

Activity-dependent synaptic GRIP1 accumulation drives synaptic scaling up in response to action potential blockade

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Synaptic scaling is a form of homeostatic plasticity that stabilizes neuronal firing in response to changes in synapse number and strength. Scaling up in response to action-potential blockade is accomplished through increased synaptic accumulation of GluA2-containing AMPA receptors (AMPA), but the receptor trafficking steps that drive this process remain largely obscure. Here, we show that the AMPAR-binding protein glutamate receptor-interacting protein-1 (GRIP1) is essential for regulated synaptic AMPAR accumulation during scaling up. Synaptic abundance of GRIP1 was enhanced by activity deprivation, directly increasing synaptic GRIP1 abundance through overexpression increased the amplitude of AMPA miniature excitatory postsynaptic currents (mEPSCs), and shRNA-mediated GRIP1 knockdown prevented scaling up of AMPA mEPSCs. Furthermore, knockdown and rescue experiments targeting either GRIP1 or GluA2 revealed that scaling up requires the interaction between GRIP1 and GluA2. Finally, GRIP1 synaptic accumulation during scaling up did not require GluA2 binding. Taken together, our data support a model in which activity-dependent trafficking of GRIP1 to synaptic sites drives the forward trafficking and enhanced synaptic accumulation of GluA2-containing AMPAR during synaptic scaling up.

synaptic scaling | synaptic plasticity | GRIP1 | AMPAR | GluA2

Proper development of neuronal circuits, as well as efficient information storage during learning and memory, are thought to depend upon the presence of homeostatic mechanisms that stabilize neuronal excitability (1–3). One such mechanism is synaptic scaling, which compensates for perturbations in average firing by scaling up or down the postsynaptic strength of all of a neuron's excitatory synapses (4). Synaptic scaling is a cell-autonomous process in which neurons detect changes in their own firing through a set of calcium-dependent sensors that then regulate receptor trafficking to increase or decrease the accumulation of AMPA receptors (AMPA) at synaptic sites and thus increase or decrease synaptic strength (4–6). Despite great recent interest, the AMPA receptor-trafficking events that underlie synaptic scaling remain largely obscure. Defects in synaptic scaling have been postulated to contribute to disorders as diverse as Alzheimer's disease (7) and epilepsy (8), so illuminating the underlying AMPAR trafficking steps could shed light into the genesis of a wide range of neurological disorders.

Most neocortical AMPAR are heteromeric receptors composed of both GluA1 and GluA2 subunits, which have unique phosphorylation sites and interact with distinct trafficking proteins (9). During synaptic scaling up in response to action potential blockade, synaptic strength is increased through enhanced synaptic accumulation of GluA1 and GluA2-containing AMPAR (5, 10–13) and requires the C-terminal domain of the GluA2 subunit (12), but which subunit-specific interactions underlie synaptic scaling remain controversial (12, 14). Several trafficking proteins are known to interact with the GluA2, but not the GluA1, C-tail, including glutamate receptor interacting protein-1 (GRIP1) (15) and protein interacting with C-kinase-1 (PICK1) (16). Many studies have examined the role of GRIP1 and PICK1 in AMPAR trafficking and surface accumulation (15, 17–22), but little is

known about their potential roles in regulating AMPAR synaptic accumulation during synaptic scaling. It was recently shown that deletion of PICK1, which competes with GRIP1/2 for binding to GluA2, enhances AMPAR accumulation and occludes synaptic scaling up (23), suggesting GRIP1/PICK1-GluA2 interactions as possible critical players in synaptic scaling.

GRIP1 was one of the first AMPAR-binding proteins identified (15), and yet its exact function in synaptic transmission and plasticity remains controversial. GRIP1 is an abundant multi-PDZ domain-containing protein that interacts with GluA2 through its fourth and fifth PDZ domains (15) and has known interactions with several other signaling and trafficking proteins, including itself (24), ABP (25), EphB receptors (26); the rasGEF GRASP-1 (27), the scaffold protein liprin- α (28), and the microtubule motor protein KIF5, or kinesin 1 (29). The role of GRIP1 in AMPAR trafficking is complicated and may involve AMPAR trafficking to and stabilization at synapses (17), as well as microtubule-based transport into dendrites (29) and the regulation of AMPAR movement between intracellular recycling compartments and the cell surface (22, 30). How GRIP1 influences basal AMPAR trafficking is not entirely clear. Overexpression of GRIP1 or gain-of-function GRIP1 mutants have been consistently observed to enhance surface AMPAR levels (21, 31), but knockout or dominant-negative GRIP1 constructs have had inconsistent effects, with slower synaptic AMPAR accumulation

Significance

Brain circuits need plasticity mechanisms that stabilize activity to function properly. Although such homeostatic plasticity mechanisms have been widely described in a number of brain circuits, little is known about the molecular pathways that mediate them. Here, we show that an important form of homeostatic synaptic plasticity at excitatory synapses, synaptic scaling, relies on the activity-dependent accumulation of the glutamate receptor-binding protein glutamate receptor-interacting protein-1 (GRIP1) at synaptic sites. Our data show that GRIP1 is recruited to synapses under conditions of hypoactivity and, through a direct interaction with AMPA-type glutamate receptors, in turn, recruits more AMPA receptors to the synapse. These findings generate molecular insight into the mechanisms that adjust excitatory synaptic strength in response to perturbations in firing.

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observed in one study (17) but no effects on basal AMPAR recycling and transmission in others (22, 32). Interestingly, GRIP1 and -2 are critical for the expression of cerebellar long-term depression (LTD), where they play redundant roles in regulated AMPAR endocytosis (33, 34). Currently no direct role for GRIP1 in activity-dependent synaptic strengthening or homeostatic plasticity has been established.

Here, we show that the GRIP1–GluA2 interaction plays an essential role in the activity-dependent synaptic AMPAR accumulation and enhanced excitatory synaptic strength that underlies synaptic scaling up. Activity blockade with TTX increased the accumulation of GRIP1 at synaptic sites, whereas directly enhancing synaptic GRIP1 accumulation through overexpression (OE) was sufficient to mimic synaptic scaling. GRIP1 was necessary for synaptic scaling, because scaling up was prevented by shRNA-mediated knock down (KD) of endogenous GRIP1 and rescued by replacement with an RNAi-insensitive (RNAiI) GRIP1 but not a GRIP1 mutant that lacks the GluA2 interaction domain. We showed previously that GluA2 KD blocks synaptic scaling (12). Here, we show that synaptic scaling after GluA2 KD can be rescued by wild-type (RNAiI) GluA2 or point mutants that do not

interfere with GRIP1 binding but not by GluA2 point mutants (Y876E and S880E) that reduce GluA2–GRIP1 binding, strongly suggesting that GRIP1 mediates synaptic scaling through interactions with GluA2. Finally, TTX still induced GRIP1 synaptic accumulation even when AMPAR accumulation was prevented by expression of GluA2 Y876E; thus, during synaptic scaling GluA2 synaptic accumulation depends on GRIP1 binding, but GRIP1 translocation and synaptic accumulation occur independently of GluA2 binding. Together our data show that activity-dependent regulation of synaptic GRIP1 abundance is critical for the forward trafficking and accumulation of AMPA receptors at synapses during synaptic scaling.

