The role of GRIP1 in synaptic scaling

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ABSTRACT

Neural circuits develop based on homeostatic mechanisms that stabilize the excitability of neurons about a set point. “Scaling up” occurs when a cell increases synaptic strength to compensate for decreased network activity. Underlying these adjustments of excitability are changes in synaptic strength, which is mediated by AMPA receptors. In particular, glutamate receptor-interacting protein 1 (GRIP1) is a protein that binds GluR2 subunits and has been shown to be necessary for synaptic scaling. Here we show that during scaling, GRIP1 accumulates at exocyst sites. Furthermore, we demonstrate a method of assessing the reliability of detecting sites of colocalization. Additionally, we determined that during synaptic scaling, the rates of recycling exocytosis are unaffected. We further establish that GRIP1 is required for recycling processes, but not sufficient to drive it.
INTRODUCTION

1. Synaptic Scaling

Neural circuits develop based on homeostatic mechanisms that stabilize the excitability of neurons about a set point (Turrigiano and Nelson, 2004). Synaptic scaling, a particular form of homeostatic plasticity, was first characterized by considering the effects of chronic perturbations of network activity on miniature excitatory postsynaptic currents (mEPSCs) in culture (Turrigiano, Leslie, Desai, Rutherford, & Nelson, 1998). “Scaling up” occurs when a cell increases synaptic strength to compensate for decreased network activity. Accordingly, “scaling down” occurs in an opposite way where synaptic strength decreases in response to increased network activity (Turrigiano et al., 1998). Interestingly, this phenomenon differs from the classical form of Hebbian plasticity since it occurs at single synapses that strengthen or weaken from activity (Turrigiano & Nelson, 2004). Changes due to synaptic scaling occur globally rather than locally in order to stabilize the overall firing rates of the cell (O'Brien et al., 1998). In fact, synaptic scaling cannot be locally induced in the dendrite, which further supports that these are global changes (Ibata, Sun, & Turrigiano, 2008). These changes in synaptic strength depend on the surrounding network activity and allow the cell to maintain a particular level of excitability.

Network activity can be manipulated by the addition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antagonists, tetrodotoxin (TTX) or CNQX, to inhibit network activity or a gamma-aminobutyric acid (GABA$_A$) receptor antagonist, bicuculline, to excite network activity (Turrigiano et al., 1998; Turrigiano & Nelson, 2004). Since mEPSC amplitude changes were first determined by manipulating
AMPA-mediated currents, it was proposed that synaptic scaling arises from changes in receptor number or function (Turrigiano et al., 1998). In agreement with this idea, another study found that changes in network activity can regulate the expression of synaptic surface receptors (Lissin et al., 1998). Additionally, two lines of thinking arose about the combined influence of pre- and postsynaptic changes during synaptic scaling. Pre- and postsynaptic changes could either be coupled or work independently of one another. It was later determined that increased AMPAR accumulation occurred independently of presynaptic changes and primarily in postsynaptic locations (Wierenga, Ibata, & Turrigiano, 2005). Much research has since focused on the accumulation of postsynaptic AMPARs and how it drives synaptic scaling.

The initial experiments were performed with a 48-hour activity blockade (Turrigiano et al., 1998). Further characterization of this blockade was conducted to establish an effective time course for the induction of synaptic scaling (Ibata et al., 2008). Within one hour, a significant increase of synaptic AMPAR accumulation was detectable and five to six hours of incubation was sufficient to increase AMPAR puncta intensity (Ibata et al., 2008). Our current experiments are conducted within these parameters of a 6-hour TTX incubation.

II. AMPA receptors and Recycling

AMPA receptors are a subtype of ionotropic receptors that the neurotransmitter, glutamate, acts upon. AMPARs vary in either a homomer or heteromer composition of four types of subunits: GluR1, GluR2, GluR3, and GluR4 (Anggono & Huganir, 2012). Early experiments focused primarily on the GluR1 subunit since previous work showed
that it is essential for expression of long-term potentiation (LTP), a Hebbian form of plasticity (Zamanillo et al., 1999). Increases of GluR1 due to network activity blockade were localized to postsynaptic surface locations. Interestingly, this accumulation occurred without any effect on the number of GluR1 synapses or total number of synapses (O'Brien et al., 1998). Along with an increase of GluR1, a later study also noted that GluR2 accumulation increases proportionally to GluR1 after activity blockade (Wierenga et al., 2005). Notably, AMPARs containing GluR2 subunits are Ca\(^{2+}\)-impermeable whereas subunits that lack GluR2 are Ca\(^{2+}\)-permeable, which gives them a unique function in conducting network activity (Anggono & Huganir, 2012). Dominant-negative experimental approaches showed that blocking GluR1’s binding region (the C-tail) had no effect on synaptic scaling, but blocking the GluR2 C-tail did. Furthermore, replacing endogenous GluR2 with a GluR1 C-tail blocked scaling. These results indicated that GluR2, not GluR1, is required for synaptic scaling (Gainey, Hurvitz-Wolff, Lambo, and Turrigiano, 2009).

