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Disruption of the interaction between myosin VI and SAP97 is associated with a reduction in the number of AMPARs at hippocampal synapses

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Abstract

Myosin VI is an actin-based motor protein that is enriched at the postsynaptic density and appears to interact with alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate-type glutamate receptors (AMPARs) via synapse associated protein 97 (SAP97). Here, we find that a Flag epitope-tagged dominant negative construct that inhibits the interaction between SAP97 and myosin VI (Flag-myoVI-DN) causes a dramatic reduction in the number of synapses and the surface expression of AMPARs in cultured hippocampal neurons. Furthermore, we find that Flag-myoVI-DN also prevents the rapid delivery of AMPARs to synapses that can be induced by the transient activation of *N*-methyl-D-aspartate receptors. The Flag-myoVI-DN induced decrease in surface AMPARs is not because of reduced AMPAR subunit protein synthesis. Using whole-cell recording, we show that Flag-myoVI-DN also prevents the activity-induced increase in miniature excitatory postsynaptic current frequency that is normally associated with recruitment of AMPARs to the cell surface at synaptic sites that lack these receptors (i.e. 'silent' synapses). Together, these results indicate that myosin VI/SAP97 plays an important role in trafficking and activity-dependent recruitment of AMPARs to synapses.

Keywords: AMPA, GluA1, hippocampal neurons, myosin VI, SAP97, synaptic plasticity.

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In the hippocampus, synaptic plasticity has been linked with learning and memory of spatial information. Hippocampal ionotropic glutamate receptors play a key role in basal synaptic transmission, as well as in long-term potentiation (LTP) and long-term depression (LTD) (Collingridge *et al.* 1983, 2004; Bear and Malenka 1994). It is believed that LTP involves the insertion of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs) into the plasma membrane, followed by their lateral diffusion to the synapse, whilst LTD involves lateral diffusion away from the synapse followed by endocytosis (Ashby *et al.* 2004; Groc and

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Abbreviations used: AMPAR, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor; DIV, days *in vitro*; Flag-myoVI-DN, Flag epitope-tagged truncated C-terminal cargo domain of myosin VI; GFP, green fluorescent protein; GK, guanylate kinase; HBS, HEPES-based saline buffer; LTD, long-term depression; LTP, long-term potentiation; mEPSC, miniature excitatory postsynaptic current; MT, mitotracker; NMDAR, *N*-methyl-D-aspartate receptor; NT, non-transfected; PBS, phosphate-buffered saline; SAP97, synapse-associated protein 97; SH3, Src homology 3.

Choquet 2006; Derkach *et al.* 2007). There are four AMPAR subunits named GluA1–4 (IUPHAR nomenclature of subunits previously referred to as GluR1–4 or GluRA–D) (Collingridge *et al.* 2009). The GluA2 subunit is particularly important for *N*-methyl-D-aspartate receptor (NMDAR)-dependent LTD. Through the study of the proteins that interact with GluA2 subunits, some of the molecular events underpinning these processes are beginning to be understood. For example, LTD involves interactions between the GluA2 subunit with *N*-ethylmaleimide-sensitive fusion protein (Luthi *et al.* 1999), the clathrin adaptor protein AP2 (Carroll *et al.* 1999; Lee *et al.* 2002b) and protein interacting with kinase 1 (Xia *et al.* 2000; but see Daw *et al.* 2000). Second order interactors have also been identified in this LTD process; for example, hippocalcin binds to AP2 and functions as a high affinity Ca^{2+} sensor (Palmer *et al.* 2005). The molecular mechanisms responsible for LTP expression are less clear, although it appears that GluA1 subunit-driven delivery of AMPARs to the synapse is required (Shi *et al.* 2001). While studies have shown that various membrane-associated guanylate kinases (MAGUKs) modulate GluA1-linked synaptic plasticity, synapse-associated protein 97 (SAP97) is the only family member that binds directly to GluA1 (via an interaction between the C terminus of GluA1 and PDZ2 domain in the N-terminal region of SAP97; Leonard *et al.* 1998).

In a previous study, we found that an actin-based motor protein, myosin VI, binds to the N terminus of SAP97 (Wu *et al.* 2002). Furthermore, SAP97, myosin VI and GluA1 form a complex in rat brain (Wu *et al.* 2002; Osterweil *et al.* 2005). This suggests that by interacting with SAP97, myosin VI may be involved in the trafficking of AMPARs, either under basal conditions or during activity-dependent alterations in their synaptic distribution. Consistent with this possibility, AMPA- and insulin-stimulated AMPAR endocytosis is impaired in mice lacking myosin VI (Osterweil *et al.* 2005).

In this study, we have used the flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN; lacking the motor domain; Fig. 1a), that acts as a dominant negative, to further investigate the role of SAP97 interactions in AMPAR trafficking at basal and increased activity levels in hippocampal neurons. We found that expression of Flag-myoVI-DN caused a dramatic loss of GluA1-containing AMPARs at synaptic sites under basal conditions with no reduction in total NMDAR surface expression. Furthermore, transient depolarisation of hippocampal neurons, a treatment that has previously been shown to increase AMPAR insertion into the plasma membrane (Pickard *et al.* 2001) and induce an increase in synaptic activity (Fitzjohn *et al.* 2001), had no effect on the surface expression of GluA1 in neurons expressing Flag-myoVI-DN. These findings suggest that the interaction between myosin VI and GluA1 via SAP97 may be important for both basal trafficking and activity-induced delivery of AMPARs to synapses.

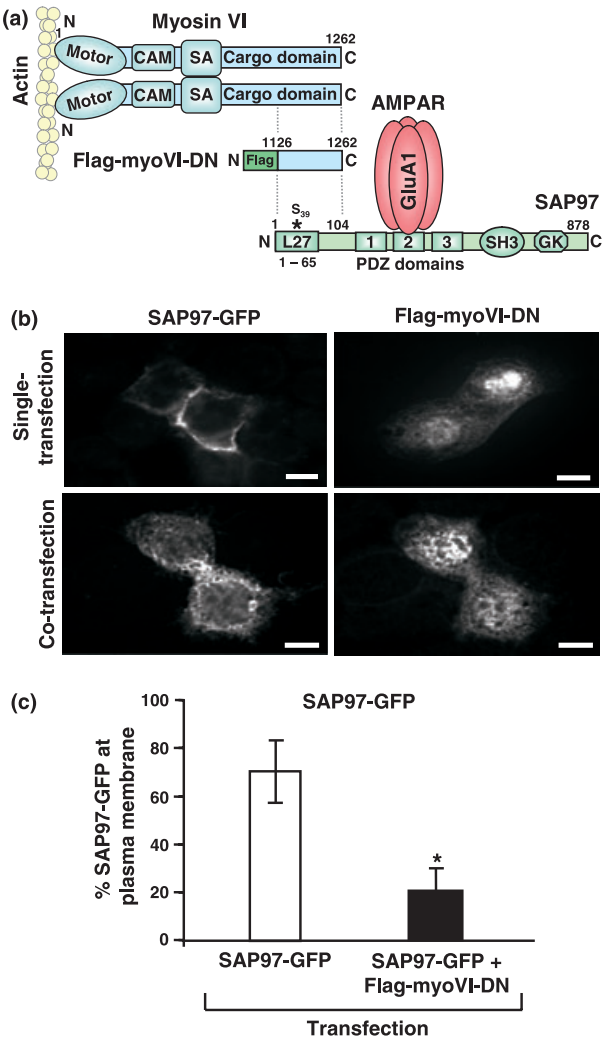

Fig. 1 Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN) alters the subcellular distribution of synapse-associated protein 97–green fluorescent protein (SAP97-GFP) in CACO-2 cells. (a) Schematic representations of myosin VI, Flag-myoVI-DN and SAP97. The relative positions of the myosin VI motor-, calmodulin binding (CAM), coiled–coiled self-association (SA) and cargo domains are indicated. The myosin VI and GluA1 binding regions of SAP97 are highlighted as well as the L27, PDZ1-3, Src homology 3 (SH3) and guanylate kinase (GK) domains and serine 39 residue (S_{39}). (b) Characteristic distribution pattern of SAP97-GFP and Flag-myoVI-DN following single and co-transfection. Scale bars: 5 μm. (c) Comparison of the plasma membrane localisation of SAP97-GFP when transfected alone or together with Flag-myoVI-DN. Plasma membrane localisation was expressed as mean percentage of total SAP97-GFP fluorescence ± S.D. (*$p < 0.05$, Student's *t*-test, $n = 4$).