Results

We showed previously that the GluA2 C-tail is required for TTX-induced synaptic scaling up (12). Here, we wished to determine the role of the GluA2 C-tail binding protein, GRIP1, in synaptic scaling. Experiments were performed on cultured postnatal rat visual cortical neurons on 7–10 d in vitro (DIV). Synaptic scaling was induced with a 6-h TTX treatment as

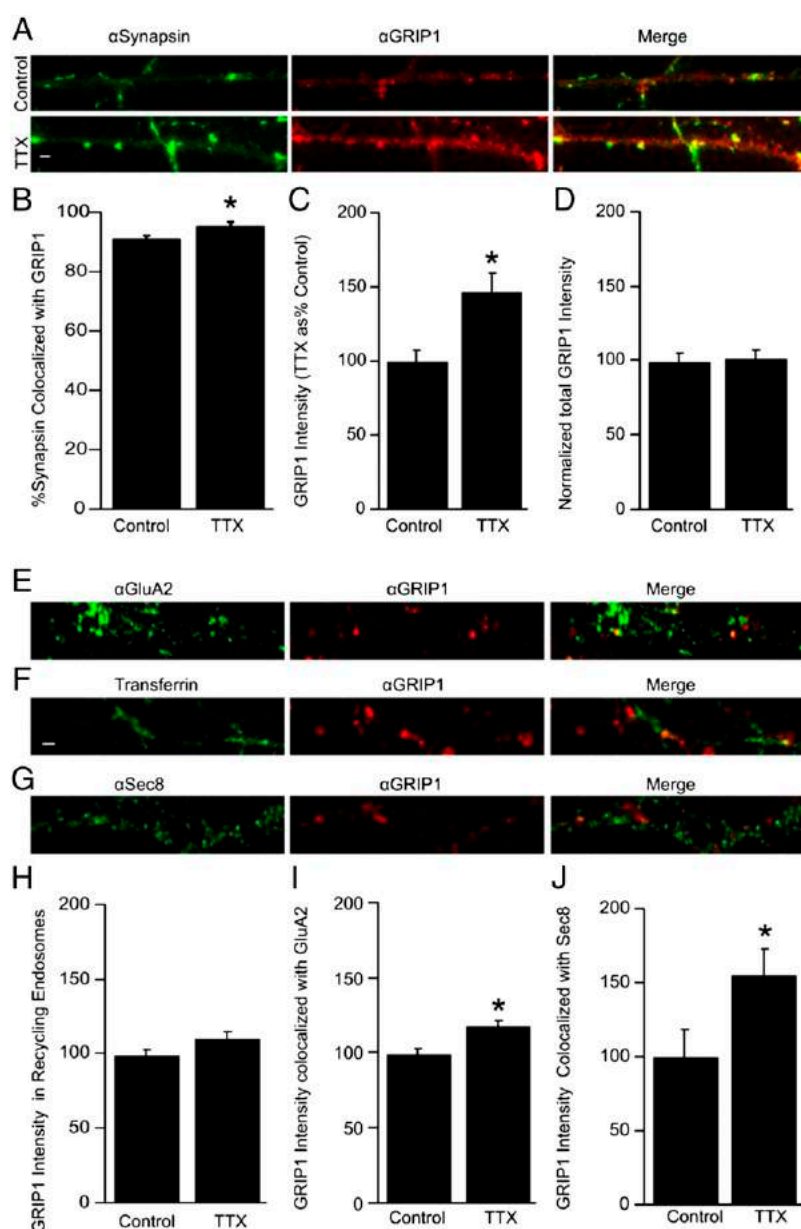


Fig. 1. Activity blockade drives increased synaptic GRIP1 accumulation. (A) Examples of staining against synapsin (green) and GRIP1 (red) in control (top images) (upper images) and 6-h TTX conditions (lower images). (B) Percentage of synapsin puncta colocalized with GRIP1 puncta in control and TTX conditions. (C) Average Intensity of GRIP1 puncta that colocalized with synapsin; values from TTX-treated neurons expressed as percentage of untreated controls. (D) Analysis of the total GRIP1 dendritic signal (normalized to dendritic area) for control and TTX conditions; values from TTX-treated neurons expressed as percent of untreated controls. (E, F, and G) Example dendritic images showing colabeling against GRIP1 and GluA2 (Top), transferrin (Middle), or sec8 (Bottom), respectively. (H, I, and J) Intensity of the GRIP1 signal at puncta that colocalize with Transferrin (H), GluA2 (I), or Sec8 (J); values from TTX-treated neurons expressed as percentage of untreated control. (Scale bar: 1 μ m.) *Different from control, $P < 0.05$. Here and below, all n values are given in the results section, and all data are presented as means \pm SEM for the number of neurons (n) indicated.

plays a role in trafficking GluA2 to synapses during synaptic scaling.

Overexpression of GRIP1 Is Sufficient to Increase mEPSC Amplitude.

Because GRIP1 accumulates at synapses during activity blockade, we next asked whether directly increasing GRIP1 levels through OE was sufficient to drive more GRIP1 to synapses and to increase mEPSC amplitude. OE of GRIP1 for 2 d significantly enhanced the intensity of the GRIP1 signal at puncta that colocalize with postsynaptic density-95 (PSD-95) (Fig. 3*A* and *B*; control, $n = 10$; GRIP1 OE, $n = 9$; $P < 0.01$). Furthermore, baseline mEPSC amplitude (but not frequency) was significantly increased in GRIP1 OE neurons compared with control (Fig. 3*C–E*; control, $n = 8$; GRIP1 OE, $n = 7$; mEPSC amplitude in the GRIP1 OE condition was $141.2 \pm 19\%$ of control; $P < 0.05$). Together, these results indicate that OE of GRIP1 is sufficient to increase the accumulation of GRIP1 at synaptic sites and to drive an increase in AMPA mEPSC amplitude.

GRIP1 Is Necessary for the Expression of Synaptic Scaling Up. The data described above suggest that activity blockade induces synaptic scaling by enhancing the synaptic accumulation of GRIP1. If so, then reducing neuronal GRIP1 should attenuate or abolish synaptic scaling. GRIP1 KO is embryonic lethal (38, 39), so to reduce GRIP1 levels acutely and in a cell-autonomous manner, we used an shRNA against GRIP1 (40) to selectively KD GRIP1 in individual cultured cortical pyramidal neurons. A 2-d expression of the GRIP1 shRNA (KD) reduced GRIP1 puncta intensity to 46% of control and also produced a 37% reduction in dendritic length density of GRIP1 puncta (Fig. 4*A* and *B*; control, $n = 14$ neurons; GRIP1 KD, $n = 13$ neurons), giving a total KD to $\sim 35\%$ of control levels. This degree of KD was sufficient to completely block synaptic scaling; a 6-h treatment with TTX significantly enhanced mEPSC amplitude in control neurons as described previously (5, 35) but not in GRIP1

KD neurons (Fig. 4*C* and *D*; control, $n = 46$; TTX, $n = 55$; TTX was $129.6 \pm 7.3\%$ of control; $P < 0.0001$; control GRIP1 KD, $n = 13$; TTX GRIP1 KD, $n = 8$; TTX was $103.8 \pm 10.8\%$ of control; $P = 0.73$).

To control for any potential off-target effects of the hairpin, we cotransfected neurons with the GRIP1 shRNA and an RNAi-resistant GRIP1 construct (Rescue) and asked whether we could rescue synaptic scaling. Indeed, scaling was normal in neurons transfected with the shRNA and the rescue construct (Fig. 4*C* and *D*; control GRIP1KD & Rescue, $n = 13$; TTX GRIP1KD & Rescue, $n = 11$; TTX was $132.5 \pm 12.9\%$ of control; $P < 0.01$). GRIP1 interacts with GluA2 through PDZ domains 4–5 (9); a mutated form of RNAi GRIP1 missing PDZ domains 4–6 (40) was unable to rescue scaling (Fig. 4*D*). These data demonstrate that GRIP1 is essential for synaptic scaling up, and raise the possibility that scaling may require a direct interaction between GRIP1 and GluA2.

Manipulating GRIP1 Expression Does Not Affect mEPSC Kinetics or Passive Properties.