The AMPAR synaptic accumulation observed is likely dependent upon underlying trafficking of these receptors through the neuron to the cell membrane. Synaptic AMPARs have been established to recycle through mechanisms of exocytosis and endocytosis. AMPAR endocytosis has previously shown to regulate long-term depression (LTD), another form of Hebbian plasticity. This provides an attractive mechanism by which postsynaptic AMPAR distribution changes (Man et al., 2000). Disruption of recycling mechanisms, such as exo- or endocytosis, has shown to affect excitatory postsynaptic currents and the levels of surface AMPAR expression (Luscher et al., 1999). Recycling endosomes have, in particular, been identified to have a major role
in AMPAR transportation to the cell membrane during LTP (Park, Penick, Edwards, Kauer, & Ehlers, 2004). Indeed, endocytosis of AMPARs was confirmed to be a requirement for the induction of synaptic scaling (Grunwald, Mellem, Strutz, Maricq, & Kaplan, 2004).

These mechanisms of AMPAR trafficking have been highlighted as a particular area of interest in order to untangle the underlying players involved in synaptic scaling. Regarding the AMPAR trafficking involved in LTP and LTD, a three-step model has been proposed. First, intracellular AMPAR-containing vesicles are transported to the membrane surface followed by lateral diffusion to synaptic sites. The final step is driven by scaffolding interactions that anchor the AMPARs to the synapses (Opazo & Choquet, 2011). Building upon this model, various ideas about the methods of AMPAR trafficking have been highly researched as well as debated. Currently, there is much known about these trafficking mechanisms, but still much more to investigate. Studies regarding LTP and LTD have shown the key stages in regulating AMPAR trafficking lie in the rates of exo- and endocytosis, retention of AMPARs in intracellular compartments, and delivery of recycling endosomes back to the cell membrane (Anggono & Huganir, 2012). Therefore, these areas of trafficking are attractive to investigate regarding the mechanisms of AMPAR accumulation that drive synaptic scaling.

Four proteins have been identified to interact with the GluR2 subunit and influence AMPAR trafficking: N-ethylmaleimide-sensitive fusion protein (NSF), AMPA receptor-binding protein (ABP), protein interacting with protein kinase C (PICK1), and glutamate receptor interacting protein (GRIP). The protein NSF is known to be involved in membrane fusion events. The blocking of GluR2/NSF interaction has shown to result
in an increase of AMPAR endocytosis (Braithwaite, Xia, & Malenka, 2002). The proteins ABP, PICK1, and GRIP bind the same PDZ-domain of the C-terminus of GluR2/3, which suggests competitive binding and regulation (Dong, Zhang, Liao, & Huganir, 1999). PICK1 has been shown to retain intracellular GluR2, implicating it in the recycling of AMPARs to the cell membrane. Knock out of PICK1 results in accelerated GluR2 recycling and dissociation of binding with GRIP1 (Lin & Huganir, 2007). ABP is a short splice variant of GRIP that contains the only PDZ1-6 domains, but is also capable of binding GluR2 (Srivastava et al., 1998). Finally, GRIP1 knockdown has been shown to inhibit the accumulation of surface AMPARs when synaptic scaling is induced, which makes it a particular protein of interest in regards to synaptic scaling (unpublished data).

III. Glutamate receptor interacting protein (GRIP)

GRIP was first identified as a 135kDa protein with seven PDZ domains. Specifically, PDZ4 and PDZ5 interact with the C-terminus of GluR2/3 AMPAR subunits (Dong et al., 1997). Since then, two variants of GRIP have been identified: GRIP1 (135kDa) and GRIP2 (130kDa). These variants mostly differ at the linker regions between PDZ domains while the domains themselves remained highly homologous (Dong et al., 1999). Further investigation into GRIP1 and GRIP2 expression revealed drastic developmental differences, suggesting that GRIP is able to perform a variety of functions. GRIP1 was detectable at embryonic development and peaked at postnatal days 6-8. Interestingly, GRIP2 is only detectable at postnatal stages and peaks at day 14, which parallels GluR2 expression (Dong et al., 1999).
The distribution of GRIP1 was found to be in both excitatory and inhibitory synapses, specifically GABA<sub>A</sub> receptors (Dong et al., 1999). In fact, the percentage of GABA<sub>A</sub> receptors colocalized with GRIP1 was similar to AMPA receptors, specifically the GluR2/3 subunits. These interactions between GRIP1 and GABA<sub>A</sub> receptors were localized both pre- and postsynaptically. Interestingly, GRIP1 had a higher density in presynaptic terminals of GABAergic synapses whereas it had a higher density in the postsynaptic terminals of glutamatergic synapses (Li et al., 2005). Finding GRIP1 in both excitatory and inhibitory synapses further supports the emerging idea that GRIP is a multi-functional protein found throughout the cell (Dong et al., 1999). Further splice variants of GRIP1, GRIP1a and GRIP1b, were later identified to have differences of an 18-19 amino acid region on the N-terminus. Distribution of GRIP1b was primarily membrane-associated whereas GRIP1a has the ability to change subcellular locations, which further suggests the functional diversity of GRIP (Yamazaki et al., 2001).

