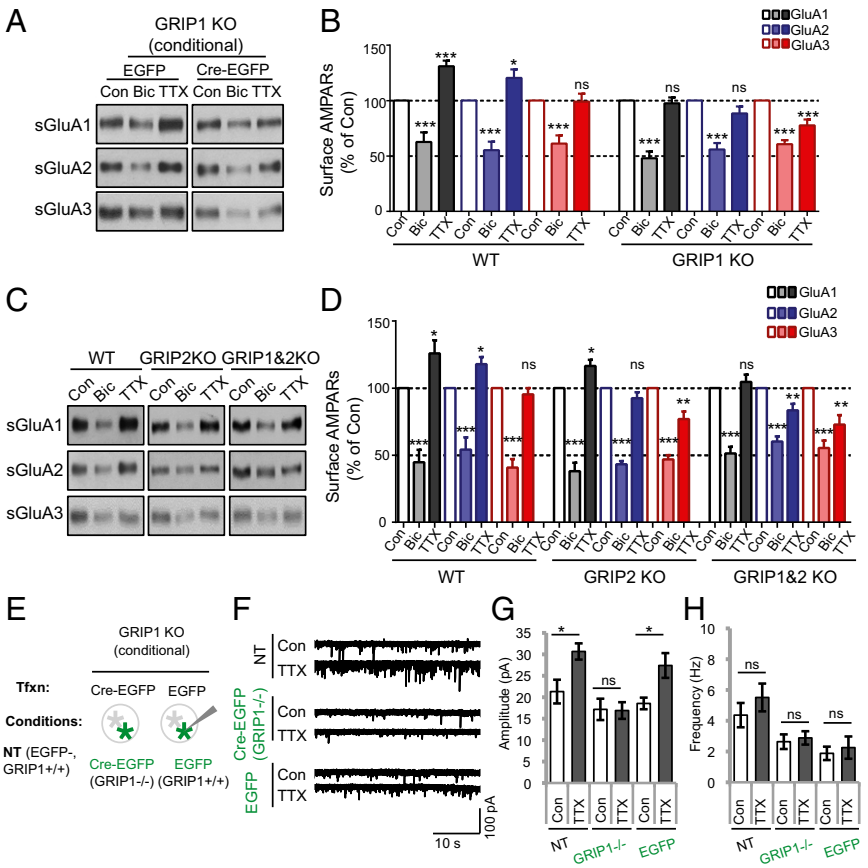

Fig. 3. TTX-induced synaptic scaling is blocked in GRIP knockout neurons. (*A* and *C*) Cortical neurons from GRIP knockout or WT mice were treated with Con/Bic/TTX for 48 h and were then subjected to surface biotinylation assay. The relative amount of surface AMPARs was assessed by Western blot using specific antibodies against GluA1, GluA2, and GluA3 subunits. (*B* and *D*) Quantification of the surface of GluA1, GluA2, and GluA3 in GRIP knockout and WT neurons. Data represent mean ± SEM of band intensities normalized to control values of WT or GRIP knockout neurons (ANOVA, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 5$–10). (*E*) Schema of electrophysiological recording. (*F*) Representative whole-cell recording sample traces of mEPSC events from cultured cortical neurons derived from GRIP1 knockout and WT neurons after treatment with Con/TTX for 48 h. (*G* and *H*) Quantification of mean mEPSC amplitude (*G*) and frequency (*H*) for each population (ANOVA, $*P < 0.05$; $n = 10$–15).

trafficking was observed, and surface AMPARs were significantly decreased during elevated activity (Fig. 3 *A*–*D*).

We considered that the scaling-up defect in GRIP1 or GRIP1/2 knockout neurons may be due to an inability to up-regulate AMPAR subunit protein expression. However, no significant difference in the total expression of GluA1 or GluA2 subunits was found between WT neurons and GRIP knockout neurons during synaptic scaling. Bicuculline treatment down-regulated total expression of all three subunits, and chronic TTX treatment selectively increased total GluA1, but not GluA2 or GluA3 (Fig. S1). A slight decrease of total GluA3 subunit was observed in GRIP2 knockout and double GRIP1/2 knockout neurons following TTX incubation (Fig. S1). Collectively, these data demonstrate that loss of GRIP function blocked TTX-induced synaptic scaling by specifically impairing the trafficking of AMPARs.