Longer KD of GRIP1 (4 d) has been reported to impact dendritic elaboration in cultured hippocampal neurons (40), raising the possibility that changes in electrotonic filtering could contribute to the changes in mEPSC amplitude we observe upon manipulations of GRIP1 levels. To address this possibility, we measured the mEPSC kinetics, and the passive properties of neurons, upon OE or KD of GRIP1. Neither OE nor KD of GRIP1 had any significant effect on mEPSC rise time or decay (Fig. 5*A*: control, $n = 8$; GRIP1 OE, $n = 7$; for rise, $P = 0.25$; for decay, $P = 0.31$; Fig. 5*B*: control, $n = 5$; GRIP1 KD, $n = 13$; for rise, $P = 0.59$; for decay, $P = 0.46$). Thus, changes in mEPSC amplitude are independent of changes in electrotonic filtering.

In addition, dendritic morphologies appeared normal in OE and KD neurons, and these manipulations had no significant impact on whole cell capacitance or input resistance (Fig. 5*C*;

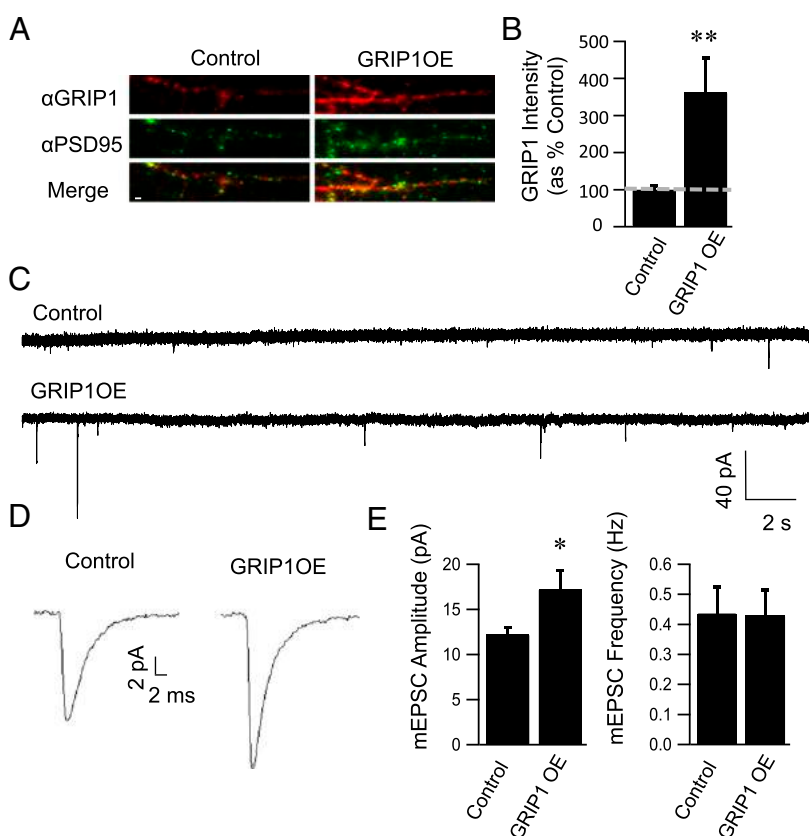


Fig. 3. Overexpression of GRIP1 increases synaptic GRIP1 accumulation and mEPSC amplitude. (A) Example of control (Left) and GRIP1 OE (Right) dendrites labeled against PSD-95 (in green) and GRIP1 (in red; endogenous in control, both endogenous and exogenous in GRIP1 OE condition). (Scale bar: 1 μ m.) (B) Average Intensity of GRIP1 puncta colocalized with PSD-95 for control and GRIP1 OE conditions; values from GRIP1 OE neurons expressed as percentage of control neurons. (C) Example mEPSC recordings from control and GRIP1OE neurons. (D and E) Average mEPSC waveform (D) and average mEPSC amplitude (E) (Left) and frequency (Right) for control and GRIP1 OE conditions. *Different from control, $P < 0.05$, ** $P < 0.01$.

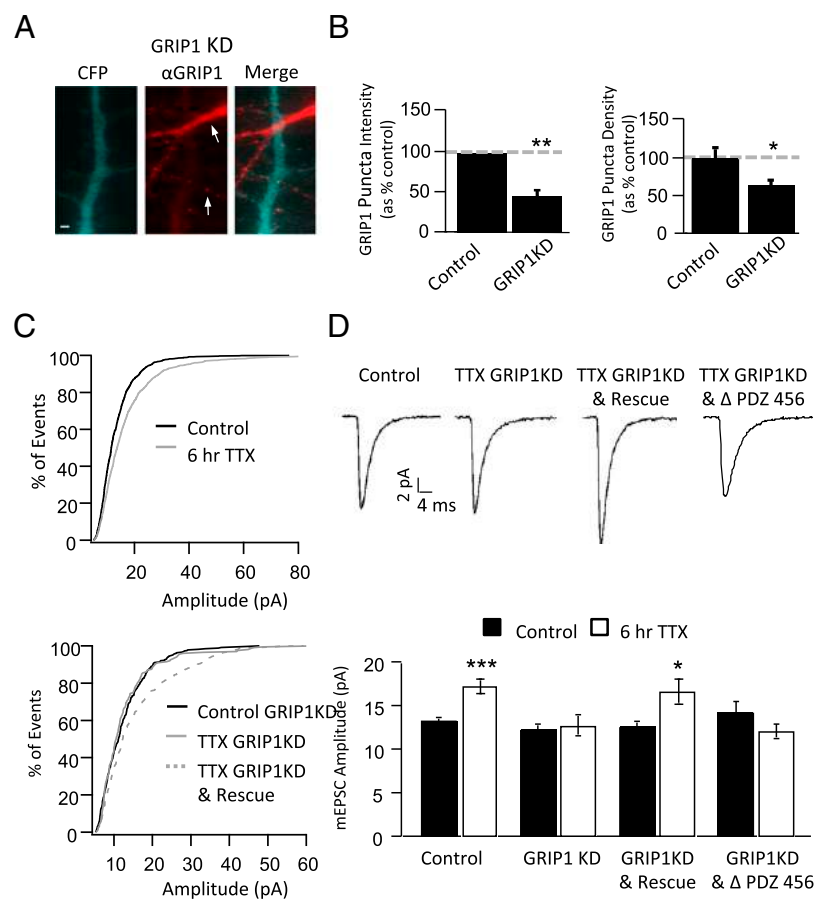


Fig. 4. GRIP1 KD blocks synaptic scaling. (A) Sparse transfection of cultured neurons with the GRIP1 shRNA and soluble CFP (blue) (to label transfected neurons and dendrites) and α GRIP1 (red); the dendrite from the hairpin-expressing neuron shows little GRIP1 signal relative to the nontransfected dendrite (arrows). (B) Puncta Intensity (Left) and length density (puncta per μm) (Right) of GRIP1 puncta for control or shRNA-transfected dendrites; values from GRIP1KD neurons expressed as percentage of control. (C) Cumulative histograms of mEPSC amplitude from neurons in control and TTX conditions (Upper) and control GRIP1KD, TTX GRIP1KD, and TTX GRIP1KD & Rescue (Lower). (D) Average mEPSC waveform (Upper) and average mEPSC amplitudes from indicated conditions (Lower). (Scale bar: 1 μm .) *Different from control: $P < 0.05$; ** $P < 0.001$.

ANOVA, $P = 0.58$ for capacitance and $P = 0.39$ for input resistance), together suggesting that cell size and overall dendritic branching is unperturbed by a two day manipulation of GRIP1 expression. Thus, it is unlikely that the failure to scale in GRIP1KD neurons is a secondary effect of changes in dendritic morphology.