Methods

Antibodies

Anti-GluA1 rabbit polyclonal antibody was raised against the extracellular N-terminal residues 253–267 (Molnár *et al.* 1993). Anti-GluA2 mouse monoclonal antibody to the N-terminal domain

(Vissavajjhala *et al.* 1996) was obtained from Zymed Laboratories. Anti-NMDA receptor subunit 1, GluN1, rabbit polyclonal antibody was raised against extracellular N-terminal residues 436–450 (Molnár *et al.* 1995). Anti-Flag mouse monoclonal antibody was obtained from Eastman Kodak (New Haven, CT, USA). The rabbit and guinea pig polyclonal anti-GluA1-4 antibodies were raised against residues 724–781 of the extracellular M3–M4 loop region of the $GluA1_{flop}$ sequence (Pickard *et al.* 2000, 2001).

Synthesis of constructs and adenoviruses

The Flag-myoVI-DN construct was generated by a prey clone identified using yeast two hybrid (Wu *et al.* 2002) into pCMV-tag 2B (Stratagene, La Jolla, CA, USA) with EcoRI. Sindbis virus expressing recombinant Flag-tagged myosin VI-DN or green fluorescent protein (GFP) was synthesised using pSinRep5 (Stratagene). Sindbis virus expressing GFP alone was used as a control rather than Flag alone because of the small size of the Flag tag.

Transfection and imaging of CACO-2 cells

The human carcinoma cell line CACO-2 (American Type Culture Collection # HTB 37) was maintained in minimum essential medium (Life Technologies. Grand Island, NY, USA), supplemented with 10% foetal bovine serum (Life Technologies) and 40 u/μL of penicillin/streptomycin (Life technologies) at 37°C in 5% CO_2. The SAP97-GFP construct has been previously described (Wu *et al.* 1998). To assess the effect of the C terminus of myosin VI on the localisation of SAP97, constructs were transiently transfected into CACO-2 cells (grown to 60–70% confluency) using lipofectamine (Life Technologies). Eighteen hours later, epifluorescent images were taken using a Nikon Diaphot 300 microscope equipped with a Photometric CH250 cooled camera. Images were captured with IP lab spectrum software (Signal Analytics Inc., Palo Alto, CA, USA) and presented in Adobe Photoshop. In non-confluent CACO-2 cells, SAP97 is diffuse, when cells become confluent, cell–cell adhesion results in the recruitment of SAP97 at the lateral cortico cytoskeleton (Reuver and Garner 1998). The re-localisation of SAP97 is quite dramatic in that the distribution is either almost completely cytosolic or localised at the plasma membrane. To determine the effect of Flag-myoVI-DN on SAP97-GFP localisation, 100 cells from each replicate ($n = 1$) were chosen that moderately expressed SAP97-GFP, and localisation at the plasma membrane was assessed. Threshold was set as the level of background fluorescence on the coverslip in the absence of cells. The percentage plasma membrane distribution in cells co-expressing SAP97-GFP and Flag-myoVI-DN was compared with levels in SAP97-GFP alone in four independent experiments.

Neuronal cultures

Cultures of dissociated hippocampal neurons were prepared from postnatal day one old (P1) rats (Malgaroli and Tsien 1992). Rats were killed by decapitation and the brains rapidly removed. The CA1–CA3 regions of the hippocampus were isolated, and cells were recovered by trypsin digestion and mechanical dissociation before subsequent plating on 22 mm glass coverslips coated with poly-D-lysine (Sigma, St Louis, MO, UK). Cultures were maintained at 37°C in a humidified atmosphere of 95% O_2, 5% CO_2 in Neurobasal Medium (Gibco, Rockville, MD, USA; Life Technologies); glucose (38.9 mM), glutamine (2 mM), HEPES (15 mM), gentamicin (2 μg/mL), 10% foetal horse serum. After 24 h, media were supplemented with 1% Ara-C (Sigma) to prevent glial proliferation and foetal horse serum concentration was reduced to 3%. Culture media was changed three times per week.

Immunohistochemistry

Immunofluorescence labelling of surface expressed AMPARs and NMDARs were performed in non-permeabilised hippocampal neurons as described previously (Noel *et al.* 1999; Pickard *et al.* 2000, 2001). AMPAR proteins were identified with either a rabbit (Pickard *et al.* 2000) or a guinea pig (Pickard *et al.* 2001) polyclonal antibody recognising the same conserved extracellular loop region of all four AMPAR subunits (GluA1-4, both *flip* and *flop*). Individual AMPAR subunits were analysed with extensively characterised primary antibodies raised against the extracellular epitopes of GluA1 (1 μg/mL; Molnár *et al.* 1993) and GluA2 (5 μg/mL; Zymed Laboratories, South San Francisco, CA, USA; Vissavajjhala *et al.* 1996) subunit proteins. NMDARs were immunolabelled with a rabbit polyclonal antibody that binds to an extracellular N-terminal epitope of the GluN1 subunit, common to all splice variants (1 μg/mL; Molnár *et al.* 1995; Noel *et al.* 1999; Pickard *et al.* 2000). To detect synapses, mitotracker (MT), a rhodamine derivative of a mitochondrial marker (red deep MT; Molecular Probes, Inc., Eugene, OR, USA; Groc *et al.* 2004) was utilised, using the manufacturer's protocol for adherent cells. Anti-Flag mouse monoclonal antibody (1 : 50 dilution; Eastman Kodak) was used to visualise Flag-myoVI-DN distribution in permeabilised hippocampal neurons.

Unless otherwise stated, neuronal cultures adhered to coverslips were washed with HEPES-based saline (HBS: 119 mM NaCl, 5 mM KCl, 25 mM HEPES, 33 mM D-glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 μM glycine, 0.1 mM picrotoxin, 0.5 μM tetrodotoxin, 300–310 mOsm, pH 7.4), and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 10 min, then incubated in PBS supplemented with 10 mM glycine for 10 min to reduce auto-fluorescence. Before immuno-staining, cells were blocked in 5% bovine serum albumin in PBS for 15 min at room temperature (~20°C). Primary antibodies were visualised with the appropriate secondary antibodies (Alexa 647 or Alexa 568 labelled goat anti-mouse or goat anti-rabbit antibodies, 10 μg/mL, Molecular Probes, Inc) in 5% bovine serum albumin containing PBS. Non-specific labelling and the method specificity of the antibodies were determined by the use of pre-immune sera, blocking antibody labelling with the antigenic peptide or fusion protein used for the production of antibodies (Molnár *et al.* 1993, 1995; Pickard *et al.* 2000) and by performing immunolabelling with the secondary antibodies alone. Immunofluorescent staining was visualised using a Zeiss LSM510 Meta confocal microscope (Oberkochen, Germany). Fluorophores were excited with 488, 543 or 633 nm wavelengths and emission was detected through 505–530 band-pass, Meta detector set to detect wave length between 580 and 610 nm and 650 long-pass filters.