To determine the functional role of GRIP at synapses, we recorded miniature excitatory postsynaptic currents (mEPSCs) from WT or GRIP knockout neurons. Because our biotinylation results indicate that GRIP1 plays a major role in homeostatic upscaling, we used GRIP1 conditional knockout mouse neurons for this purpose. Neurons were transfected with either EGFP or EGFP-IRES-Cre to remove GRIP1, and were subsequently treated at DIV 11–13 with TTX for 48 h. As has previously been described (4), TTX treatment increased mEPSC amplitude in untransfected neurons (Fig. 3 *E*–*H*; Tables S1 and S2). However, we saw no increases in synaptic strength following TTX treatment in EGFP-Cre–expressing (GRIP1 knockout) neurons from the same dish (Fig. 3 *E*–*H*). Basal synaptic transmission was decreased in GRIP1 knockout neurons, supporting our findings of reduced surface AMPAR expression in these cells (Fig. 2). To control for the effects of transfection, we recorded mEPSCs from conditional GRIP1 knockout neurons expressing EGFP alone. EGFP-transfected neurons with intact GRIP1 showed a normal homeostatic response to TTX (Fig. 3 *E*–*H*; Tables S1 and S2). These functional data, together with the biochemical results, indicate that GRIP1 is indispensable for scaling up synaptic strength during chronic activity suppression.

Synaptic Scaling Changes GRIP1 Subcellular Distribution and Its Association with GluA2. GRIP1 interacts with the C termini of GluA2/3, and the interaction is critical for AMPAR trafficking (7). Previous studies indicate that there may be two distinct pools of GRIP1 within neurons (29–31). One pool of GRIP1 anchors AMPARs on the cell surface and at synapses, whereas the other pool remains in the cytoplasm and retains AMPARs within intracellular compartments. To test whether GRIP1 regulates the trafficking of AMPARs through these two pools during synaptic scaling, we first examined the effects of synaptic scaling on GRIP1 subcellular distribution. Using a biochemical subcellular fraction method (Fig. 4*C*), we found that TTX treatment decreased GRIP1 in the cytoplasmic fraction (S2), while increasing GRIP1 in the membrane fraction (P2) (Fig. 4 *A* and *B*). We further purified postsynaptic densities (PSD) and found that synaptic GRIP1 was significantly increased following TTX treatment, which parallels the change in synaptic GluA2 levels (Fig. 4 *D*–*F*). In contrast, bicuculline treatment up-regulated GRIP1 expression in both membrane and cytoplasmic fractions (Fig. 4 *A* and *B*), but decreased GRIP1 in the postsynaptic density (Fig. 4 *D*–*F*). Collectively, these data demonstrate that synaptic scaling causes a subcellular redistribution of GRIP1. TTX-induced scaling up results in an overall decrease in GRIP1 protein levels, but the remaining GRIP1 protein becomes enriched at synapses, suggesting that GRIP1 is selectively depleted from cytoplasmic pools. Conversely, bicuculline-mediated scaling down increases GRIP1 in the cytoplasm and membrane fractions, but decreases GRIP1 at synapses.