Expression of Synaptic Scaling Requires Association Between GluA2 and GRIP1. Next, we wished to determine whether direct interactions between GRIP1 and GluA2 are required for the expression of synaptic scaling. To address this possibility, we used site-directed mutagenesis to create four GluA2 C-tail phosphorylation mutants that either disrupt binding between GluA2 and GRIP1 (Y876E, S880E) or leave GRIP1/GluA2 binding unaffected (Y876F, K882A). The S880E mutant is a phosphomimetic mutation known to reduce the interaction between GluA2 and GRIP1 (32, 41, 42). It has been shown that phosphorylation of tyrosine 876 after glutamate stimulation causes a reduced interaction between GluA2 and GRIP1 in cultured neurons (43), so we designed a second phosphomimetic mutation for the tyrosine 876 residue (Y876E) as a second means of reducing GRIP1/GluA2 binding. The Y876F mutation renders tyrosine 876 nonphosphorylatable and does not affect binding between GluA2 and GRIP1 (43). The K882A mutant blocks phosphorylation of serine 880 by protein kinase C (PKC) and also does not perturb binding between GluA2 and GRIP1 (41).

We have previously shown that synaptic scaling is blocked by GluA2 KD and rescued by coexpression of an RNAi-resistant WT GluA2 subunit (12). Here, we use this KD and replace strategy to determine which of the GluA2 phosphorylation mutants are able to rescue synaptic scaling. Neurons were cotransfected with the GluA2 shRNA (GluA2KD) and various GluA2 subunits resistant to the hairpin for 2 d and then treated with

TTX for 6 h to induce synaptic scaling. In control neurons, 6 h of TTX significantly enhanced mEPSC amplitude (Figs. 4C and 6B; control, $n = 46$; TTX, $n = 55$; $P < 0.0001$). On a GluA2 KD background, the two phosphomimetic GluA2 mutants with reduced GRIP1 binding, Y876E and S880E failed to rescue scaling (Fig. 6A and B; Y876E: control, $n = 8$; TTX, $n = 8$; $P = 0.7$; S880E: control, $n = 8$; TTX, $n = 8$; $P = 0.75$). In contrast, the two nonphosphorylatable mutants that do not disrupt GRIP binding, Y876F and K882A, were able to rescue scaling (Fig. 6A and B; Y876F: control, $n = 12$; TTX, $n = 9$; $P < 0.05$; K882A: control, $n = 6$; TTX, $n = 8$; $P < 0.05$). Together, these data suggest that the interaction between GluA2 and GRIP1 is required for the expression of synaptic scaling. Because there is some evidence that GRIP1 is important for basal trafficking of AMPA receptors (21, 32, 44), we also examined whether the GluA2 mutants on a GluA2 KD background affected the baseline amplitude of mEPSCs. None of these mutations on a GluA2KD background induced any significant change in mEPSC amplitude compared with nontransfected neurons, suggesting that basal trafficking is unperturbed (Fig. 6C; ANOVA $P = 0.98$).

The failure of Y876E and S880E to rescue synaptic scaling could be explained by a defect in the targeting of these subunits to synaptic membranes. To confirm that the mutant GluA2 subunits are able to traffic to synapses, we performed live cell immunolabeling against a GFP tag in the extracellular N-terminal domain under nonpermeable conditions, fixed, and then counterstained against the postsynaptic marker PSD-95. All of the mutant GluA2 subunits on a GluA2 KD background colocalized with PSD-95 to a similar extent as the wild-type RNAi rescue construct (GluA2) (Fig. 6D; $n = 6$ neurons per condition; ANOVA, $P = 0.46$). Furthermore, the intensity of the surface GFP signal at synaptic sites was not significantly different between these various mutants (Fig. S4), indicating that the failure

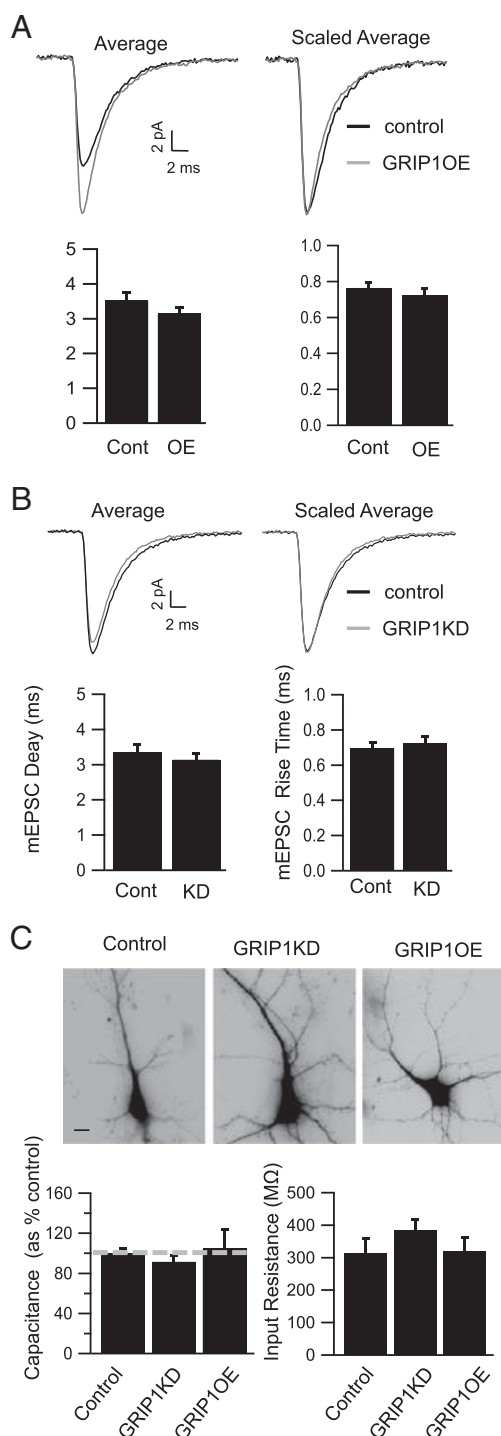


Fig. 5. Manipulations of GRIP1 do not affect mEPSC kinetics or passive neuronal properties. (A) Average mEPSC waveforms (Left) and peak-scaled waveforms (Right) (to allow comparison of kinetics) from control and GRIP1 OE neurons (Upper) and average mEPSC decay tau and rise times for the indicated conditions (Lower). (B) Average mEPSC waveforms (Left) and peak-scaled waveforms (Right) from control and GRIP1KD neurons (Upper) and average mEPSC decay tau and rise times for the indicated conditions (Lower). (C) Representative images of dendritic morphology for control, GRIP1KD, and GRIP1OE neurons (Upper) and whole-cell passive properties for indicated conditions (Lower). (Scale bar: 10 μ m.)

of Y876E and S880E to rescue scaling is not attributable to general (basal) trafficking defects but rather to blockade of a