Significantly, GRIP1a contains two cysteine residues at positions 8 and 10 while GRIP1b contains one cysteine residue at position 11. These cysteine residues are locations of palmitoylation (Yamazaki et al., 2001), which is a reversible lipid modification that often occurs within neuronal cells. A well-studied protein that undergoes palmitoylation is PSD-95, which is also found in the post-synaptic density (el-Husseini Ael & Bredt, 2002). GRIP1b has recently been identified to undergo palmitoylation, which was suggested to occur at a higher percentage than PSD-95 (Thomas, Hayashi, Chiu, Chen, & Huganir, 2012). Through the manipulation of the ability to palmitoylate, it was determined that nonpalmityoable GRIP1b was confined to the cell soma and proximal dendrites with a diffuse pattern while palmitoyable GRIP1b
extended into the distal dendrites with a punctated pattern (Thomas et al., 2012; Yamazaki et al., 2001). Additionally, palmitoylatable GRIP1b was identified to colocalize with recycling endosomes and not early endosomes. This further supports the idea that GRIP, and by extension GluR2, is trafficked to the cell membrane through recycling processes (Thomas et al., 2012).

Much research has focused on the interaction between GRIP1 and GluR2 regarding its impacts on the intrinsic mechanisms of the cell. One approach of blocking the interaction between the GluR2 C-terminus with PDZ-dependent proteins was accomplished by a dominant negative approach using a short peptide containing the C-terminus of GluR2 (Watt, van Rossum, MacLeod, Nelson, & Turrigiano, 2000). Several sequences of peptides were identified to have varying impacts. For example, a peptide sequence of NVYGIESVKI (GluR2-SVKI) blocks the binding of GRIP1, ABP, and PICK1. However, the peptide sequence of NVYGIEEVKI (GluR2-EVKI) blocked the binding of only PICK1, but allows GRIP1 and ABP to interact (Daw et al., 2000). Unfortunately, since a peptide to directly block only GRIP1 and GluR2 interaction has yet to be discovered, this dominant-negative approach is not an adequate system to study the specifics of GRIP1 function.

Knockout (Zamanillo et al., 1999) mice models have also been attempted and have revealed a significant difference between GRIP1 and GRIP2. GRIP1 KO mice are embryonically lethal whereas GRIP2 are viable (Takamiya, Mao, Huganir, & Linden, 2008). To get around this obstacle, a Cre-loxP system has been utilized in hippocampal and cortical neuron cultures to conditionally knockout GRIP1 expression (Takamiya et al., 2008). Additionally, a knockdown (KD) of GRIP1 using a small interfering RNA
(siRNA) has been found to work effectively in reducing GRIP1 expression (Hoogenraad, Milstein, Ethell, Henkemeyer, & Sheng, 2005). This GRIP1 KD has also been shown to effectively block synaptic scaling from occurring, which makes it a sufficient model to study the reduction of GRIP1 (unpublished).

A third system of disrupting GRIP1/GluR2 protein interactions utilizes two sites of mutations on the C-terminus of GluR2. The first identified was the serine residue at position 880, which is a site of phosphorylation (Dong et al., 1997) performed by protein kinase C (PKC) (Chung, Xia, Scannevin, Zhang, & Huganir, 2000). A portion of endogenous GluR2 of was phosphorylated at Ser880 in vivo. In fact, the phosphorylation of Ser880 blocks GRIP1-GluR2 interaction, but binding of PICK1 is unaffected (Chung et al., 2000). Another site of phosphorylation mediated by protein tyrosine kinase (PTK) was utilized to block GRIP1 binding: the tyrosine residue at position 876. Similar to the Ser880 mutation, phosphorylation of the tyrosine blocks the binding of GRIP to the GluR2 tail while not affecting the binding of PICK1. The mutation of Tyr876, specifically to phenylalanine (Y876F) leaves GluR2 non-phosphorylatable, but does not block the binding of GRIP1. This Y876F mutation was found to reduce the amount of surface expression and synaptic targeting of GluR2, implicating the phosphorylation of GluR2 as important in the regulation of AMPAR trafficking (Hayashi & Huganir, 2004). In our experiments, we have chosen to utilize the mutation of Tyr876 to a glutamate residue (Y876E), which has been found to block GRIP and GluR2 binding.

GRIP1 has also been characterized to colocalize and interact with a variety of other proteins. One of the first identified was liprin-α1, a scaffolding protein, that has been implicated to function in cell-matrix interactions. Staining of liprin-α1 revealed a
similar distribution to GRIP1 in both glutamatergic and GABAergic synapses both pre- and postsynaptically. Two experimental approaches, a dominant-negative approach and the transfection of a liprin-α1 construct unable to bind to GRIP, showed that the disruption of GRIP1/liprin-α1 interaction resulted in a reduction of endogenous GluR2 surface expression. However, the disruption of the GRIP1/liprin-α1 interaction did not affect the GRIP1/GluR2 interaction. These researchers proposed the model that liprin-α1 could be a “docking” site for GRIP1 at the synapse that keeps the whole complex tethered (Wyszynski et al., 2002).

Neuron-enriched endosomal protein 21kDa (NEEP21) has also been established as a binding partner of GRIP1 (Steiner et al., 2005). NEEP21 is an endosomal protein that has been shown to be critical for vesicle recycling and sorting (Steiner et al., 2002). Disruption of the GRIP1 and NEEP21 interaction leads to a decrease of GluR2 surface expression. Instead, AMPARs accumulate in early endosomes and lysosomes (Steiner et al., 2005), further suggests that GRIP1 plays a significant role in recycling.