Quantitative analysis of immunofluorescent receptor clusters was carried out in 3D using confocal microscopy and Volocity software (Improvision, Coventry, UK). Twice the level of background fluorescence was used as the threshold, and only clearly identifiable dendrites were selected for analysis. Puncta between 0.3 and 0.8 μm^3 that were above threshold intensity were measured. For quantitative comparisons, the number of puncta per 100 μm length

of dendrite was expressed for GluA1, GluA2, pan-GluA1-4 and GluN1 antibodies within a given field. The less punctuate GluN1 immunolabelling was analysed by quantification of pixel intensities in dendrites using the ImageJ software (NIH, http://rsbweb.nih.gov/ij) as described previously (Corrêa *et al.* 2009). All analysis was performed blind to the experimental manipulation. A single value was obtained from each independent experiment and used to construct the mean and standard error. The number of independent experiments was the number of observations used for statistical analysis.

Infection of primary cultures

Sindbis virus (2 μL/plate) containing the construct for Flag-myoVI-DN or GFP was added to neuronal cultures twelve hours before cells were used for experiments. The efficiency of the Sindbis virus infectivity of postnatal neuronal cultures was typically around 30–40%.

Induction of transient depolarisation for immunohistochemistry

Transient depolarisation was induced using three 1 s applications of 90 mM extracellular K^+ as described previously (Fitzjohn *et al.* 2001; Pickard *et al.* 2001). Briefly, neuronal cultures (grown on cover slips) were transferred into HBS. 90 mM K^+ in HBS (Na^+ adjusted to maintain osmolarity) was applied for 3 × 1 s periods with 10 s intervals in the presence and absence of the NMDAR antagonist L-689,560 (5 μM; Tocris Cookson Ltd, Bristol, UK).

Whole-cell recordings

Neurons were used 21-28 days after plating, and whole-cell electrophysiological recording was carried out as described previously (Fitzjohn *et al.* 2001). In brief, cells were perfused with HBS containing 0.5 μM tetrodotoxin at approximately 2 mL/min at room temperature. Pyramidal cells were voltage-clamped at –70 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Intracellular solution comprised (mM): Cs Methane sulphonate (110); HEPES (40); EGTA (0.6); NaCl (10); Mg-ATP (4); Na_2-GTP (0.3); 290 mOsm, pH 7.2. Once a stable baseline had been obtained for 5 min, 90 mM KCl was perfused directly via a sewer pipette as a series of 3 × 1 s pulses, each separated by an interval of 10 s (Fitzjohn *et al.* 2001). Movement of the sewer pipette was controlled by a fast step perfusion system allowing complete change of the perfusion solution in less than 20 ms (Warner Instruments, Hamden, CT, USA). Data were filtered at 5 kHz and collected continuously, digitised at 10 kHz, and converted to Axon Binary Files using Axoscope software (Axon Instruments, USA). Miniature excitatory postsynaptic currents (mEPSCs) were identified using amplitude and area threshold detection using Mini Analysis software (Synaptosoft, Inc., Decatur, GA, USA). Events were detected by setting the threshold value for detection at three times the root mean square of background noise, followed by visual confirmation of mEPSC detection. For the construction of cumulative probability plots, at least 200 successive events were used from the period immediately preceding application of high K^+. Data obtained no less than 30 min following transient depolarisation were normalised to the median value obtained prior to induction. Access resistance was monitored throughout using LTP software (Anderson and Collingridge 2001) and recordings disregarded if this varied more than 20% throughout the experiment.

Statistics

Statistical analysis was performed using an unpaired Student's *t*-test. For non-equal '*n*' numbers, a two sample equal variance *t*-test was performed. For comparison of multiple sets of data, an ANOVA (SPSS) was used. A p value less than 0.05 was considered significant (*) and a p value less than 0.01 was considered highly significant (**). Results are presented as mean ± SEM. For the electrophysiological experiments, ANOVA with Tukey multiple comparisons test *post hoc* was performed.

Results

Expression of the truncated C-terminal domain of myosin VI alters the sub-cellular distribution of SAP97

Previous studies have shown that SAP97, myosin VI and GluA1 form a complex, and this is thought to be important for normal AMPAR function (Wu *et al.* 2002; Osterweil *et al.* 2005). It has also been shown that the C-terminal region of myosin VI (amino acids 1126–1262) binds to the N-terminal region (amino acids 1–104) of SAP97 (Wu *et al.* 2002). To determine the role of the C-terminal domain of myosin VI on SAP97 and AMPAR distribution, a Flag epitope-tagged truncated construct containing amino acids 1126–1262 of myosin VI (Flag-myoVI-DN) was produced (Fig. 1a). In line with previous studies (Wu *et al.* 1998), transient transfection of GFP-SAP97 alone resulted in localisation primarily at the plasma membrane of CACO-2 cells (81 ± 12%; $n = 4$; Fig. 1b and c). Transfection of Flag-myoVI-DN alone showed a diffuse staining pattern (Fig. 1b). Following co-transfection of Flag-myoVI-DN and GFP-SAP97, there was an approximate 60% reduction in the plasma membrane localisation of GFP-SAP97 (81 ± 12% to 21 ± 6%, $p < 0.05$, Student's *t*-test, $n = 4$) resulting in a more diffuse, perinuclear staining pattern of SAP97 (Fig. 1b and c). Therefore, Flag-myoVI-DN has a dominant negative effect on the ability of GFP-SAP97 to accumulate at the plasma membrane.

Flag-myoVI-DN reduces AMPARs on the surface of hippocampal neurons

To determine whether expression of Flag-myoVI-DN alters the surface expression of AMPARs, control (non-transfected, NT), GFP or Flag-myoVI-DN transfected, non-permeabilised hippocampal cultures were immuno-labelled with antibodies raised against the extracellular N-terminal regions of GluA1 and GluA2 (Molnár *et al.* 1993; Richmond *et al.* 1996; Vissavajjhala *et al.* 1996). There was no significant difference in the number of GluA1 and GluA2 clusters in the dendrites of neurons expressing GFP compared with NT cells (Fig. 2; immunopositive clusters/100 μm dendrite: $GluA1_{NT}$ = 19 ± 2, $GluA1_{GFP}$ = 17 ± 1, $p > 0.05$; $GluA2_{NT}$ = 18 ± 1, $GluA2_{GFP}$ = 20 ± 1, $p > 0.05$, ANOVA, $n = 6$). However, following transfection with Flag-myoVI-DN, the number of