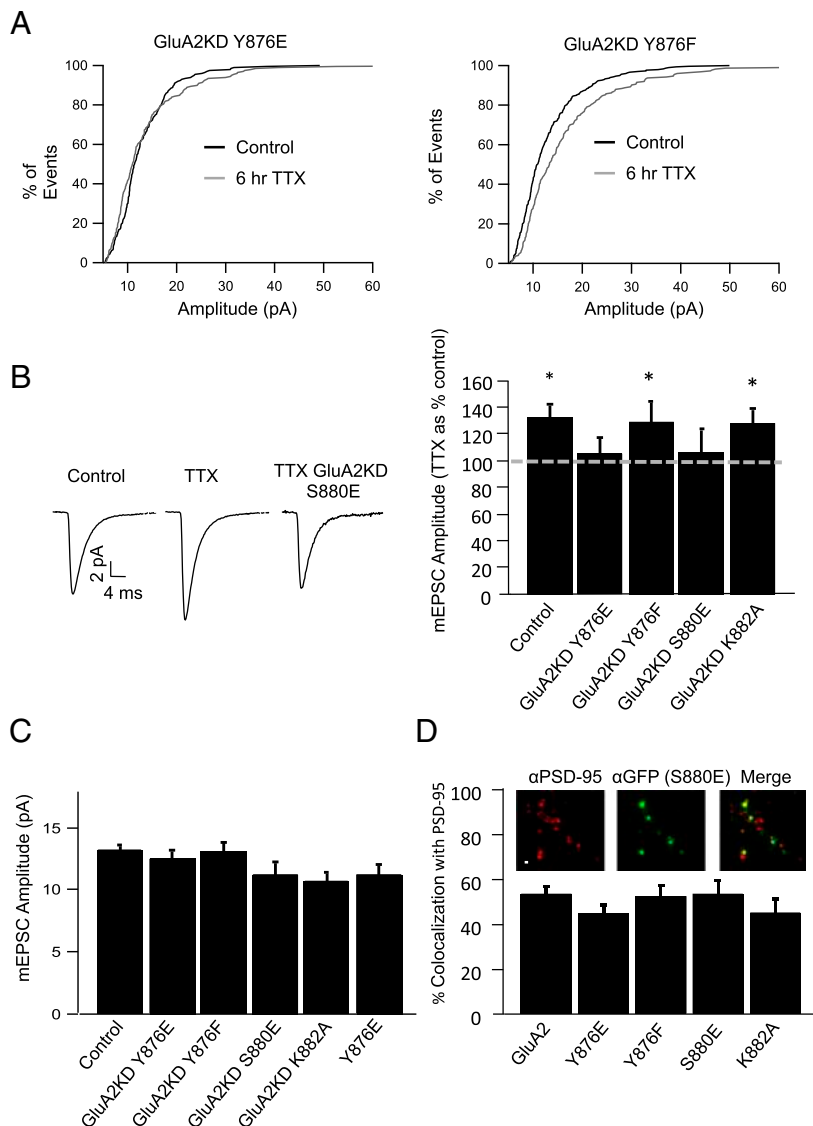
regulated interaction between GluA2 and GRIP1 that is necessary for scaling up.

Activity Blockade Leads to Synaptic GRIP1 Accumulation Independently of GluA2. Taken together, our data show that synaptic scaling up depends upon the interaction between GluA2 and GRIP1. This raises the question of whether enhanced GRIP1 accumulation at synapses drives the increase in synaptic GluA2, or vice versa. To address this question, we first asked whether we could block synaptic scaling by using the GluA2 Y876E as a dominant negative. Similar to the effects of Y876E expression on a GluA2 KD background (Fig. 6 A and B), synaptic scaling was completely blocked by expression of the Y876E construct alone (Fig. 7A; control, $n = 7$; TTX, $n = 6$; $P = 0.93$). Next, we prevented the expression of synaptic scaling by transfecting neurons with the GluA2 Y876E mutant and asked whether GRIP1 still accumulates at synapses when neurons are treated with TTX for 6 h. Neurons were immunolabeled against GRIP1 and PSD-95 and the intensity of the synaptic GRIP1 signal quantified. Consistent with our earlier observations (Figs. 1 and 2), 6 h of TTX caused a significant increase in the intensity of GRIP1 puncta colocalized with PSD-95 (Fig. 7B; control, $n = 25$ neurons; TTX, $n = 10$ neurons; $P < 0.05$). Interestingly, GRIP1 accumulation at synaptic sites was normal in Y876E-expressing neurons (Fig. 7B; control, $n = 14$; TTX, $n = 10$; $P < 0.05$), suggesting that GRIP1 is able to traffic to synapses in an activity-dependent manner independently of GluA2. These data support a model in which activity-dependent trafficking of GRIP1 to synapses drives the accumulation of GluA2 during synaptic scaling.

Discussion

Synaptic scaling is a form of homeostatic plasticity that adjusts the weights of all of a neuron's synapses in proportion to their initial strength (4). During TTX-induced synaptic scaling up, synapses are strengthened through increased synaptic accumulation of GluA1 and GluA2-containing AMPA receptors (11), in a manner that requires the GluA2, but not the GluA1, C-tail (12). Despite much recent interest in the mechanisms of synaptic scaling, the GluA2-dependent receptor trafficking steps that underlie scaling up remain largely undefined. Here, we examine the possibility that synaptic scaling requires GluA2 interactions with GRIP1, a multi-PDZ domain-containing trafficking/scafolding protein that binds the GluA2, but not the GluA1, C-tail (15). Although GRIP1 was one of the first AMPAR binding proteins to be identified, its precise role in AMPAR trafficking is still unclear, and whether it is important for homeostatic plasticity is completely unexplored. Here, we provide several lines of evidence that activity-dependent increases in synaptic GRIP1 are necessary for the induction of synaptic scaling up. We find that synaptic GRIP1 levels increase during activity blockade and that directly enhancing synaptic GRIP1 increases mEPSC amplitude. Furthermore, GRIP1 KD prevents synaptic scaling up, and both the inactivity-induced increase in mEPSC amplitude and in GluA2 trafficking require the GluA2-GRIP1 interaction. Together, our results support a model in which activity-dependent trafficking of GRIP1 to synaptic sites drives the forward trafficking and enhanced accumulation of GluA2-containing AMPAR during synaptic scaling.

GRIP1 is an abundant protein that has been strongly implicated in the transport and synaptic maintenance of GluA2-containing AMPAR, but contradictions in the literature have led to uncertainty about its precise role. GluA2 point mutants that fail to bind GRIP1 show decreased synaptic accumulation in some studies (17, 32) but not in another (19). GluA2 has reduced surface expression on a GRIP1 dominant-negative background (31) but, consistent with our data, shows normal surface expression on a GRIP1 knockout background (22). We found that acute KD of GRIP1, as well as replacement of GluA2 with point mutations that have decreased binding to GRIP1, do not significantly affect baseline mEPSC amplitude or frequency. Furthermore, GluA2 point mutants with reduced GRIP1 binding



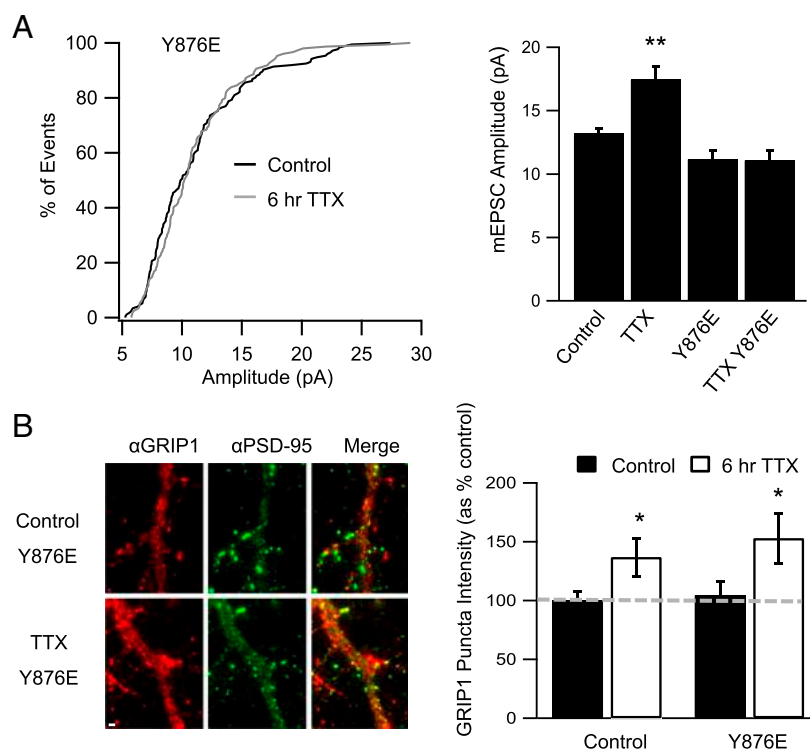


Fig. 7. Chronic inactivity induces synaptic GRIP1 accumulation independently of GluA2 binding. (A) Cumulative histogram of mEPSC amplitude in Y876E neurons in control and TTX conditions (Left) and average mEPSC amplitude for indicated conditions (Right). (B, Left) Example of Y876E-expressing neurons in control (Upper) and TTX (Lower) conditions, labeled against GRIP1 (red) and PSD-95 (green). (Scale bar: 1 μ m.) (B, Right) Average intensity of GRIP1 puncta colocalized with PSD-95, for control or Y876E-expressing neurons; TTX expressed as percentage of control for same condition. *Different from control: $P < 0.05$; ** $P < 0.001$.