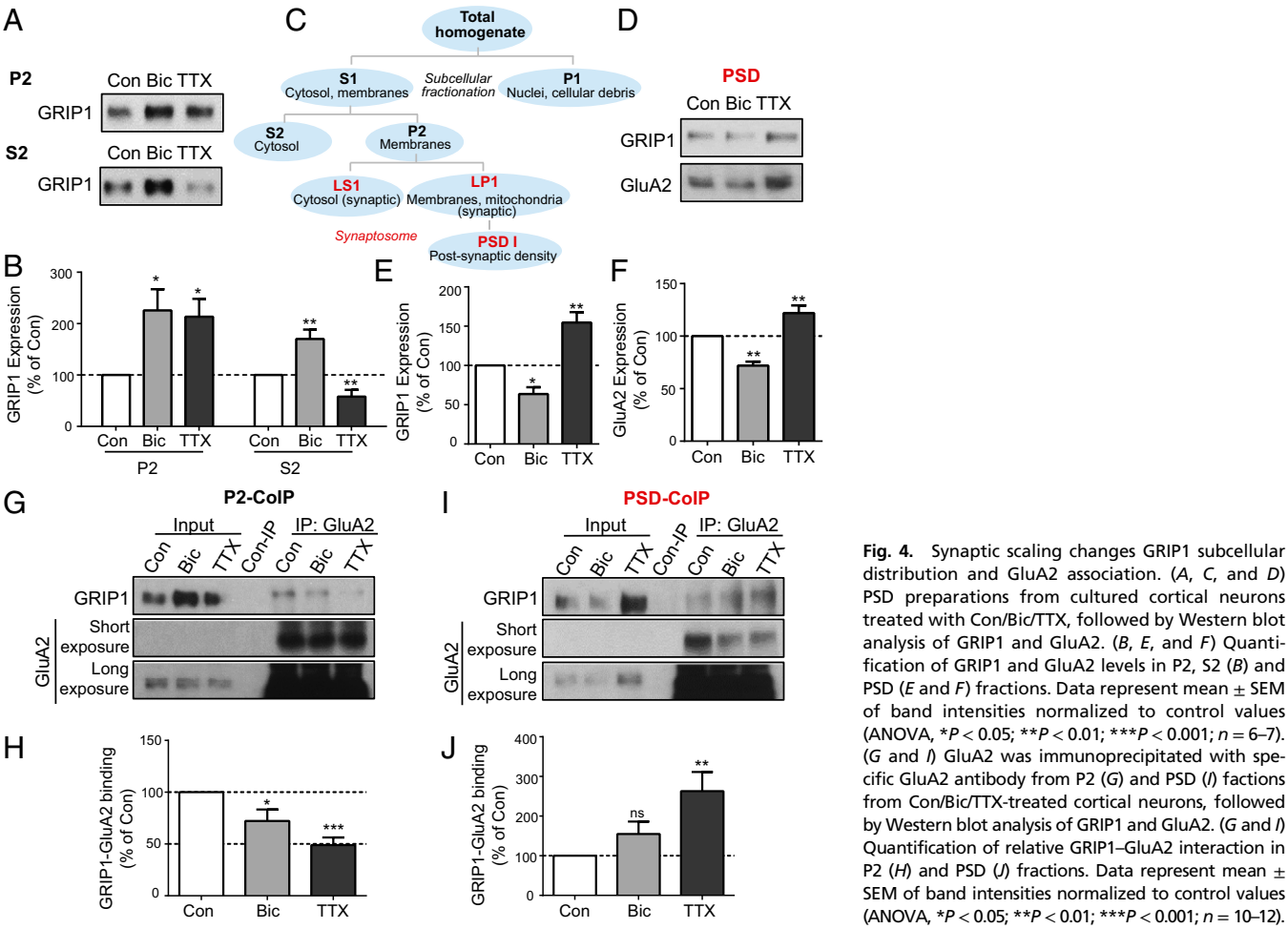

Fig. 4. Synaptic scaling changes GRIP1 subcellular distribution and GluA2 association. (*A*, *C*, and *D*) PSD preparations from cultured cortical neurons treated with Con/Bic/TTX, followed by Western blot analysis of GRIP1 and GluA2. (*B*, *E*, and *F*) Quantification of GRIP1 and GluA2 levels in P2, S2 (*B*) and PSD (*E* and *F*) fractions. Data represent mean ± SEM of band intensities normalized to control values (ANOVA, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 6–7$). (*G* and *I*) GluA2 was immunoprecipitated with specific GluA2 antibody from P2 (*G*) and PSD (*I*) factions from Con/Bic/TTX-treated cortical neurons, followed by Western blot analysis of GRIP1 and GluA2. (*G* and *I*) Quantification of relative GRIP1–GluA2 interaction in P2 (*H*) and PSD (*J*) fractions. Data represent mean ± SEM of band intensities normalized to control values (ANOVA, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 10–12$).

We next examined the interaction between GRIP1 and GluA2 in the different pools. Because GluA2 is a transmembrane protein, we used the membrane fraction (P2) to perform the co-immunoprecipitation (co-IP) experiments to avoid the artificial binding of cytoplasmic GRIP1 with GluA2. Surprisingly, bicuculline or TTX treatment each resulted in reduced coimmunoprecipitation between GRIP1 and GluA2 (Fig. 4 *G* and *H*; Fig. S2). These data indicate that the overall GRIP1–GluA2 interaction was largely disrupted during both downscaling and upscaling processes. We next examined the GRIP1–GluA2 association at synapses. Strikingly, we found that inactivity strongly increased binding of GRIP1 with GluA2 at synapses, whereas hyperactivity did not alter the synaptic association of GRIP1 and GluA2 (Fig. 4 *I* and *J*). Taken together, these data indicate that homeostatic scaling up involves an increase in the synaptic pool of GRIP1, increased GRIP1 interaction with synaptic AMPARs, and disruption of GRIP1 binding to intracellular AMPARs. Conversely, during downscaling, GRIP1 leaves the synapse, accumulating within the cytoplasm.