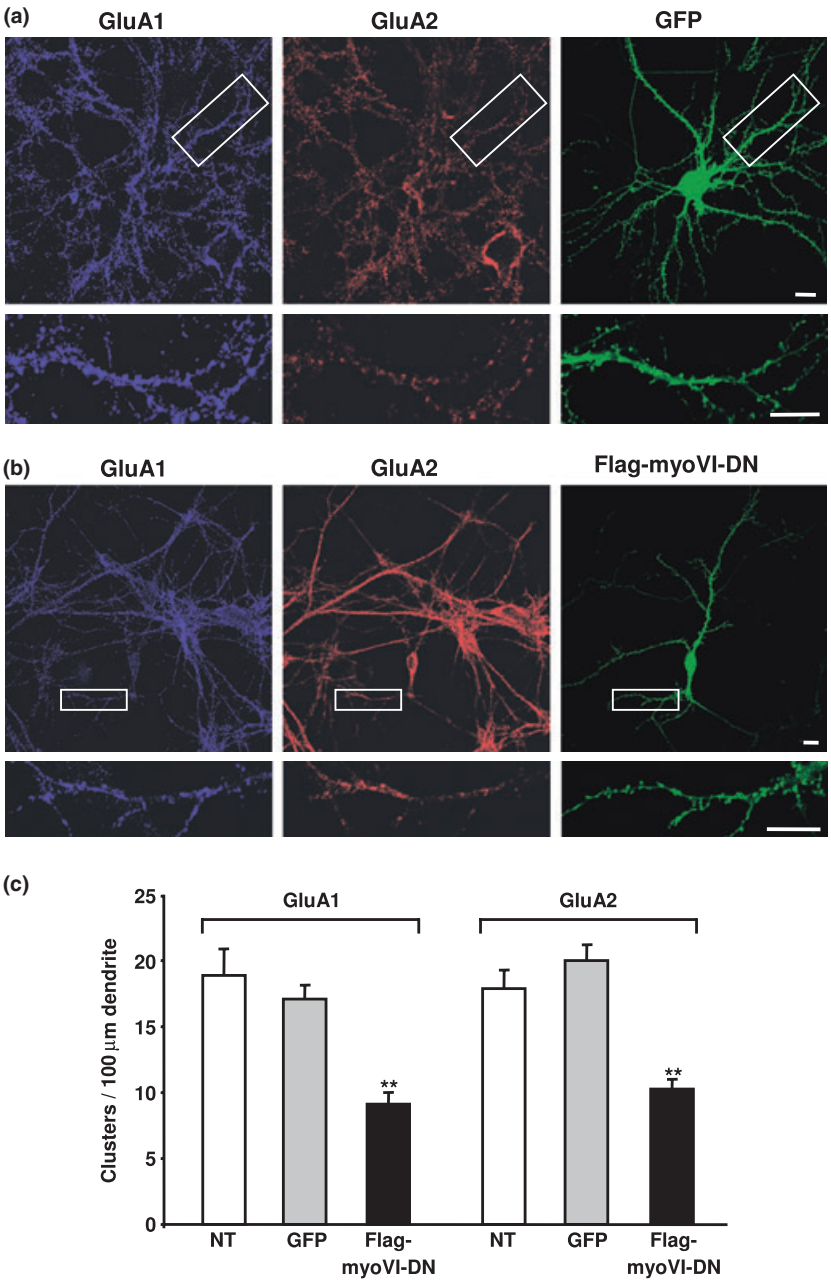

Fig. 2 Surface expression of GluA1 and GluA2 AMPAR subunits is reduced in hippocampal neurons expressing Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN). Non-transfected (NT), green fluorescent protein (GFP)-transfected (a) and Flag-myoVI-DN transfected (b) hippocampal neuronal cultures at 21 days *in vitro* (21 DIV) were fixed (without permeabilisation) and immuno-stained with GluA1 (blue) and GluA2 (red) extracellular N-terminal domain specific antibodies. Flag-myoVI-DN and GFP Sindbis viruses infected a subset of neurons therefore panels illustrate both transfected and NT neurons. Scale bars: 10 μm. (c) Quantitative comparison of NT, GFP expressing and Flag-myoVI-DN expressing neurons identified a significant reduction in GluA1 and GluA2 immunopositive cluster numbers/100 μm dendrite at the cell surface in Flag-myoVI-DN positive neurons (**$p < 0.01$, ANOVA, $n = 6$).

dendritic GluA1 and GluA2 clusters (9 ± 1 and 10 ± 1 clusters/100 μm dendrite, respectively) was significantly reduced to ~50% of NT or GFP-transfected control values ($p < 0.01$, ANOVA, $n = 6$).

Flag-myoVI-DN decreases the number of synapses in hippocampal neurons

Previous studies have shown that expression of the C-terminus of myosin VI causes a substantial decrease in the number of synapses in hippocampal cultures (Osterweil *et al.* 2005). To determine whether this was the case in the present study, 24 h following transfections with Flag-myoVI-DN, the number of synapses was assessed using MT (Groc *et al.* 2004) and compared with levels in GFP-transfected cells (Fig. 3a and b). In neurons expressing GFP, there were 46 ± 4 MT positive puncta/100 μm dendrite ($n = 8$), which was not significantly different to the levels observed in NT cells (50 ± 4 MT positive puncta/100 μm dendrite; $p > 0.05$, ANOVA, $n = 5$). Following expression of Flag-myoVI-DN, there was a significant decrease (~45%) in MT positive puncta compared with GFP-transfected neurons (25 ± 1/100 μm dendrite; $p < 0.01$, ANOVA, $n = 5$; Fig. 3b).

Flag-myoVI-DN decreases GluA1 levels at synapses

The reduction in AMPARs on the plasma membrane could therefore be simply caused by the reduction in the number of

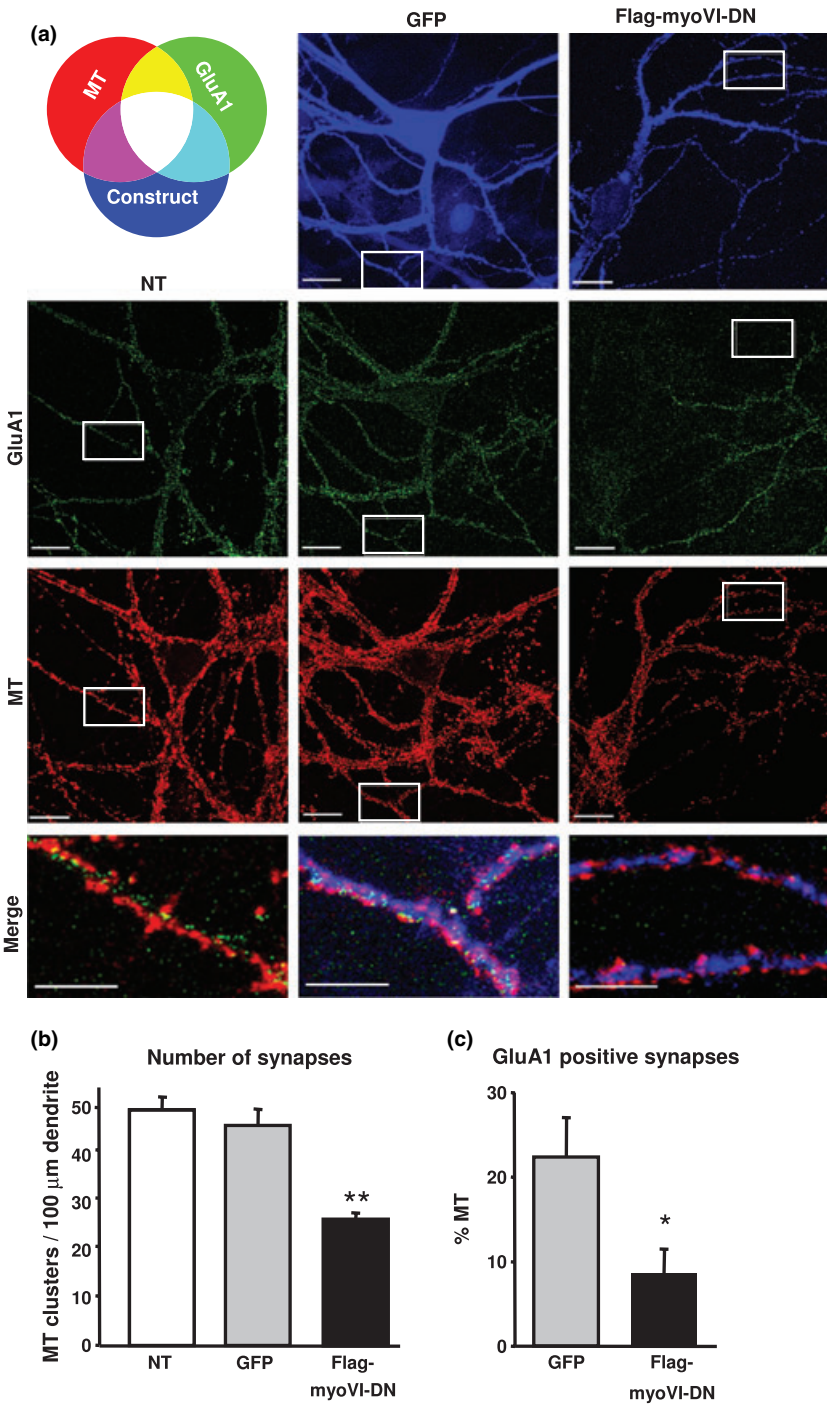

Fig. 3 Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN) causes a significant reduction in synapse number and GluA1 immuno-reactivity at synapses. (a) Non-transfected (NT), green fluorescent protein (GFP)-transfected and Flag-myoVI-DN transfected hippocampal neuronal cultures (21 DIV) were fixed and immuno-stained (prior to permeabilisation) with GluA1 extracellular N-terminal domain-specific rabbit antibody (green). Following permeabilisation of plasma membranes, neurons were re-labelled with mouse anti-flag antibody (blue, top right panel) and mitotracker (MT) (red). The Venn diagram illustrates the co-localisation patterns. Scale bars: 20 (top panels) and 5 μm (merged highlighted and enlarged regions). (b) Quantitative analysis revealed a significant decrease in the number of GluA1 positive puncta on the surface of dendrites, as well as a significantly reduced GluA1 immuno-reactivity in the remaining synapses (c) following expression of Flag-myoVI-DN compared with GFP. $^{**}p < 0.01$, $^{*}p < 0.05$, ANOVA, $n = 5$–8.