Kinesin, a motor protein that transports axonal and dendritic cargo, was found to bind GRIP1 via its heavy chains (KIF5). Eliminating KIF5 led to the mis-location of GRIP1 from the periphery of the cells to more central locations. In fact, kinesin was determined to be a binding partner of both GRIP1 and GluR2 to create a complex. GluR2 was established to be a passenger of kinesin-transport through dominant negative experiments using KIF5 constructs that disrupt its binding to GRIP1 (Setou et al., 2002). Further investigation revealed that N-cadherin, a transmembrane protein that is important for cell adhesion, also plays a role in the function of the GRIP1/KIF5/GluR2 complex. N-cadherin interacts with the PDZ2 domain of GRIP1 and has reduced surface expression
when KIF5 function is blocked. Various experiments investigated these specific protein interactions and concluded that GRIP played a significant role in coordinating N-cadherin and GluR2. These results support the idea that GRIP1 functions as an adaptor protein (Heisler et al., 2014).

A recent study has also found that GRIP1 interacts with the exocyst complex, which has been implicated in targeting vesicles to the cell membrane. A specific exocyst complex protein, Sec8, was shown to interact with GRIP1 through PDZ-domain interactions. Disruption of Sec8 expression revealed a disruption of AMPAR trafficking and recycling (Mao, Takamiya, Thomas, Lin, & Huganir, 2010), which makes it a potential mechanistic player of AMPAR accumulation induced by synaptic scaling.

Here we chose to investigate the role of GRIP1 in trafficking processes. Rates of endo- and exocytosis as well as delivery of recycling endosomes have been shown to be key stages of regulating AMPAR trafficking during LTP and LTD (Anggono & Huganir, 2012). Naturally, it is worth investigating whether these recycling processes are also implicated in synaptic scaling. We have chosen to focus on GRIP1 due to its crucial role in synaptic scaling (unpublished data). Additionally, extensive evidence has shown that GRIP1 is highly associated with recycling processes. The knockout of PICK1 has shown to increase AMPAR recycling (Lin & Huganir, 2007). Since PICK1 and GRIP1 competitively bind GluR2, this suggests that GRIP1 may also affect recycling. Specifically, palmitoylatable GRIP1b has been shown to localize at recycling endosomes (Thomas et al., 2012). Evidence has also shown that GRIP1 binds many types of proteins that have been implicated in trafficking: endosomal (NEEP21), motor (KIF5), and
exocyst (Sec8). Therefore, we have set out to further understand whether altered recycling processes may underlie synaptic scaling and in what ways.

**METHODS**

*Immunohistochemistry.* Cultured cortical neurons DIV7/8 were treated with 5uM tetrodotoxin (TTX) for 6 hours at 37°C. Fixed in 4% PFA/5% sucrose for 20 min at 25°C. Permeabilizing block (1% BSA, 10% gt serum, and 0.25% TritonX100) was then applied for 20 min at 25°C. Primary and secondary antibody mixtures were made in permeabilizing block. Cells were simultaneously incubated in Sec8 (1:250; BD Transduction Laboratories) and GRIP1 (1:250; Abcam) antibodies overnight in a humidified container at 25°C. Three washes in 1xPBS were held for 10 min in order to remove any unbound or loosely bound antibodies. Cells were then simultaneously incubated in Alexa Fluor 488 and Alexa Fluor 647 (1:300; Life Technologies) for 1 hr covered at 25°C. These secondary antibodies marked Sec8 in the 488 channel and GRIP1 in the 647 channel. Again, three 1x PBS washes were held for 10 min before slides were mounted and kept covered. Imaging was performed on a Leica SP5 Microscope using a 63x/1.4 oil immersion objective. Pyramidal cells were selected to image based on morphology in the 488 channel. A low zoom image was taken for morphology reference followed by a 3x high zoom image to be analyzed. Granularity analysis was performed in Metamorph. The range of approximate granule width was set to 0.4-2um. Regions were made to be limited to the dendrite in order to exclude background signal from glia cells. Binary masks were made for each channel as well as a logical AND mask. Four measurements were performed: a) Channel 1 granules, b) Channel 2 granules, c) AND
mask on Channel 1 granules, and d) AND mask on Channel 2 granules. Total intensity and total colocalized intensity were measured in Metamorph. Densities were calculated based on the number of granules divided by the measured region area. Percent-colocalized measurements were deemed to be the number of colocalized granules divided by each number of granules in each channel.

**Random Colocalization Analysis.** Artificial Sec8 images were made in ImageJ using ThunderSTORM plugin. An Integrated Gaussian was used setting the full width at half maximum (FWHM) range from 200-350 nm intensity range from 700-900 photons. The average density for measured regions was used to create an artificial image. Within each experiment, one artificial image was made to compare with every GRIP1 image. Again, granules were identified in order to determine the number of colocalized granules, which was deemed to occur by random. Then, the percent colocalized randomly was compared to the previously determined experimental percent colocalized.