Discussion

In this study, we found that GRIP1 protein expression and subcellular distribution are regulated during synaptic scaling. Hyperactivity increases total GRIP1, primarily in the cytoplasmic pool, whereas synaptic GRIP1 is reduced. Conversely, inactivity decreases GRIP1 expression, reflecting a loss of cytoplasmic GRIP1, and the remaining GRIP1 protein becomes enriched at synapses. Further, these changes result in altered interaction between GRIP1 and GluA2 at specific subcellular locations. Further, GRIP1 is essential for synapse strengthening during homeostatic scaling up. GRIP1 knockout neurons have reduced surface AMPARs under basal conditions and are unable to up-regulate surface AMPARs or synaptic strength during chronic activity suppression (Fig. 5).

The exact role of GRIP1 in AMPAR trafficking is a subject of some controversy. Some studies suggest that GRIP1 anchors AMPARs at synapses or delivers AMPARs to dendrites through binding to kinesin heavy chain or liprin-α (7, 25, 32, 33). Other studies rather suggest that GRIP1 retains AMPARs within intracellular compartments (22, 34). Still others provide evidence of two pools of GRIP1 (29–31), one associated with the plasma membrane to anchor AMPARs on the cell surface, and the second in the cytoplasm to retain AMPARs within the cell. Our results support the model that GRIP1 regulates AMPAR trafficking by shuttling receptors between the two distinct pools. Inactivity increases the pool of synaptic GRIP1 and strengthens the association between GRIP1 and synaptic AMPARs, stabilizing surface AMPARs at synapses. Simultaneously, cytoplasmic GRIP1–GluA2 interactions are disrupted to relieve intracellular retention, promoting additional trafficking and accumulation of AMPARs at the plasma membrane. During elevated activity, GRIP1 is removed from synapses and accumulates within the cytoplasm, consequently leading to reduced surface AMPARs (Fig. 5). Loss of GRIP1 shows a more specific defect in homeostatic scaling up. Therefore, though the majority of GRIP1 is localized to the cytoplasm under any activity condition, our data suggests that the predominant function of GRIP1 is to deliver AMPARs to the surface and retain them at synapses.

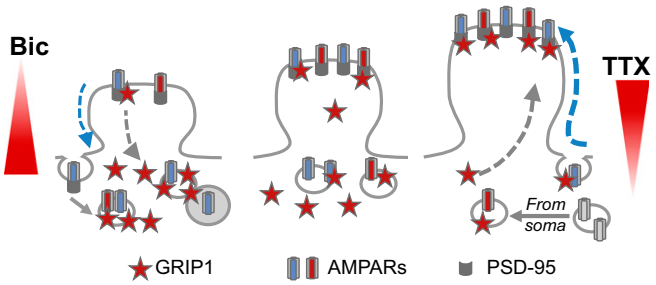

Fig. 5. Model of GRIP1 regulation of AMPARs during synaptic scaling. GRIP1 regulates AMPAR trafficking between two distinct pools. The pool associated with the plasma membrane anchors surface AMPARs, whereas the intracellular pool retains AMPARs within the cell. In inactivity-induced scaling up, synaptic pool of GRIP1 is increased to stabilize surface AMPARs at synapses while cytoplasmic GRIP1–GluA2 interactions are disrupted to relieve intracellular retention, consequently leading to accumulation of AMPARs at cell surface. In hyperactivity-induced scaling down, GRIP1 is removed from synapses and accumulates within the cytoplasm, resulting in decreased surface AMPARs.