synapses. Alternatively, there could be an additional reduction in the number of AMPARs at the remaining synapses. To determine the effect of Flag-myoVI-DN on the expression of AMPARs at synapses, surface immuno-staining of GluA1 was followed by permeabilisation of the plasma membrane and MT labelling of synapses. In GFP-transfected neurons, 23 ± 5% of MT positive regions co-localised with surface GluA1 clusters (Fig. 3c, $p > 0.05$, ANOVA, $n = 6$). Flag-myoVI-DN significantly reduced the number of GluA1 immunopositive synapses to 8 ± 2% (Fig. 3c; $p < 0.02$, ANOVA, $n = 6$). Thus, Flag-myoVI-DN caused a significant reduction (63%) in GluA1 containing synapses labelled with MT.

Flag-myoVI-DN does not alter total expression level of AMPAR proteins

To investigate the possibility that the reduced surface expression of GluA1 and GluA2 is because of a reduction

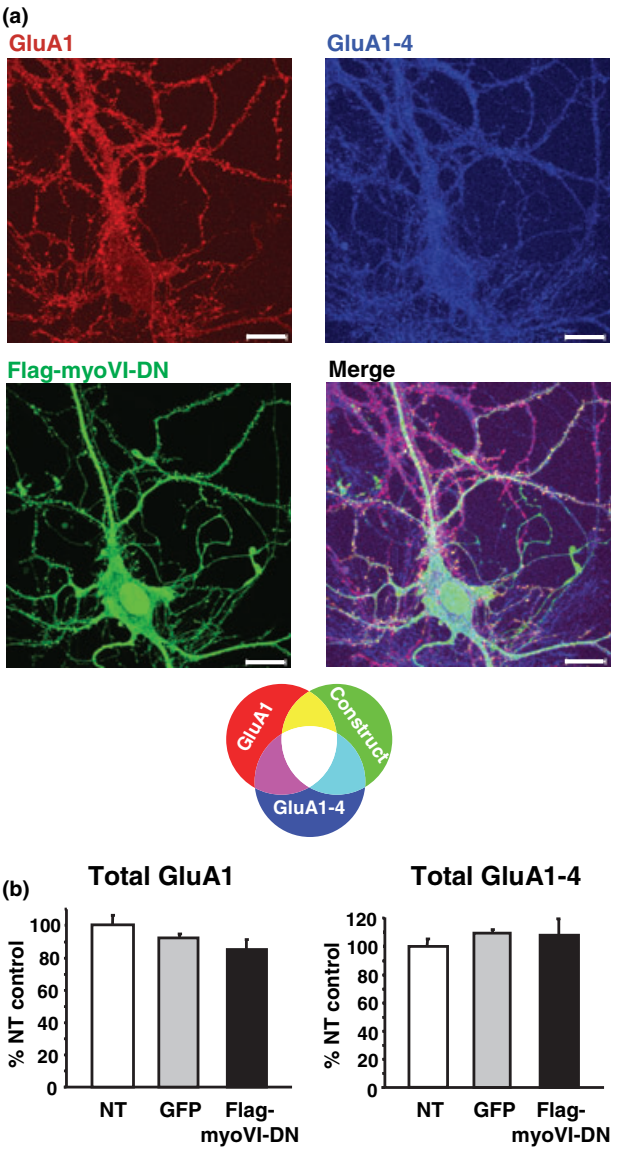

Fig. 4 Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN) does not interfere with AMPAR protein synthesis. Flag-myoVI-DN transfected hippocampal neurons (21 DIV) were fixed, permeabilised and immunostained with rabbit anti-GluA1 and guinea pig anti-GluA1–4 polyclonal and anti-Flag mouse monoclonal antibodies. The Venn diagram illustrates the co-localisation patterns. Scale bars: 10 μm. The total GluA1 and GluA1–4 immunoreactivities in Flag-myoVI-DN transfected permeabilised neurons were comparable with untransfected control neurons. Quantitative analysis (b) revealed no significant difference in total GluA1 or GluA1–4 immunoreactivities in Flag-myoVI-DN containing neurons. $p > 0.05$, ANOVA, $n = 5–8$.

in the synthesis of AMPAR subunit proteins, we immunolabelled NT and GFP or Flag-myoVI-DN transfected permeabilised hippocampal neurons with antibodies against GluA1 and GluA1-4 subunits to reveal the total AMPAR population (Fig. 4a). Expression of GFP or Flag-myoVI-DN had no apparent effect on the overall expression of GluA1 or net AMPAR subunit protein level compared with NT or GFP-transfected neurons [Fig. 4b; GluA1 immunoreactivity: NT, 100 ± 6% ($n = 8$); GFP-transfected, 92 ± 3% ($n = 5$); Flag-myoVI-DN transfected, 84 ± 6% ($n = 7$); GluA1-4 immunoreactivity: NT, 100 ± 5% ($n = 8$); GFP-transfected, 109 ± 3% ($n = 5$); Flag-myoVI-DN transfected, 107 ± 13% ($n = 7$); $p > 0.05$, ANOVA]. Thus, the Flag-myoVI-DN induced decrease in surface AMPAR population is not because of reduced AMPAR subunit protein synthesis.

Flag-myoVI-DN does not reduce NMDAR surface expression

To investigate possible changes in the cell surface expression of NMDARs, we immuno-labelled NT and GFP or Flag-myoVI-DN transfected non-permeabilised hippocampal neurons with an antibody against the GluN1 subunit protein (Fig. 5a). GluN1 is a good marker of the entire NMDAR population, because this subunit is an essential component of all known NMDAR hetero-oligomers (Molnár 2008). The punctuate distribution and density of anti-GluN1 labelling obtained in NT and GFP-transfected neuronal cultures was similar to our previous studies of NMDARs in hippocampal neurons (Fig. 5a; Noel *et al.* 1999; Pickard *et al.* 2000). Expression of GFP or Flag-myoVI-DN had no significant effect on overall surface GluN1 immunoreactivity compared with NT neurons [Fig. 5b; GluN1 immunoreactivity: NT, 100 ± 14%; GFP-transfected, 96 ± 8%; Flag-myoVI-DN transfected, 90 ± 7% ($n = 4$); $p > 0.05$, ANOVA]. These results indicate that the Flag-myoVI-DN induced decrease in surface AMPAR population is not accompanied by changes in NMDAR surface targeting. However, while GluN1 immunolabelling is clearly present on the surface of all neurons studied, distribution appears less punctate in Flag-myoVI-DN transfected neurons (Fig. 5a, enlarged regions). Quantitative analysis revealed a ~27% reduction in punctuate labelling for GluN1 [Fig. 5c; GluN1 immunopositive puncta/100 μm dendrite: NT, 10.1 ± 1.3; GFP-transfected, 10.2 ± 1.7; Flag-myoVI-DN transfected, 7.3 ± 1.6 ($n = 3$); $p > 0.05$, ANOVA]. This is consistent with the observed reduction in the number of synapses in Flag-myoVI-DN containing cells (Fig. 3b) and suggests that, because of the reduced number of synapses to populate, NMDARs accumulate on the plasma membrane of dendrites.