Our data currently cannot distinguish between a role for GRIP1 in the forward trafficking of GluA2 to synaptic membranes from intracellular compartments or as an anchoring protein for GluA2 at synaptic sites during synaptic scaling. There is evidence that GRIP1 is required for transporting internalized AMPARs to the cell surface in an activity-dependent manner, through association with NEEP21 (30) and Sec8 (22). Sec8 is a member of the exocyst complex important for vesicle fusion and is localized to a number of endosomal compartments as well as to sites of exocytosis on the plasma membrane (46). We find that TTX treatment increases GRIP1 accumulation at Sec8-positive internal sites as well as at the postsynaptic density. This raises the interesting possibility that GRIP1 might be important for the forward trafficking of AMPAR to exocytotic sites during scaling up. The increased accumulation of GRIP1 at synaptic sites suggests that GRIP1 remains associated with AMPAR after they reach the synaptic membrane, where GRIP1 could play an additional role in the stabilization of the receptors. Although mutations that prevented GluA2 from interacting with GRIP1 prevented the enhanced trafficking of GluA2 to synapses, TTX still drove GRIP1 to synaptic sites under these conditions. These data strongly suggest that, during synaptic scaling, movement of GRIP1 to synapses drives the enhanced accumulation of GluA2 rather than the other way around.

This study demonstrates that GRIP1 localization can be regulated by activity, but how this regulation is accomplished is not clear. GRIP1 can be multiply phosphorylated by PKC and other kinases (47), and AMPA or NMDA stimulation enhances the phosphorylation of GRIP1, which affects the binding of GRIP1 to NEEP21 and thus modifies the endosomal sorting of GluA2 (31), but the endogenous kinase(s) responsible for GRIP1 phosphorylation, and the signals that normally activate them, are unknown. Interestingly, we found that total dendritic GRIP1 protein is not increased by TTX treatment, suggesting that the enhanced synaptic accumulation is attributable to a redistribution of GRIP1 rather than an increase in total protein abundance. This redistribution was transcription-dependent, suggesting that it is driven by the transcriptional up-regulation of unidentified factors during activity blockade.

In addition to playing a critical role in synaptic scaling, there is much accumulating evidence that GRIP1 is important for the expression of a Hebbian form of synaptic plasticity, LTD. The GluA2 S880E mutant has previously been shown to occlude LTD in the cerebellum by triggering PICK1-dependent AMPA receptor endocytosis (41, 48), and cerebellar LTD is blocked in a GRIP1/2 knockout (34). Given that GRIP1 and GRIP2 are able to compensate for one another in cerebellar LTD (34), it is perhaps surprising that we are able to block scaling up by KD of GRIP1 alone. These data show that GRIP1 and GRIP2 do not play entirely redundant roles in synaptic plasticity, with GRIP2 able to sustain LTD, but not synaptic scaling up, in the absence of GRIP1.

Although a number of proteins and signaling pathways have been identified that play a role in synaptic scaling, the crucial factors that lead to enhanced AMPAR accumulation during scaling up have remained obscure. Furthermore, our understanding of the function of synaptic scaling has been limited by our inability to selectively interfere with synaptic scaling while leaving baseline transmission and other forms of plasticity intact (49). Here, we show that regulated synaptic GRIP1 accumulation is necessary to induce scaling up, and we provide compelling evidence that the interaction between GluA2 and GRIP1 is critical for this process. Our data support a model of synaptic scaling in which chronic activity deprivation promotes the interaction between GluA2 and GRIP1, the trafficking of GRIP1 to synapses, and thus the GRIP1-dependent synaptic trafficking and/or stabilization of GluA2-containing AMPARs.

Experimental Procedures

Neuronal Cultures, Transfections, and Drug Treatments. Dissociated cultures were prepared from the visual cortex of postnatal day 1 (P1)–P3 Long–Evans rats and plated onto glass-bottomed dishes as described previously (50). All experiments were performed after 7–10 DIV. Neurons were transfected at low efficiency with Lipofectamine 2000 2 d before recording or immunostaining, unless otherwise noted. To visualize transfected neurons, constructs were cotransfected with cyanofluorescent protein (CFP). To induce synaptic scaling, neurons were treated with 5 μ M TTX for 6 h. For ActD treatment, cultures were treated with 50 μ M ActD (Sigma–Aldrich; A 1410) \pm TTX for 6 h; ActD was dissolved in DMSO, with the final concentration of DMSO at

1:1,000; control cultures were treated with DMSO only. For all experiments, matched controls from sister cultures were obtained for each experimental condition. There were no significant differences in control mEPSC amplitude between different experiments (ANOVA, $P = 0.56$), so control values were pooled in Figs. 4 and 6. Unless stated otherwise, data are presented as the means \pm SEM for the number of neurons indicated; where data are presented as percentage of control values, the propagated SEM was used.

Construct Design. The GluA2 shRNA and RNAi GluA2 were designed as described in ref. 12. Site-directed mutagenesis was used to create the RNAi GluA2 C-tail mutants. The presence of mutations was verified with sequencing. The GRIP1 shRNA and GRIP1 rescue construct were gifts from Casper Hoogenraad, Utrecht University, Utrecht, The Netherlands.

Immunocytochemistry. Permeabilized immunostaining was performed as described (51) using antibodies against the following: PSD-95 (1:200; MA1-046, Thermo Scientific); synapsin 1 and 2 (1:1,000, 106 004, Synaptic Systems); GRIP1 [1:800, ab25963, Abcam; or 1:400, Mouse (Ms) Anti-GRIP1, 611319, BD Transduction Laboratories]; and Sec8 (1:450, 610659; BD Transduction Laboratories). To visualize surface GluA2 (1:50, MAB397, Chemicon; ms monoclonal antibody, gift of Eric Gouaux, Vollum Institute, Oregon Health & Science University, Portland, Oregon) and GFP (1:100, AB3080P; Millipore), live neurons were incubated with antibodies under nonpermeable conditions as described in ref. 11. Alexa Fluor 488, Texas red, and Alexa Fluor 657 (1:200; Invitrogen) were used as secondary antibodies. Digital images were acquired with Openlab (Improvision) or Volocity (PerkinElmer) software on an Olympus IX-81 microscope with an oil immersion 60 \times objective using an Orca ER camera. A 6% neutral density filter was used during image acquisition to prevent photobleaching. Digital images were quantified with Metamorph software (Molecular Devices). Localization of GRIP1 to recycling endosome and Sec8-labeled compartments was performed with LSM5 Leica confocal microscope under sequential scanning settings for multichannel fluorescence image acquisition. To ensure uniformity, data were acquired from the apical-like dendrite and dendritic branches off of it. Puncta were selected by thresholding above local background with the Granularity function in Metamorph. Experimental conditions were run in parallel with the control condition on sister cultures from the same dissociations; the mean total puncta intensity for experimental conditions was normalized to the mean total puncta intensity of control in sister cultures. Similar results were obtained when average puncta intensity was used.

Because the Sec8 puncta density was high, we wished to verify that the colocalization we observed between Sec8 and GRIP1 puncta was greater than expected for random colocalization of the two signals. To estimate the expected random colocalization rate, we generated artificial Sec8 images in ImageJ using the ThunderSTORM plugin. An Integrated gaussian option was used to generate artificial puncta with a similar size and intensity distribution to the Sec8 images (full width at half maximum range from 200 to 350 nm, intensity range from 700 to 900 photons). Average Sec8 puncta density values obtained from Sec8 experimental images were then used to create an artificial Sec8 image, by randomly distributing these artificial puncta at the experimental density measured for each image pair, and the colocalization rate was determined using the same criteria as for the experimental images. This "random" rate was then compared with the experimentally measured rate for each image pair.