The interaction of GRIP1 and GRIP2 with GluA2/3 regulates AMPAR trafficking and synaptic targeting, but how this regulation might manifest lacks scientific consensus and remains under active scrutiny. Our results suggest that GRIP1 is required for homeostatic up-regulation of GluA1 as well as GluA2, despite the fact that GRIP1 and 2 do not bind GluA1. Within the homeostatic plasticity literature, there is still much debate as to whether synaptic upscaling promotes homomeric GluA1–GluA1 AMPAR insertion, heteromeric GluA1–GluA2 insertion, or both. Postsynaptic recruitment of GluA1 homomers or GluA1–GluA2 heteromers has both been reported during inactivity (35–39). Studies that argue for GluA2-lacking AMPAR insertion during scaling up generally use cultured hippocampal neurons and primarily induced upscaling by a combination of TTX-mediated disruption of firing and glutamate receptor blockade (35–38). Here, we used rat or mouse cortical neurons and blocked firing alone with prolonged incubation of TTX. Consistent with previous studies using similar strategies (9, 16, 26, 39), we saw an increase of both surface GluA1 and GluA2 upon activity blockade, indicating preferential homeostatic insertion of GluA1–GluA2 heteromers. We saw no clear changes in synaptic current kinetics, making it unlikely that synaptic insertion of homomeric GluA1–GluA1 AMPARs predominated. Our data support the hypothesis that synaptic upscaling in cortical neurons occurs through GluA1–GluA2 heteromeric incorporation and that loss of GRIP1 impairs the trafficking of GluA1–GluA2 heteromers.

Emerging evidence in the field of homeostatic plasticity suggests that scaling up and scaling down involve different signaling pathways. Several molecules involved in signaling or receptor trafficking are important or required for scaling up, such as AKAP5, PICK1, and Arc (9, 11, 16), are dispensable for scaling down. Conversely, molecules like Plk2 and PSD-93, though essential for downscaling, are not required for upscaling (15, 40). Our observation that GRIP1 is indispensable for synaptic scaling up provides further evidence that synaptic homeostasis does not consist of reciprocal modulation of the same signaling pathways. Our data demonstrate that downscaling remains intact in GRIP knockout neurons despite reduced surface expression of AMPARs at rest. One possible explanation for these data could be that multiple surface pools of AMPARs are modulated by distinct sets of proteins. The pool that is regulated or anchored by GRIP1 may be primarily implicated in basal synaptic transmission and inactivity-induced scaling up, whereas hyperactivity-induced scaling down might mainly involve other surface pools that are regulated by other scaffolding proteins, such as PSD95 rather than GRIP1. Further, synaptic scaling down is associated with degradation of internalized receptors as shown by the decrease in total AMPARs (Fig. S1). This protein degradation pathway is mainly mediated via the ubiquitin–proteasome system (41–43).

Several variants of GRIP1 have been identified in patients with autism, and GRIP1/2 double-knockout mice showed abnormal social behaviors (44). Autism spectrum disorders (ASD) are associated with abnormal synaptogenesis and imbalance between excitatory and inhibitory currents (3, 45). GRIP1 could be implicated in ASD through these two processes given its role in neuronal development and expression at both glutamatergic and GABAergic synapses (19, 23, 46). Further, a wealth of studies provides molecular links between homeostatic plasticity and ASD. For example, Homer and mGluR signaling play essential roles in homeostatic plasticity and are also implicated in ASD (47, 48). Our findings that GRIP1 is required for homeostatic synaptic scaling may shed light on the mechanisms of how GRIP1 contributes to ASD. In addition, previous work indicates that synaptic size increases during upscaling process (49), it will be interesting to know whether or how GRIP1 modulates this synaptic structural plasticity because GRIP1 is necessary for both upscaling and neuronal development.

Collectively, the results presented here demonstrate that GRIP1 is essential for inactivity-induced synaptic scaling up. Our data support the notion that GRIP1 regulates AMPAR trafficking between two different pools. The GRIP1 pool associated with the plasma membrane stabilizes surface AMPARs, and the intracellular pool retains AMPARs in intracellular compartments. During neuronal inactivity, GRIP1 is redistributed to synapses, and this shift is essential for homeostatic adaptation of synaptic strength. Conversely, during neuronal hyperactivity, GRIP1 accumulates in the cytoplasm, allowing AMPARs to be removed from synapses.

Materials and Methods

Neuronal Culture. Rat or mouse embryonic (E18) cortical neurons were plated on poly-L-lysine coated tissue culture dishes or glass coverslips at a density of 65,000 cells/cm^2 and grown in glia-conditioned neurobasal media (Invitrogen) supplemented with 2% (vol/vol) B-27, 2 mM GlutaMAX, 50 U/mL Pen Strep, and 1% horse serum (Invitrogen). Cultured neurons were fed twice per week and used at DIV 13–15. To induce synaptic scaling, neurons were treated with bicuculline (20 μM) or TTX (1 μM) for 48 h.