**GRIPKD Exocytotic Assay.** Cultured cortical neurons were fed DIV7/8 regardless of transfection date. Cells were transfected 2-3 days prior to imaging. For each dish, 0.6ug Lipofectamine and 1ug of each DNA construct were added. Lipofectamine and DNA were added. A construct containing a pH-sensitive superecliptic pHluorin (SEP) fused to transferrin receptors (TfSep) was expressed in all cells. A short hairpin RNA targeting GRIP1 was used to knockdown GRIP1 expression. Each dish received 40uL of DNA/Lipofectamine mixture. On the day of imaging, cells were treated with 5uM TTX for 6 hours. Before imaging, the media was replaced with 2mL of live-imaging media
made (in mM): 117 NaCl, 5.3 KCl, 1.8 CaCl\(_2\), 0.814 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 20 HEPES, 50 dextrose, and 100mg BSA (final osmolarity between 315 and 325 mOsm, ~ pH 7.3). For TTX-treated dishes, 1uM of TTX was added when media was replaced with live-imaging media. Confocal microscopy was performed on a Marianas Spinning Disk with a 63x/1.4 oil immersion objective. A heating platform was used to maintain a media temperature of 36.5-37°C. Only transfected live pyramidal cells were imaged. Living cells were determined as cells with movement seen in real time. A total of 600 images were taken every 333ms with a 488 laser. Cell movies were analyzed using an ImageJ plugin, Octane. The number of events was measured within regions proximal to the cell body unless specifically identified as measured distally. Exocytotic events were defined as appearing punctated within 1-3 frames and then gradually disappearing within 3-5 sec. Endocytotic events, which were not counted, were characterized as appearing widespread and concentrating into a puncta that did not disappear for several 100 frames. Additionally, moving puncta that overlap with stationary puncta were not counted as events. Ambiguous flashes were not counted either. Events within same-sized regions were analyzed both proximal distal to the cell body. The frequency of exocytotic events was determined by the number of events divided by the time interval of 180s.

**GRIPOE Exocytotic Assay.** The same protocol for these experiments was used as previously described for GRIPKD neurons besides the following details. A GRIP1a overexpression plasmid (Ziff lab) was transfected instead of the GRIPKD shRNA alongside TfSep. All imaging and analytic procedures were the same.
**Statistical Normalization and Analysis.** Control values were normalized to a value of 1. The average of all control values was calculated. Each experimental value was then calculated as a percentage of the average control. Significance was calculated using two-tailed, two-sample unequal variance t-test in Excel.

**CypHer Trafficking Assay.** Cells were transfected with either WTGluA2 or Y876E-GluA2 constructs in order to block binding of GRIP1 and GluA2. CypHer pHluorin-tagged FAB fragments (Synaptic Systems) were pre-incubated with GluA2 antibodies for 20 min. A ratio of 2.5x FAB fragments was pre-incubated for 1 hr GluA2 antibody. Cells were then incubated in the mixture of for 40 min prior to imaging in order to allow the tagged primary antibodies to bind to surface receptors and be internalized. The CypHer secondary fluoresces only at pHs within endosomal compartments. Cells were then washed briefly with live-imaging media before being replaced with fresh 2 mL of live-imaging media. During imaging, cells were placed on a heating stage under an Olympus IX-81. Only transfected pyramidal cells were chosen for imaging. Images were taken every 5 min with a 63x Oil objective in Volocity. Images taken in velocity were then exported as TIF files to be evaluated in ImageJ. First, these images were registered using the plug-in, StackReg, to make compensate for cell bodies drifting between timepoints. Tracks of CypHer signals were measured by using an ImageJ plug-in called, TrackMate. Here, a Laplacian of Gaussian (LoG) detector was used to pick particular puncta to track. An estimated blob diameter of 5 pixels was used with a threshold of 0.5 pixels. A sub-pixel localization was also incorporated. The HyperStack Distplayer view was chosen and no further filtering was performed. An Linear Assignment Problem
(LAP) tracker was chosen. Tracks were limited to having a max distance of 20 pixels and no gap closing. Statistics about the Links, Spots, and Tracks were saved in Excel to further be compiled. Average track duration, displacement and speed were then evaluated for each cell. In these averages, tracks of 2 or 1 frame length were excluded.

RESULTS

GRIP1 accumulates at Sec8 sites

GRIP1 has seven different PDZ domains, which suggests many binding partners. Sec8, a member of the exocyst complex, has previously been identified as a binding partner of GRIP1 (Mao et al, 2010). To examine the role that the interaction between GRIP1 and Sec8 may play in synaptic scaling, we examined colocalization of these proteins via immunostaining. Synaptic scaling was induced by treatment of tetrodotoxin (TTX) for 6 hrs. The total intensity of each signal was representative of the total amount of protein present in each neuron (Figure 1a, b). Between control and scaled cells, the intensity of Sec8 did not increase. However, an increase of GRIP1 intensity was observed with TTX, which was consistent with previous findings (Figure 1b) (Gainey et al, unpublished). Also, the density of GRIP1 puncta increased in TTX, but the density of Sec8 puncta did not (Figure 1d). The intensities of each signal were examined at these colocalized spots to determine the effects of scaling up. Sec8 total intensity colocalized with GRIP1 did not increase between conditions, indicating that there was the same amount of Sec8 found at each colocalized spot (Figure 1c). However, the total intensity of GRIP1 did increase with TTX, which indicates that the amount of GRIP1 colocalized
with Sec8 increases when scaling is induced (Figure 1c). These results suggest that with scaling, more GRIP1 is associated with Sec8, and by extension, the exocyst complex.