Transferrin Labeling. To label recycling endosomes, cells were incubated with 50 ng/ μ L Alexa Fluor 546-labeled transferrin in neuronal culture media for 30 min at 37 $^{\circ}$ C in the incubator. After incubation, cells were removed from the incubator and washed three times in phosphate buffered saline (PB) followed by fixation with 3.7% (wt/vol) paraformaldehyde in PB. Cells were then permeabilized and stained using various antibodies as described (51).

Immunoelectron Microscopy. Neurons were plated on gridded coverslips under normal culture conditions (catalog no. P35G-2-14-GCRD; Matek); 7- to 9-DIV pyramidal neurons were identified based on morphology and their location on the dish was noted. Cells were fixed in 4% paraformaldehyde, labeled with guinea pig anti-VGLUT1 antibody (1:600, catalog no. 135 304; Synaptic Systems) followed by biotinylated anti-guinea pig secondary antibody (1:400, catalog no. BA-7000; Vector Labs) and then immersed in a 1% DAB solution in the presence of H_2O_2 solution for 1–2 min. Next neurons were dehydrated through a series of ethanol and acetone mixtures. After dehydration, a BEEM capsule was placed over the region of interest, filled with EPON resin (catalog no. 14120; EMS), and placed in a 60 $^{\circ}$ C oven to polymerize. After polymerization, blocks containing pyramidal neurons were quickly immersed in liquid N_2 to remove the EPON block from the culture dish. Blocks were then trimmed, imaged on a dissection scope to correlate with imaging on the electron microscope, and sectioned on an ultramicrotome to 50- to 70-nm thickness. Ultrathin sections containing identified pyramidal neurons were placed on nickel formvar-coated slot grids and sections were postembedding labeled with a rabbit anti-GRIP1 antibody (1:200; ab25963, Abcam), followed by anti-rabbit 5-nm gold-conjugated secondary antibody (1:25; catalog no. 15725; Ted Pella). Identified pyramidal neurons were relocated under electron microscopy and examined for the presence of VGLUT1 DAB-labeled presynaptic boutons forming synapses onto pyramidal spines, dendrites, or somata. DAB-labeled synapses were deemed "VGLUT1-positive" if they contained at least 3 DAB-labeled presynaptic vesicles, had parallel alignment of pre- and postsynaptic membranes, and contained a postsynaptic density. VGLUT1-positive synapses were imaged at 16,000 \times to 20,000 \times magnification, corresponding to a pixel size of 1.4–0.9 nm, respectively. Captured images of positive synapses were analyzed for the distribution and density of GRIP1-gold particles. The centroid and edges of the postsynaptic density were used to define the midpoint and lateral edges of synapses, and the centroid of the synaptic cleft was defined as the midpoint for axospinous/dendritic distribution measurements. GRIP1-gold particle density was calculated as: number of gold particles within 40-nm radius of the synaptic cleft centroid/synapse length (as defined by the postsynaptic density). Statistical significance was determined with Mann–Whitney U test with significance set at $P < 0.05$.

Electrophysiology. Whole-cell AMPA-mediated mEPSC recordings were obtained and analyzed as described previously (4, 11) from visually identified pyramidal neurons at 25 $^{\circ}$ C from a holding potential of -70 mV in the following artificial CSF (ACSF): 126 mM NaCl, 5.5 mM KCl, 2 mM $MgSO_4$, 1 mM NaH_2PO_4 , 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 14 mM dextrose, 0.1 μ M TTX, 0.025 mM picrotoxin, and 0.025 mM D-amino-5-phosphovaleric acid. The internal recording solution contained the following (in mM): 120 KMeSO₄, 10 KCl, 2 $MgSO_4$, 10 K-Hepes, 0.5 EGTA, 3 K_2ATP , 0.3 NaGTP, and 10 Na_2 phosphocreatine. Recordings with resting membrane potential (V_m) greater than -55 mV, series resistance (R_s) >20 M Ω , input resistance (R_{in}) <150 M Ω , a change in R_{in} or V_m $>30\%$, or <25 mEPSCs were excluded. Cumulative amplitude histograms of mEPSC amplitude were created using the first 25 events from each neuron, as described (4).

Statistical Analysis. Data are reported as means \pm SEM for the number of neurons indicated. Unless otherwise indicated, statistical tests were two-tailed Student t tests or, for multiple comparisons, single-factor ANOVAs followed by two-tailed Student t tests or post hoc Tukey tests.

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Supporting Information

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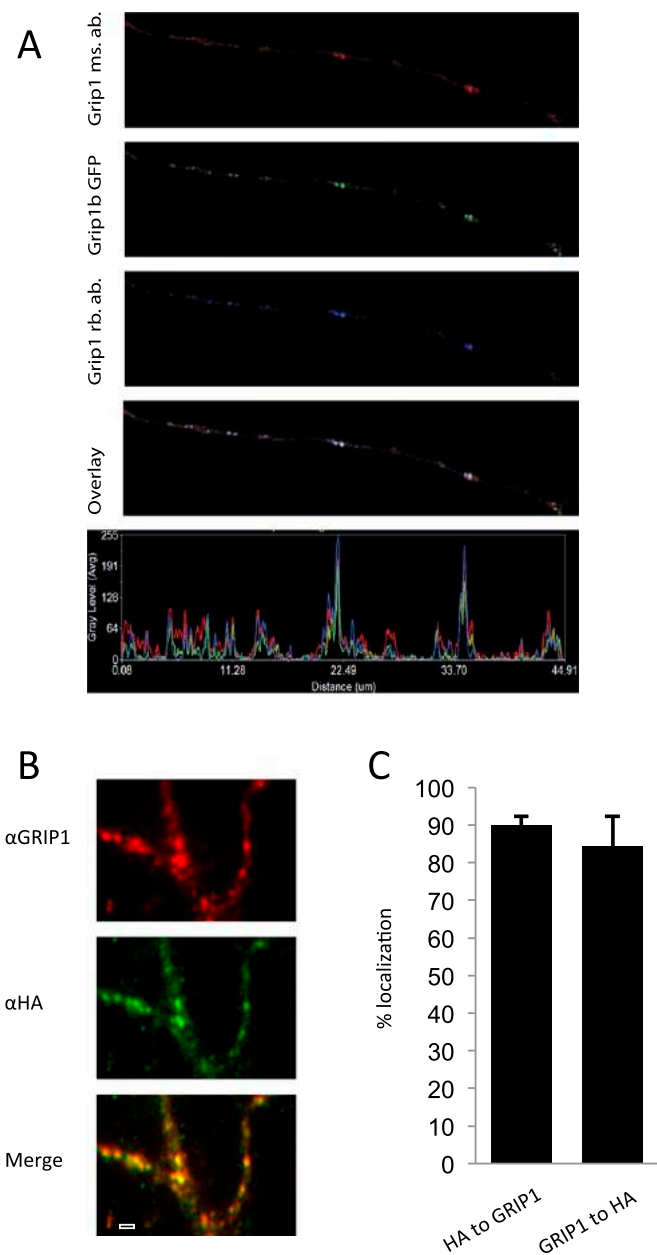


Fig. S1. Characterization of GRIP1 antibodies. (A) Neurons transfected with GRIP1b GFP (green) were stained with αGRIP1 Ms 611319 (BD Transduction Laboratories) (red), and αGRIP1 rb ab25963 (Abcam) (blue). The bottom graph demonstrates intensity values of all three signals from a line scan that was drawn along the dendrite. (B and C) Neurons transfected with HA-tagged GRIP1b (green) were stained with αGRIP1 antibody rb ab25963 (red) (B), and the colocalization rates of the two signals were determined (C).