Surface Biotinylation. Neurons rinsed once with ice-cold PBSCM [PBS-calcium-magnesium: 1 × PBS, 1mM $MgCl_2$, 0.1mM $CaCl_2$ (pH 8.0)] were incubated with Sulfo-NHS-SS-biotin (0.5 mg/mL, Thermo Scientific) for 30 min at 4 °C. Neurons were then washed with PBSCM and incubated in 20 mM glycine twice for 5 min to quench unreacted biotinylation reagent. Neurons were lysed in lysis buffer [PBS containing 50 mM NaF, 5mM sodium pyrophosphate, 1% Nonidet P-40, 1% sodium deoxycholate, 0.02% SDS, and protease inhibitor mixture (Roche)]. Equal amounts of proteins were incubated overnight with NeutrAvidin agarose beads (Thermo Scientific) and then washed with lysis buffer four times. Biotinylated proteins were eluted using 2× SDS loading buffer. Surface or total proteins were then subjected to SDS/PAGE and analyzed by Western blot.

PSD Fractionation. Rat cortical neurons were harvested in buffer [320 mM sucrose, 5 mM sodium pyrophosphate, 1 mM EDTA, 10 mM Hepes (pH 7.4), 200 nM okadaic acid, protease inhibitor mixture (Roche)] and homogenized using a 26-gauge needle. Homogenate was then centrifuged at 800 × *g* for 10 min at 4 °C to yield P1 and S1. S1 was centrifuged at 20, 000 × *g* for 20 min to yield P2 and S2. P2 was then resuspended in water adjusted to 4 mM Hepes (pH 7.4) followed by 30-min agitation at 4 °C. Suspended P2 was centrifuged at 25,000 × *g* for 20 min at 4 °C. The resulted pellet was resuspended in 50 mM Hepes (pH 7.4), mixed with an equal volume of 1% Triton X-100, and agitated at 4 °C for 10 min. The PSD fraction was generated by centrifugation at 32,000 × *g* for 20 min at 4 °C.

Co-IP. P2 membrane and PSD fractions were prepared as described previously and then lysed in PBS containing 50 mM NaF, 5 mM sodium pyrophosphate, 1% Nonidet P-40, 1% sodium deoxycholate, 1 μM okadaic acid, and protease

inhibitor mixture (Roche). The IP antibody or control antibody was precoupled to Protein-A Sepharose beads and incubated with 200 μg of P2 proteins or 120 μg of PSD proteins in lysis buffer at 4 °C for 2 h. The beads were then washed in lysis buffer 6× followed by 2× SDS loading buffer elution. Bound proteins were resolved by SDS/PAGE for Western blot analysis.

Antibodies. The following antibodies were used: anti–β-tubulin mAb (Sigma), anti-GluA1 N-terminal antibody mAb (4.9D, made in-house), anti-GluA2 N-terminal antibody mAb (032.19.9, made in-house), anti-GluA2 phospho-880 specific mAb (02.22.4, made in house), anti-PSD95 mAb (NeuroMab), anti-GluA3 pAb (JH4300, made in-house), anti-GRIP1 mAb (BD Biosciences), anti-GRIP1 pAb (Chemicon), and anti-GRIP1 pAb (JH2260, made in-house).

Immunocytochemistry. Cortical neurons fixed in PBS containing 4% (vol/vol) paraformaldehyde/4% (wt/vol) sucrose were incubated with primary antibodies overnight at 4 °C in 1× GDB buffer [15 mM phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.3% Triton X-100, and 0.25 M NaCl], followed by secondary antibodies for 1 h at room temperature. Confocal z-serial image stacks of neurons were taken with an LSM510 confocal microscope system (Zeiss).

Electrophysiology. On the day of recording, neurons were transferred into room temperature artificial cerebrospinal fluid containing (in mM): 145 NaCl, 5 KCl, 5 Hepes, 5 glucose, 1 $CaCl_2$, 2 $MgCl_2$ (pH 7.4). Single-barrel glass pipettes (World Precision Instruments) were pulled to 3–6 MΩ (Sutter Instruments Flaming/Brown Micropipette Puller) and filled with internal solution (in mM): 145 K gluconate, 5 EGTA, 5 $MgCl_2$, 10 Hepes, 5 NaATP, 0.2 NaGTP (pH 7.2). Excitatory neurons were visualized with an upright Zeiss Examiner fluorescent microscope and voltage-clamped at −70 mV (MultiClamp 700B; Axon Instruments). Synaptic currents were recorded at 5 kHz in the presence of 0.5 μM TTX and 50 μM pertussis toxin (PTX), digitized (Digidata 1440A; Axon Instruments), and analyzed offline using the event detection function in Clampfit 10.5 (Molecular Devices). Miniature EPSCs were automatically detected (template search, 5 pA baseline, template match threshold is 2) and manually verified.