**Random Colocalization Analysis**

To verify that the colocalized puncta between GRIP1 and Sec8 were in fact due to functional interactions (e.g. GRIP1 localizing to exosomes) rather than signals that happened to randomly overlap, a random colocalization method was developed. An artificial image of one signal was compared to actual experimental images. The percent of colocalization found with the artificial image was deemed to be due to chance that the puncta randomly overlapped. Therefore, by comparing the random percent colocalized to the experimental percent colocalized values, we were able to determine if our colocalized spots were due to biological interactions (Figure 2b). An artificial image of Sec8 puncta was randomly generated using ThunderSTORM because it had higher raw density values (Figure 2a). When compared, the experimental percent colocalization of GRIP1 with Sec8 was significantly above random values (Figure 2c).

**GRIP1 is required for exocytotic events**

To determine whether GRIP1 plays a role in exocytosis, a live-cell imaging assay of recycling endosomes was performed. For our purposes, a construct with SEP (superecliptic pHluorin protein) fused to transferrin receptors was used. A (SEP) protein was utilized due to its ability to fluoresce when exposed to extracellular pH levels found outside the cell. Additionally, transferrin marks recycling endosomes that have been shown to carry GluRs, which allowed us to indirectly measure rates of AMPAR
trafficking (Kennedy et al, 2010). Therefore, every time a recycling endosome was exocytosed, a flash of fluorescent signal was observed (Figure 3a, d, Movie 1). Cells were transfected with this construct for 2 days before live imaging. Images were taken every 0.33 sec for a total of 180 sec for each cell. An exocytotic event was deemed to be a puncta that appeared within 0.33-0.67 sec followed by a diffusion of the signal within 2-5 sec (Figure 3d, Movie 2). At many of these sites of exocytosis, the level of fluorescence was also slightly higher than before the event (Figure 3e). In contrast, endocytotic events were determined to be a dispersed signal concentrating at one point and disappearing over 30 or more sec (not shown). The frequency of exocytotic events that occurred proximal to the cell body was compared to those that occurred distally. No significant difference was observed between the frequency of proximal to distal events (Figure 3b). However, a trend emerged that less distal events occurred than proximal events. The rates of exocytosis were evaluated after scaling was induced, but no significant change was observed (Figure 4). Cells were co-transfected with a short-hairpin RNA to knockdown GRIP1 expression. A significant reduction in the frequency of exocytotic events was observed, indicating that the expression of GRIP1 is necessary for exocytosis (Figure 5a).

GRIP1 is not sufficient to drive exocytosis

Next, we sought to determine if the overexpression of GRIP1 would lead to an increased rate of exocytosis. Although there was an increased trend observed in exocytotic events in GRIP overexpression cells, it was not a significant (Figure 5b).
Therefore, GRIP1 seems to be required, but not sufficient to increase the levels of exocytosis.

**Trafficking of GluA2-containing recycling remains unchanged**

In order to evaluate the rates of trafficking of GluA2, CypHer-tagged GluA2 antibodies were used to mark internalized compartments. Live-cell images were taken every 5 min and compiled (Figure 6a, Movie 3). Puncta were tracked between frames to examine any differences in duration, displacement, or speed (Figure 6b, Movie 4). No differences were found between control and scaled cells (Figures 6c, d, e). Additionally, in cells expressed a mutated form of GluA2 (Y876E) that is unable to bind GRIP1, there was also no significant difference in these three parameters (Figure 6c, d, e).

**DISCUSSION**

In the phenomenon of synaptic scaling, synapse-specific changes occur alongside functional changes to strengthen or weaken the neuron in response to its surrounding network activity (Turrigiano, 2012). These synapse-specific changes have been further identified as an accumulation of GluA2-containing AMPA receptors at synapses (Wierenga et al 2005, Gainey et al 2009). The specific mechanisms that result in GluA2 receptor accumulation in response to scaling are largely unknown. Previous unpublished work has identified that the interaction between GRIP1 and GluA2 receptors is necessary for successful scaling (Gainey et al 2009). Here, we report a change in protein colocalization between GRIP1 and the exoyst complex via Sec8. Furthermore, we
demonstrate that GRIP1 plays a critical role in basal exocytotic processes. These findings may be further used to understand the fundamental roles that GRIP1 plays within cells.

Although previous findings have identified Sec8 as a binding partner of GRIP1, it has yet to be implicated in synaptic scaling. We found that the levels of Sec8 did not change in the cell during scaling, which suggests that the abundance of these exocyst complexes is not altered. Furthermore, there was no change in overall density of Sec8, which eliminates the possibility that the existing exocyst population was redistributed. Most notably, we observed an increased association between Sec8 and GRIP1 after scaling was induced. At colocalized sites, we found an accumulation of GRIP1 proteins, but an unchanged amount of Sec8. This suggests that an increased amount of GRIP1 molecules have trafficked to the exocyst complexes. The high density of Sec8 immunostained puncta raised the possibility that the overlap between Sec8 and GRIP1 puncta signal may have been to chance levels. Therefore, we developed a methodology to quantitatively test whether the colocalization observed reflected a real biological interaction. Using this methodology, we were able to demonstrate that the localization of GRIP1 to these exocyst sites was not explained by chance alone. This methodology can now be used by future researchers to identify false colocalization. Often in colocalization analysis, when a low fraction of two proteins are colocalized, it is considered to not be significant. Utilizing our methodology, researchers can now identify biologically relevant interactions, even when only a small fraction of proteins are colocalized. We used this methodology to confirm our findings that GRIP1 proteins accumulate at Sec8 sites.