Flag-myoVI-DN blocks the NMDAR-dependent increase in AMPARs at synapses

Thus far, we have established that Flag-myoVI-DN significantly reduces the number of GluA1 and GluA2 subunits at synapses during basal activity, without altering AMPAR subunit protein expression or surface targeting of NMDARs in hippocampal neurons. Next, we investigated the effect of Flag-myoVI-DN on activity-induced recruitment of AMPARs to synapses. We have applied our previously developed

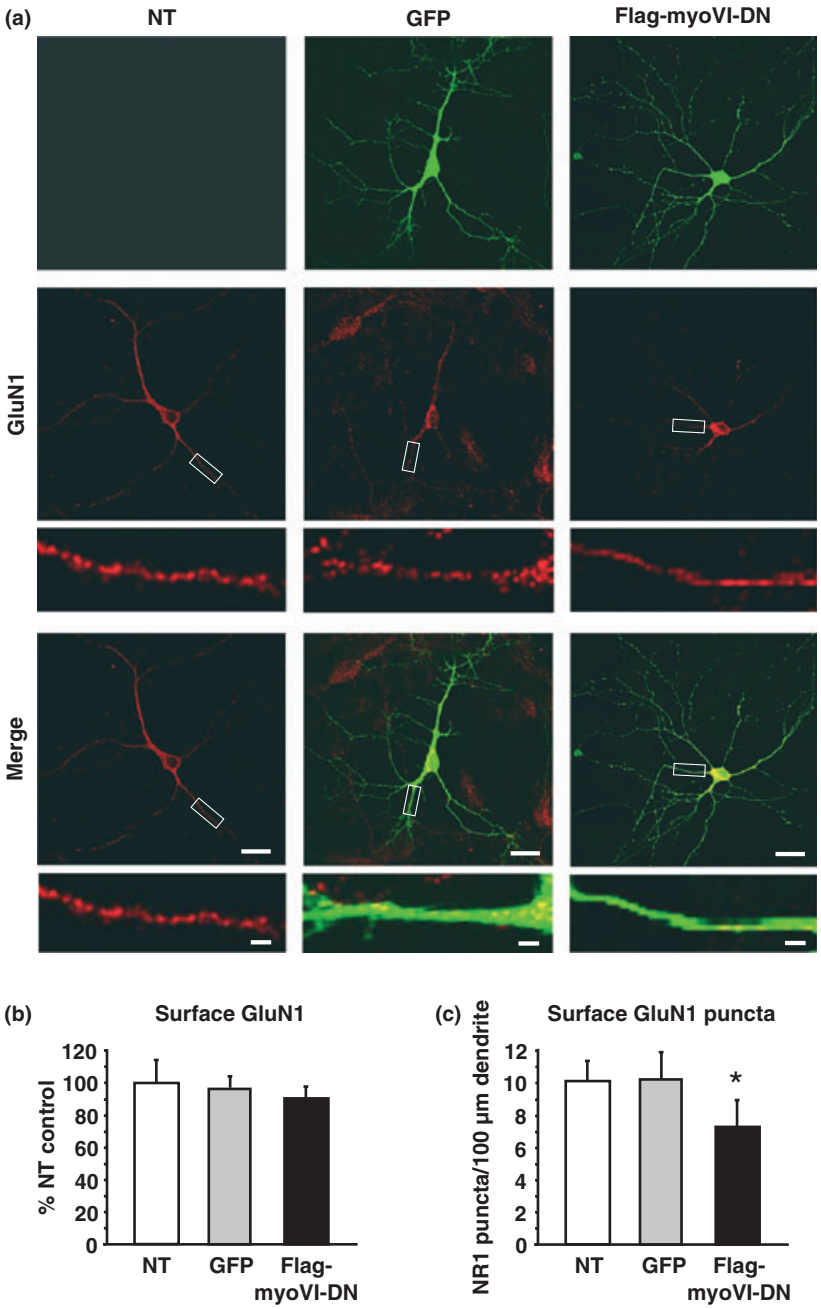

Fig. 5 Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN) does not alter total cell surface expression of NMDARs. (a) Non-transfected (NT), green fluorescent protein (GFP)-transfected (and Flag-myoVI-DN transfected hippocampal neurons (21 DIV) were fixed and immunostained (without permeabilisation) using a rabbit anti-GluN1 polyclonal antibody. Flag-myoVI-DN-containing cells (right panels) were identified by an anti-Flag mouse monoclonal antibody labelling following permeabilisation of anti-GluN1 stained neurons. Panels illustrate stacked confocal images of representative GluN1-labelled neurons. Scale bars: 10 (top panels) and 1 μm (highlighted and enlarged regions). Because of the homogeneous, less punctuate distribution of GluN1 in Flag-myoVI-DN transfected neurons surface immunolabelling was compared by quantification of pixel intensities in dendrites (b). Quantitative analysis revealed no significant differences in total GluN1 immunoreactivities at the cell surface in Flag-myoVI-DN expressing neurons. $p > 0.05$, ANOVA, $n = 4$. (c) There was a reduction in punctuate labelling of surface GluA1 following expression of Flag-myoVI-DN, which is consistent with the reduction in the number of synapses (Fig. 3). $^*p < 0.05$, ANOVA, $n = 3$.

procedure in which brief (3 × 1 s) transient depolarisation of cultured hippocampal neurons results in the rapid insertion of AMPARs at the plasma membrane (Pickard *et al.* 2001). The recruitment of new AMPARs from intracellular compartments to the cell surface is associated with an increase in synaptic transmission (Fitzjohn *et al.* 2001) and, like conventional LTP in hippocampal slices, requires the activation of NMDARs and an elevation of postsynaptic Ca^{2+} (Fitzjohn *et al.* 2001; Pickard *et al.* 2001). In this study, we performed interleaved experiments to compare the effects of transient depolarisation of NT and GFP or Flag-myoVI-DN transfected hippocampal neurons on AMPAR surface expression in the presence or absence of the NMDAR antagonist L-689,560 (5 μM; Pickard *et al.* 2001). The application of K^+ (90 mM; 3 × 1 s) caused an approximately 2.5-fold increase in the number of GluA1 immunopositive clusters at the cell surface in NT and GFP-transfected neurons ($p < 0.01$, ANOVA) compared with neurons treated with L-689,560 (Fig. 6). Furthermore, there was a significant increase in the percentage of GluA1 immunopositive synapses in NT (1.8-fold increase, $p < 0.05$, ANOVA, $n = 6$) and GFP-transfected (2.4-fold increase, $p < 0.05$, ANOVA, $n = 6$) primary hippocampal