Statistical Analysis. All statistical analysis was performed in GraphPad Prism 5. For biochemical results, statistical significance was determined by unpaired two-tailed Student *t* test or one-way ANOVA as indicated in the figure legends. Synaptic current and recording parameters (amplitude, frequency, rise time, etc.) were analyzed for normality with a D'Agostino and Pearson omnibus test. The effect of genotype (WT v. $GRIP^{-/-}$) and treatment (NT v. TTX) were determined using two-way ANOVA and, where applicable, Bonferroni posttest.

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1. Neves G, Cooke SF, Bliss TVP (2008) Synaptic plasticity, memory and the hippocampus: A neural network approach to causality. *Nat Rev Neurosci* 9(1):65–75.
2. Turrigiano GG (2008) The self-tuning neuron: Synaptic scaling of excitatory synapses. *Cell* 135(3):422–435.
3. Davis GW (2013) Homeostatic signaling and the stabilization of neural function. *Neuron* 80(3):718–728.
4. Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB (1998) Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature* 391(6670): 892–896.
5. Shepherd JD, Huganir RL (2007) The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol* 23:613–643.
6. Huganir RL, Nicoll RA (2013) AMPARs and synaptic plasticity: The last 25 years. *Neuron* 80(3):704–717.
7. Anggono V, Huganir RL (2012) Regulation of AMPA receptor trafficking and synaptic plasticity. *Curr Opin Neurobiol* 22(3):461–469.
8. Kim S, Ziff EB (2014) Calcineurin mediates synaptic scaling via synaptic trafficking of Ca2+-permeable AMPA receptors. *PLoS Biol* 12(7):e1001900.
9. Diering GH, Gustina AS, Huganir RL (2014) PKA-GluA1 coupling via AKAP5 controls AMPA receptor phosphorylation and cell-surface targeting during bidirectional homeostatic plasticity. *Neuron* 84(4):790–805.
10. Gainey MA, Hurvitz-Wolff JR, Lambo ME, Turrigiano GG (2009) Synaptic scaling requires the GluR2 subunit of the AMPA receptor. *J Neurosci* 29(20):6479–6489.
11. Shepherd JD, et al. (2006) Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron* 52(3):475–484.
12. Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. *Nature* 440(7087):1054–1059.
13. Steinmetz CC, Turrigiano GG (2010) Tumor necrosis factor-α signaling maintains the ability of cortical synapses to express synaptic scaling. *J Neurosci* 30(44):14685–14690.
14. Cingolani LA, et al. (2008) Activity-dependent regulation of synaptic AMPA receptor composition and abundance by β3 integrins. *Neuron* 58(5):749–762.
15. Sun Q, Turrigiano GG (2011) PSD-95 and PSD-93 play critical but distinct roles in synaptic scaling up and down. *J Neurosci* 31(18):6800–6808.
16. Anggono V, Clem RL, Huganir RL (2011) PICK1 loss of function occludes homeostatic synaptic scaling. *J Neurosci* 31(6):2188–2196.
17. Dong H, et al. (1997) GRIP: A synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* 386(6622):279–284.
18. Srivastava S, et al. (1998) Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP. *Neuron* 21(3):581–591.
19. Geiger JC, et al. (2014) The GRIP1/14-3-3 pathway coordinates cargo trafficking and dendrite development. *Dev Cell* 28(4):381–393.
20. Takamiya K, Mao L, Huganir RL, Linden DJ (2008) The glutamate receptor-interacting protein family of GluR2-binding proteins is required for long-term synaptic depression expression in cerebellar Purkinje cells. *J Neurosci* 28(22):5752–5755.
21. Osten P, et al. (2000) Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor. *Neuron* 27(2):313–325.
22. Daw MI, et al. (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. *Neuron* 28(3):873–886.
23. Hoogenraad CC, Milstein AD, Ethell IM, Henkemeyer M, Sheng M (2005) GRIP1 controls dendrite morphogenesis by regulating EphB receptor trafficking. *Nat Neurosci* 8(7):906–915.