Three hypotheses follow based on increased GRIP1 at exocyst sites: 1) Sec8 is able to bind multiple GRIP1 molecules or 2) GRIP1 molecules accumulate by self-
association and 3) GRIP1 binding sites on Sec8 and the exocyst complex are increased during scaling. Regardless, we have illuminated a key step of GRIP1 accumulation at exocyst complexes during synaptic scaling.

It is compelling to imagine that the accumulation of GluA2 receptors at the cell surface is a product of increased GRIP1-GluA2 bound molecules recruited to the exocyst complex. Alternatively, unbound GRIP1 may accumulate at Sec8 sites followed by binding with GluA2. However, further investigation is necessary to determine if the GRIP1 proteins accumulating at Sec8 sites are bound or unbound. GRIP1 is a multi-PDZ protein that has multiple binding partners function. Following this model, GRIP1 could act as an adaptor protein that targets GluA2 receptors to the exocyst complex.

Since we have identified the importance of GRIP1 association with exocyst complexes, we decided to try to further understand the role of GRIP1 in the exocytotic processes and whether these are altered in synaptic scaling. Previous methods have utilized a pH-sensitive GluA2 fluorophore to investigate rates of endo- and exocytosis. However, it has recently been shown that the pH sensitivity of this fluorophore is unreliable to differentiate internal and external environments (Rathje et al., 2013). Therefore, we utilized a reliable pH-sensitive fluorophore attached to transferrin to examine the rate of exocytosis (Kennedy et al., 2010). Previous methods also examined recycling processes based on the recovery of fluorescence signal after bleaching over several minutes. However, with this transferrin-based method, we were able to observe exocytotic events in real time through the emergence of fluorophores within seconds.

We found that the rate of exocytosis for recycling endosomes remains unaltered during synaptic scaling. Combined with the unchanged amount of Sec8-containing
exocyst complexes, we have established that rates of exocytotic processes are unaltered both in protein measure and function during synaptic scaling. This steers our model of AMPA trafficking away from the idea of faster receptor recycling rates via increased number of vesicles being exocytosis. Instead, we may consider that accumulation occurs upstream of exocytosis. One possibility is that more GRIP1 and GluA2 receptors could accumulate at the exocyst complex for regular exocytosis during synaptic scaling. The rates of exocytosis remain unchanged, but the amount of receptors in each event is altered.

It is worth noting that these observations were obtained from measurements of proximal areas. However, when analysis of the same data set was performed with distal regions, no significant differences were detected. The ratio between proximal and distal events remains the same between control and scaled cells. This dissuades the possibility that the distribution of exocytotic events is altered during synaptic scaling.

Up to this point, GRIP1 has been discussed as a passive binding partner to the exocyst complex. However, we found that the rates of exocytosis were significantly reduced when GRIP1 was knocked down, which indicates a more active and critical role for GRIP. Rather than imagining GRIP as a protein that just hitchs onto the exocyst complex, it may play a more complicated role upstream of proper exocytosis in neurons.

With GRIP1 overexpression, we set out to determine whether exocytosis is bidirectionally GRIP1-dependent. However, we did not see a significant change in rates of exocytosis, but we detected a slight increased trend. A potential caveat of this experiment was that when transfected cells were fixed after live-imaging and stained for GRIP1 levels, a population of cells were found to have the same levels of expression as control
cells. However, when ranked, all overexpression cells did have a higher GRIP1 expression than control (Figure 5c). Something else to consider in this experiment is that the construct only overexpresses one isoform, GRIP1a. The GRIP1a overexpression construct was used because it has been reported that this isoform is able to change subcellular location whereas GRIP1b was primarily at membrane-associated areas (Yamazaki et al., 2001). Naturally, we predicted that the isoform involved in the dynamic process of exocytosis might be the GRIP1a form. However, recent studies have shown that palmitoylable GRIP1b is found in recycling endosomes (Thomas et al., 2012), suggesting that it may be worth investigating whether GRIP1b overexpression changes the rate of exocytosis. However, it is possible that the overexpression of GRIP1 of either isoform is not sufficient to increase the amount of exocytosis in cells. Therefore, GRIP1 may be necessary for exocytosis, but is not sufficient to drive it.

With these results, we have attempted to further illuminate the role that GRIP1 plays in regards to exocytosis. First we established that when synaptic scaling is induced, GRIP1 accumulates at exocyst complexes. This implicates an important role for GRIP1 in exocytosis. Next, we determined that the rate of recycling endosome exocytosis remains unchanged during scaling. This leads us to emphasize the importance of protein accumulation rather than altered recycling rates in synaptic scaling. Additionally, we have shown that the presence of GRIP1 is crucial for proper exocytosis, but does not drive it.