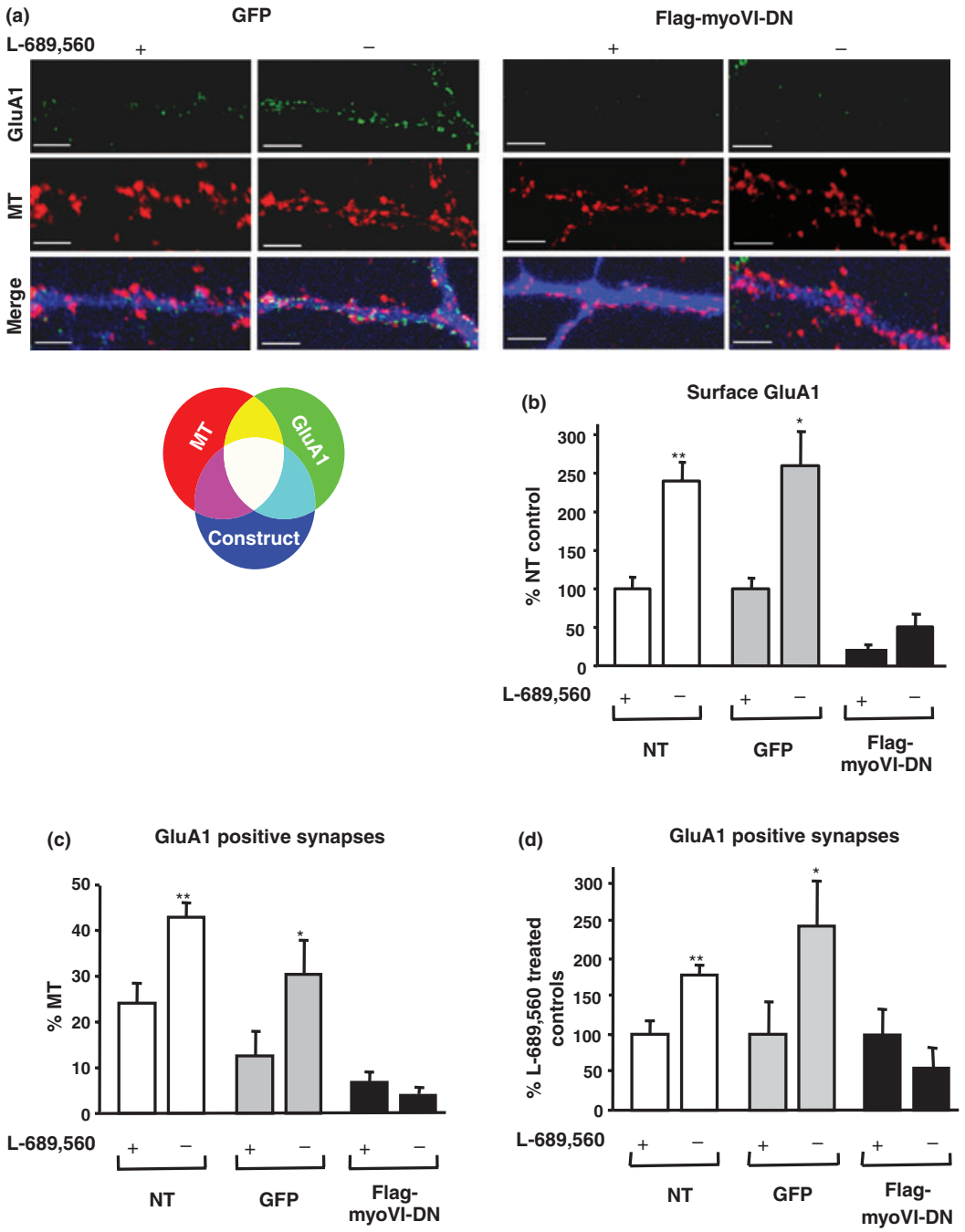

Fig. 6 Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN) blocks the NMDAR-dependent increase in cell surface GluA1 immunoreactivity at synapses. (a) Green fluorescent protein (GFP) (left panels) and Flag-myoVI-DN (right panels) transfected neurons were depolarised with 3 × 1 s pulses of 90 mM KCl in the presence (+) and in the absence (–) of 5 μM L-689,560 (NMDAR antagonist). Following exposure to high [K^+], non-permeabilised neurons were immuno-stained with GluA1 extracellular N-terminal domain-specific antibodies (green). After permeabilisation of plasma membranes, neurons were stained with mitotracker (MT, red) and anti-Flag. The Venn diagram illustrates the co-localisation patterns. Scale bars: 5 μm. Quantitative analysis revealed a significant increase in the number of GluA1 positive clusters at the cell surface along dendrites (b) and a similar increase in the number of synapses immunopositive for GluA1 (c, d) following 90 mM KCl treatment in non-transfected (NT) or GFP-transfected cells compared with corresponding control cells (i.e. 90 mM KCl treatment in the presence of L-689,560). (c) Percentage of MT-positive synapses also positive for GluA1. (d) Percentage change in the number of GluA1-positive synapses compared with the corresponding L-689,560-treated control groups. The increased cell surface and synaptic expression of GluA1 was prevented by Flag-myoVI-DN (b–d). *$p < 0.05$, **$p < 0.01$, ANOVA, $n = 6$ versus corresponding control.

cultures compared with neurons exposed to K^+ (90 mM; 3 × 1 s) in the presence of the NMDAR antagonist L-689,560 (Fig. 6). In interleaved experiments, there was no significant increase in GluA1 surface (Fig. 6b) or synaptic (Fig. 6c and d) recruitment in Flag-myoVI-DN transfected sister hippocampal neurons. These results show that activity-induced, NMDAR-dependent surface/synaptic recruitment of GluA1-containing AMPARs is dramatically impaired in the presence of Flag-myoVI-DN.

Flag-myoVI-DN blocks the activity-induced increase in mEPSC frequency

Previously we have established that activity-dependent recruitment of AMPARs from intracellular compartments to the cell surface (Pickard *et al.* 2001) is associated with an increase in the frequency of mEPSCs (Fitzjohn *et al.* 2001). To investigate whether Flag-myoVI-DN blocked this increase in synaptic activity, which would be consistent with its effect on the activity-dependent increase in AMPAR surface expression detected using immunocytochemistry, we performed whole cell recordings from sister cultures in parallel with the immunocytochemical experiments. Flag-myoVI-DN caused a decrease in spontaneous synaptic activity, as shown by an increase in the inter-mini-interval frequency of EPSCs compared with the control (GFP; Fig. 7). Exposure of cells expressing GFP to high K^+ caused an approximately twofold increase in the frequency of mEPSCs compared with baseline (Fig. 7, $*p < 0.05$, ANOVA, with Tukey multiple comparisons test *post hock*, $n = 5–6$). The increase in the frequency of mEPSCs persisted for at least 30 min, and was not associated with a change in mEPSC amplitude (Fitzjohn *et al.* 2001; Fig. 7). In contrast, Flag-myoVI-DN expression prevented the high K^+ -induced increase in mEPSC frequency. Indeed, there was a tendency for mEPSC frequency to be reduced,