24. Mao L, Takamiya K, Thomas G, Lin D-T, Huganir RL (2010) GRIP1 and 2 regulate activity-dependent AMPA receptor recycling via exocyst complex interactions. *Proc Natl Acad Sci USA* 107(44):19038–19043.
25. Setou M, et al. (2002) Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. *Nature* 417(6884):83–87.
26. O'Brien RJ, et al. (1998) Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21(5):1067–1078.
27. Bladt F, Tafuri A, Gelkop S, Langille L, Pawson T (2002) Epidermolysis bullosa and embryonic lethality in mice lacking the multi-PDZ domain protein GRIP1. *Proc Natl Acad Sci USA* 99(10):6816–6821.
28. Takamiya K, et al. (2004) A direct functional link between the multi-PDZ domain protein GRIP1 and the Fraser syndrome protein Fras1. *Nat Genet* 36(2):172–177.
29. DeSouza S, Fu J, States BA, Ziff EB (2002) Differential palmitoylation directs the AMPA receptor-binding protein ABP to spines or to intracellular clusters. *J Neurosci* 22(9): 3493–3503.
30. Hanley LJ, Henley JM (2010) Differential roles of GRIP1a and GRIP1b in AMPA receptor trafficking. *Neurosci Lett* 485(3):167–172.
31. Thomas GM, Hayashi T, Chiu SL, Chen CM, Huganir RL (2012) Palmitoylation by DHHC5/8 targets GRIP1 to dendritic endosomes to regulate AMPA-R trafficking. *Neuron* 73(3):482–496.
32. Wyszynski M, et al. (2002) Interaction between GRIP and liprin-α/SYD2 is required for AMPA receptor targeting. *Neuron* 34(1):39–52.
33. Shin H, et al. (2003) Association of the kinesin motor KIF1A with the multimodular protein liprin-α. *J Biol Chem* 278(13):11393–11401.
34. Braithwaite SP, Xia H, Malenka RC (2002) Differential roles for NSF and GRIP/ABP in AMPA receptor cycling. *Proc Natl Acad Sci USA* 99(10):7096–7101.
35. Ju W, et al. (2004) Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat Neurosci* 7(3):244–253.
36. Sutton MA, et al. (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* 125(4):785–799.
37. Aoto J, Nam CI, Poon MM, Ting P, Chen L (2008) Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron* 60(2):308–320.
38. Thiagarajan TC, Lindskog M, Tsien RW (2005) Adaptation to synaptic inactivity in hippocampal neurons. *Neuron* 47(5):725–737.
39. Wierenga CJ, Ibata K, Turrigiano GG (2005) Postsynaptic expression of homeostatic plasticity at neocortical synapses. *J Neurosci* 25(11):2895–2905.
40. Seeburg DP, Feliu-Mojer M, Gaiottino J, Pak DTS, Sheng M (2008) Critical role of CDK5 and Polo-like kinase 2 in homeostatic synaptic plasticity during elevated activity. *Neuron* 58(4):571–583.
41. Mabb AM, et al. (2014) Triad3A regulates synaptic strength by ubiquitination of Arc. *Neuron* 82(6):1299–1316.
42. Fu AKY, et al. (2011) APC(Cdh1) mediates EphA4-dependent downregulation of AMPA receptors in homeostatic plasticity. *Nat Neurosci* 14(2):181–189.
43. Scudder SL, et al. (2014) Synaptic strength is bidirectionally controlled by opposing activity-dependent regulation of Nedd4-1 and USP8. *J Neurosci* 34(50):16637–16649.
44. Mejias R, et al. (2011) Gain-of-function glutamate receptor interacting protein 1 variants alter GluA2 recycling and surface distribution in patients with autism. *Proc Natl Acad Sci USA* 108(12):4920–4925.
45. Bourgeron T (2009) A synaptic trek to autism. *Curr Opin Neurobiol* 19(2):231–234.
46. Dong H, et al. (1999) Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. *J Neurosci* 19(16):6930–6941.
47. Kelleher RJ, 3rd, et al. (2012) High-throughput sequencing of mGluR signaling pathway genes reveals enrichment of rare variants in autism. *PLoS One* 7(4):e35003.
48. Hu JH, et al. (2010) Homeostatic scaling requires group I mGluR activation mediated by Homer1a. *Neuron* 68(6):1128–1142.
49. Keck T, et al. (2013) Synaptic scaling and homeostatic plasticity in the mouse visual cortex in vivo. *Neuron* 80(2):327–334.