Even though we have shown that the rate of exocytosis for recycling endosomes is unaltered, it does not rule out the possibility that AMPAR trafficking rates could be changed (Figure 7). Further investigation will be required to understand whether GluA2-
containing endosomes have altered dynamics during scaling. Preliminary experiments have utilized a pH-sensitive fluorophore that fluoresces when internalized (CypHer) to mark internal GluA2 (Figure 6). This work has shown that there was not a change in displacement or speed. However, these observations were made over much longer time intervals in cells live-imaged every 5 min for a length of 30 min. Efforts are currently being made to image internalized GluA2 at similar time intervals as the transferrin-labeled imaging: seconds rather than minutes. We hope to further understand two major questions. The first asks, are there differences in GluA2-containing endosomal trafficking in scaled cells? Perhaps the overall rate of exocytosis for recycling endosomes is the same when scaling is induced, but the amount or rate of GluA2-containing endosomes is altered. The second considers whether there are any differences in exocytotic rates when GluA2 is unable to bind GRIP1 in cells expressing a mutant GluA2 form (Y876E). This would address whether binding of GRIP1 and GluA2 influences recycling processes.

Further research will be required to elucidate whether trafficking mechanisms underlie synaptic scaling. We must first consider whether there is a change in GluA2-containing recycling endosomal trafficking (Figure 6, 7). Secondly, there may be a change in the location of exocytotic events on the surface. One could imagine that GluA2-containing vesicles may be targeted to locations closer to the synapse during scaling. Thirdly, there may be a change in the packaging of cargo. Perhaps more GluA2 or GRIP1 is being trafficked along unaltered exocytotic processes during scaling. Lastly, we must consider the possibility that scaling is mediated by trafficking processes outside of the recycling pool (Figure 7).
References


**Figure 1.** GRIP1 accumulation at exocyst complexes during synaptic scaling. 

A) Representative images of Sec8 (green) and GRIP1 (red) staining. Top, images under control conditions. Bottom, images after 6 hrs of TTX treatment. Right panels, merge of channels with arrows indicating points of colocalization. Scale bar, 2um. 

B) Total intensities of each channel in control and TTX conditions. Normalized to the control values. 
C) Total intensities at colocalized puncta normalized to control. 
D) Density of puncta for each channel normalized to control. Statistics were performed as two-tailed Student’s t-tests. * Different from control, p < 0.05.
Figure 2. Detected colocalization is above random values. A) Example of an experimental Sec8 image (left) and an artificially generated Sec8 image (right). Red box designates inset for B. Scale bar, 10um. B) Experimental staining for Sec8 (green) and GRIP1 (red). Artificially generated Sec8 image (blue). Merge of experimental GRIP1 and random Sec8 with arrows indicating points of colocalization. Scale bar, 2um. C) Percent of total puncta that are colocalized in random and experimental conditions normalized to control values. Statistics were performed as two-tailed Student’s t-tests. * Different from control, p < 0.05.
Figure 3. Detection of exocytotic events of recycling endosomes with TfSEP. A) Representative first frame image of a control pyramidal cell. Insets for part C (red:proximal, blue:distal) and part D (green). Scale bar, 10um. B) Frequency of exocytotic events per second after proximal and distal analysis of the same cells. Statistics were performed as two-tailed Student's t-tests. No significance was found. C) PALM rendering of exocytotic events (red) throughout movie overlaid on first frame background signal (green). Scale bar 2um. D) Montage of a single exocytotic event for ten frames from 0-3 seconds of the same control cell. Scale bar, 2um. D) Line scan across montage showing intensity levels of the exocytotic event over time.
Figure 4. Frequency of exocytotic events does not change during synaptic scaling. A) Frequency of exocytotic events per second between control and TTX conditions. Statistics were performed as two-tailed Student’s t-tests. No significance was found. B) Top, PALM rendering of exocytotic events (red) overlaid on first frame signal (green). Scale bar, 2um. Bottom, first frame image of control (left) and TTX (right) example cells with insets indicated (red boxes). Scale bar, 10um.
Figure 5. **GRIP1 is necessary for proper exocytosis.** A) Frequency of exocytotic events per second between control and GRIP1 knockdown conditions. B) Frequency of exocytotic events per second between control and GRIP1a overexpression conditions. Statistics were performed as two-tailed Student’s t-tests. ** Different from control, p < 0.01. C) Top, PALM rendering of exocytotic events (red) overlaid on first frame signal (green). Scale bar, 2um. Bottom, first frame image of control (left), GRIP1 knockdown (middle), and GRIP1a overexpression (right) with insets indicated (red boxes). Scale bar, 10um.
Figure 6. GluA2 trafficking is unaffected in measured parameters in scaling and blockage of GluA2-GRIP interaction. A) First frame of a representative cell. Scale bar, 10um. B) Three example images with each frame made a single color and then overlaid. Scale bar, 2um. C-E) Parameter evaluated between control and TTX conditions. Cells were also transfected with wild-type GluA2 and Y876E GluA2 constructs. C) Mean displacement of particles over 30 min D) Mean duration of detected tracks during the 30 min movie. E) Mean speed of particles during 30 min movie. Statistics were performed as two-tailed Student’s t-tests. No significance was found.
Figure 7. Schematic of possible mechanisms underlying scaling. A) In our experiments, overall recycling exocytosis was evaluated through the marking of transferrin receptors (red triangles). However, only a subpopulation of these recycling vesicles contain GluA2 receptors (blue rectangles). Therefore, even though overall recycling processes do not change with synaptic scaling, perhaps this subpopulation does. B) Another possibility is that scaling is independent of recycling processes. Perhaps it is mediated by some other type of trafficking process (green teardrop).