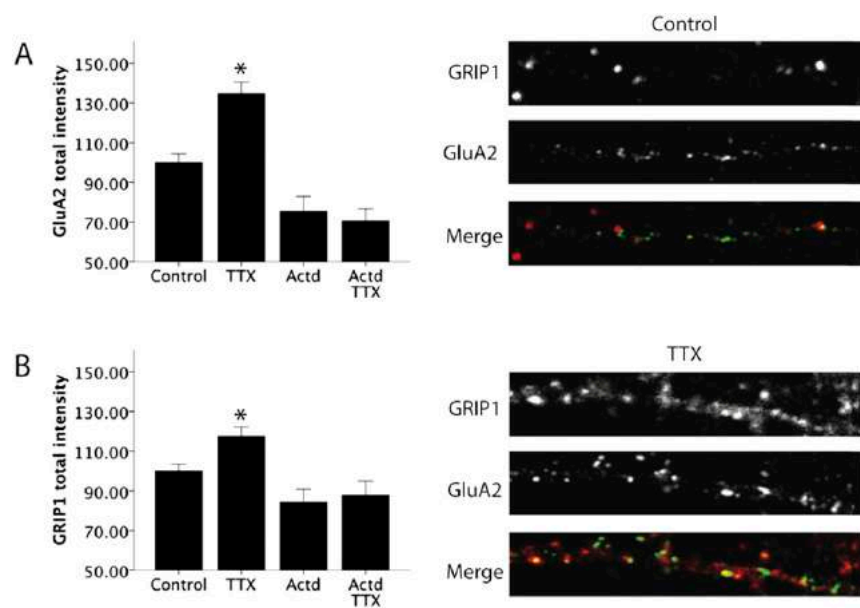


Fig. S2. The TTX-induced increase in GluA2 and GRIP1 signal at colocalized puncta is transcription-dependent. Neurons were treated with TTX for 6 h with or without ActD and then were fixed, permeabilized, and stained against GluA2 and GRIP1. At right are representative images of GRIP1/GluA2 staining for control (*Upper*) and TTX-treated (*Lower*) dendrites. (A) The intensity of the signal at colocalized puncta was then quantified for each condition and expressed as percentage of untreated control neurons; for GluA2 (A) and for GRIP1 (B). Control, $n = 83$ neurons; TTX, $n = 91$; neurons Act D, $n = 43$; Act D TTX, $n = 41$. (Scale bar: 1 μm .)

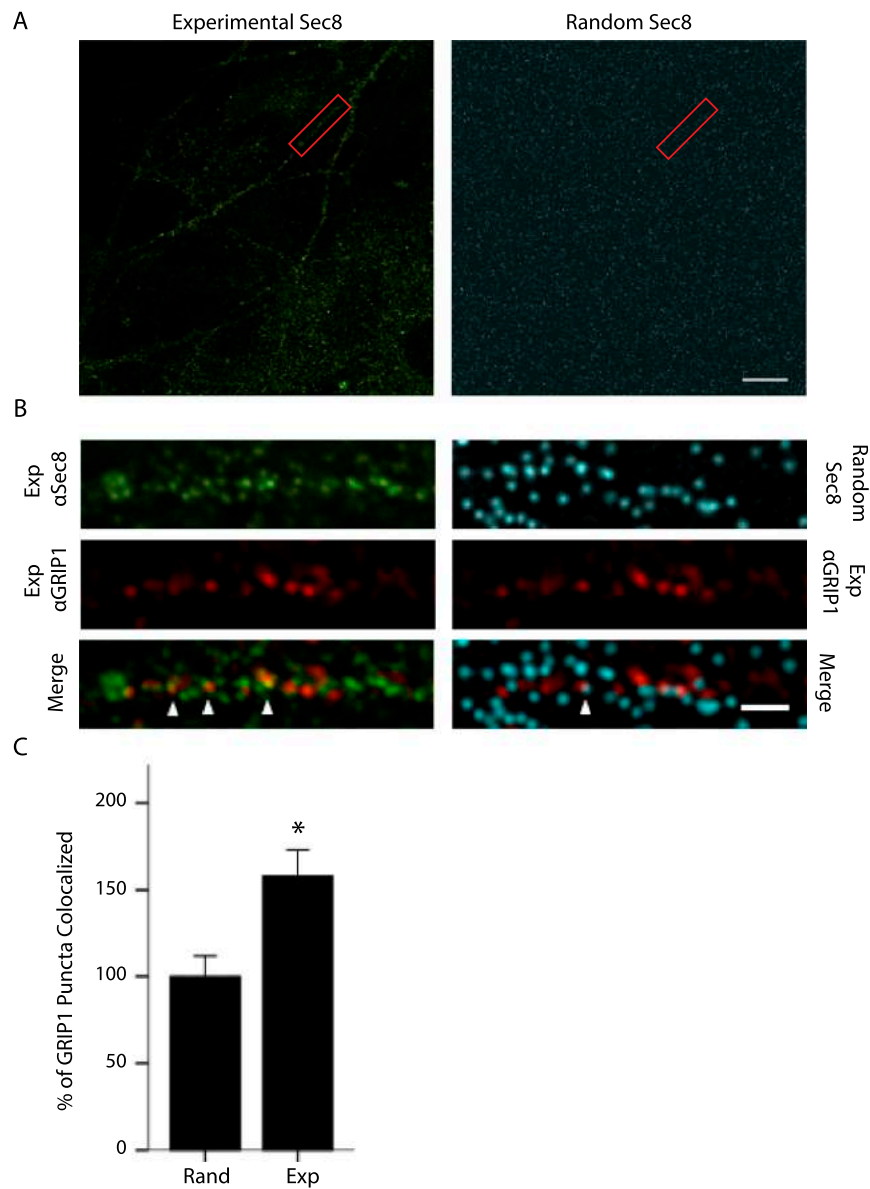


Fig. S3. Quantification of expected random colocalization rates. (A) Example of an experimental Sec8 image (Left) and an artificially generated Sec8 image (Right), in which puncta with the same size and intensity distribution as in the real data are randomly distributed throughout the field of view. Red box designates inset for B. (Scale bar: 10 μm .) (B, Left) Representative images for Sec8 (green) and GRIP1 (red) staining, with the merge shown below. (B, Right) Artificially generated Sec8 image (blue) and real GRIP1 image (red), with merge shown below. Arrows mark sites of colocalization. (Scale bar: 2 μm .) (C) Percentage of GRIP1 puncta that were colocalized with Sec8 in the random and experimental conditions ($n = 71$ images). *Experiment different from random, $P < 0.05$.

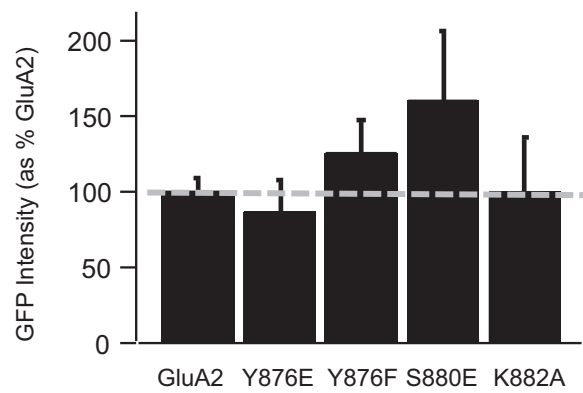


Fig. S4. Quantification of the synaptic surface expression of various GluA2 mutants. Receptors were N-terminal-tagged with GFP, and surface GFP fluorescence was detected using a GFP antibody under nonpermeant conditions. The intensity of the GFP signal at puncta colocalized with PSD-95 was determined. There were no significant differences across mutants (ANOVA) ($n = 6$ neurons per condition).