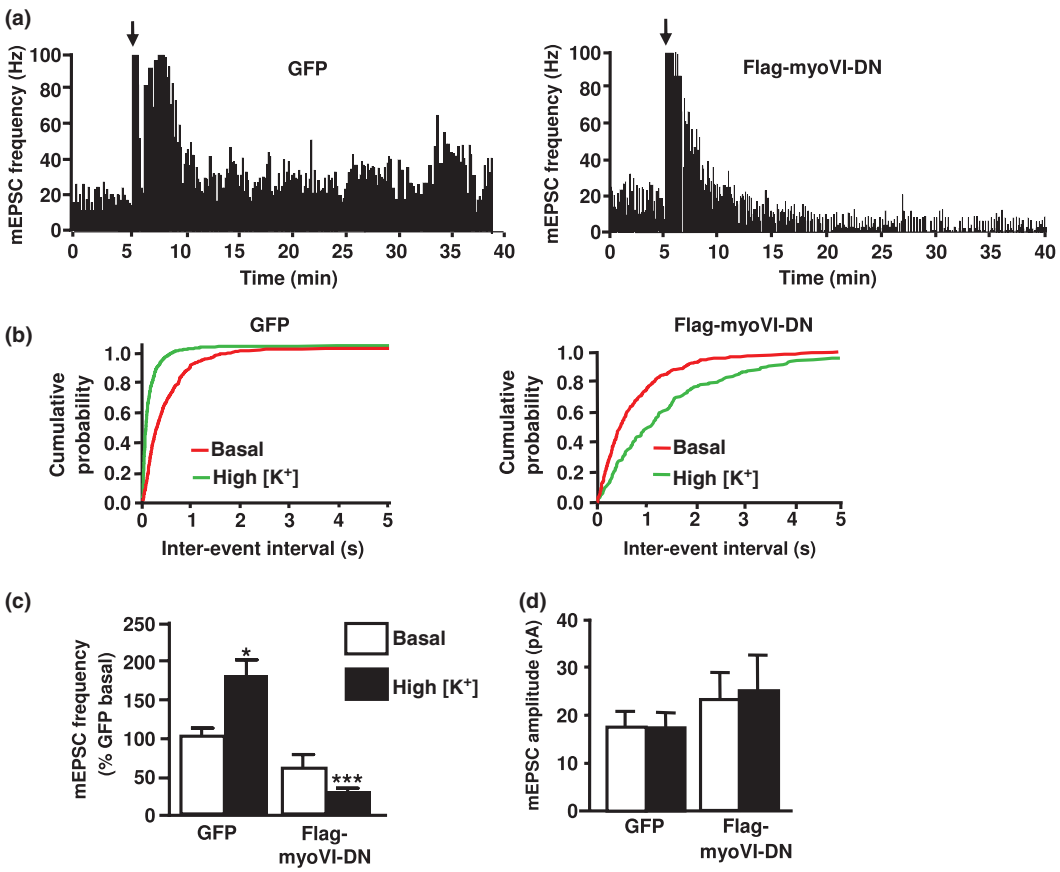

Fig. 7 Flag epitope-tagged truncated C-terminal cargo domain of myosin VI (Flag-myoVI-DN) blocks depolaristion-induced increase in synaptic activity in hippocampal neurons. (a) Representative experiments showing mEPSC frequency in neurons expressing green fluorescent protein (GFP) or Flag-myoVI-DN. Transient depolarisation with high K^+ (90 mM, 3 × 1 s, 10 ms interval) was delivered at the point indicated by the arrow. (c) Cumulative probability plots showing a shift to shorter inter-event intervals (i.e. increase in mEPSC frequency) following high K^+ application in a GFP expressing neuron, but a shift to longer intervals in a Flag-myoVI-DN expressing neuron. Pooled data for mEPSC frequency (c) and amplitude (d) for GFP and Flag-myoVI-DN expressing neurons ($n = 6$ for each group). Baseline was taken at time 0–5 min. Frequency following K^+ treatment was taken at time 30–40 min. Whole cell recordings were performed in the presence of 0.5 μM tetrodotoxin. $*p < 0.05$, $***p < 0.001$, ANOVA with Tukey multiple comparisons test *post hoc*, $n = 5–6$ for each group.

with a significant difference between control and Flag-myoVI-DN expressing cells following exposure to high K^+ (***$p < 0.001$, ANOVA, with Tukey multiple comparisons test *post hoc*, $n = 5–6$).

Discussion

In this study, we have found that expression of a dominant negative construct (Flag-myoVI-DN) that inhibits the interaction between SAP97 and myosin VI causes a dramatic reduction in the number of synapses and surface expression of AMPARs at synapses in cultured hippocampal neurons. We also found that this treatment prevents the rapid delivery of synaptic AMPARs that can be induced by the transient synaptic activation of NMDARs.

Effects on synapse number and AMPARs under basal conditions

A previous study, using a similar dominant negative truncated myosin VI construct, found a substantial reduction in the number of synapses (Osterweil *et al.* 2005). We observed a similar decrease in synapse number (Fig. 3b). The dominant negative construct that we used (Flag-myoVI-DN) was designed to disrupt the interaction between myosin VI and SAP97. Both this construct and a similar one used by Osterweil *et al.* (2005) are able to function in this capacity. Furthermore, a reduction in synapse number is also observed in the myosin VI knockout (Osterweil *et al.* 2005), which supports the idea that the effect is due to inhibition of normal myosin VI function.

In this study, we showed that the reduction in synapse number was not sufficient to account for the decrease in surface AMPARs because we observed a substantial reduction of AMPARs expressed at remaining MT-positive sites in Flag-myoVI-DN expressing neurons (Fig. 3c). The simplest explanation for these observations is that the construct reduces the surface expression and synaptic targeting of AMPARs. Indeed, the reduction in synapse number could be a secondary consequence of this effect (Hanley 2008). It has been shown that the synaptic targeting of SAP97 is necessary for proper dendritic maturation (Zhou *et al.* 2008). Therefore, by inhibiting the interaction between myosin VI and SAP97, and hence the membrane delivery of SAP97, Flag-myoVI-DN could affect synapse stability.

Effects on activity-dependent trafficking of AMPARs

Previous studies established that SAP97, myosin VI and GluA1 play an important role in endocytosis of GluA1-containing AMPARs (Osterweil *et al.* 2005). In this study, we have investigated whether these proteins are also involved in the activity-dependent synaptic delivery of AMPARs. We used a method which involves the transient depolarisation of cultured hippocampal neurons (Fitzjohn *et al.* 2001; Pickard *et al.* 2001). This method was developed to enable the subcellular distribution of native AMPARs to be monitored using antibodies that were raised against extracellular epitopes (Molnár *et al.* 1993; Richmond *et al.* 1996; Vissavajjhala *et al.* 1996; Pickard *et al.* 2000, 2001). Like conventional LTP, studied in more intact brain preparations, this neuronal culture model of rapid AMPAR trafficking requires the activation of NMDARs and an elevation of postsynaptic Ca^{2+} (Fitzjohn *et al.* 2001). It probably recapitulates one aspect of LTP, namely the 'unsilencing' of 'silent' synapses. Thus, the effect is observed both as the insertion of AMPAR clusters at sites that lack detectable AMPARs (Pickard *et al.* 2001) and as an increase in mEPSC frequency, with no change in mEPSC amplitude (Fitzjohn *et al.* 2001).

The ability of the dominant negative Flag-myoVI-DN to inhibit both the appearance of new AMPAR clusters and the increase in mEPSC frequency in response to transient depolarisation suggests that it is indeed interfering with the 'unsilencing' of 'silent' synapses. Flag-myoVI-DN therefore is an important molecular tool for exploring this component of LTP. Precisely how it functions in this regard remains a matter of speculation.

Possible sites of action of Flag-myoVI-DN

Myosin VI is an unusual member of the myosin family in that it is a minus-end directed motor protein (Wells *et al.* 1999). Usually in cells, the minus ends of cytoskeletal components are pointed inwards, thus, it would seem surprising if disruption of myosin VI function interferes with the synaptic delivery of AMPARs in mature dendrites. However, in mature dendrites, the polarity of cytoskeletal elements is not uniform, so it is possible that Flag-myoVI-DN is disrupting synaptic delivery of AMPARs (Baas *et al.* 1988, 1989). Alternatively, Flag-myoVI-DN might disrupt myosin VI-mediated delivery of AMPARs from early endosomes to recycling endosomes. In this context, lemur tyrosine kinase 2 is a serine/threonine-specific protein kinase, that binds to the WWY domain positioned at amino acids 1184 on the C-terminus of myosin VI, enabling the transition of endocytosed vesicles from early endosomes to recycling endosomes (Chibalina *et al.* 2007; Inoue *et al.* 2008). If lemur tyrosine kinase 2 sub serves this role in hippocampal neurons, then expression of Flag-myoVI-DN may prevent recycling of GluA1 receptors from early endosomes to the reserve pool in dendritic shafts. This could then explain the decrease in the number of GluA1-containing AMPARs at the dendritic membrane, and the inhibition of their activity-dependent delivery. In this way, myosin VI could act in concert with myosin V, which appears to be involved in the trafficking of AMPARs into dendritic spines (Correia *et al.* 2008; Wang *et al.* 2008).

By binding to SAP97, the dominant negative Flag-myoVI-DN construct is likely to prevent the binding of other proteins that interact with this molecule, in addition to interfering with its interaction with myosin VI. SAP97

regulates several aspects of AMPAR function, via binding to GluA1 at its PDZ2 domain, which include trafficking to the synapse (Rumbaugh *et al.* 2003; Nakagawa *et al.* 2004), stabilisation at the membrane (Waites *et al.* 2009), retention in the biosynthetic pathway (Sans *et al.* 2001) and modulation of downstream signalling by interacting with adaptor proteins such as A-kinase anchoring protein (AKAP) (Dell'Acqua *et al.* 2006). The first 65 amino acids of the N-terminus of βSAP97 contain an N-L27 domain (Fig. 1a) which multimerises either with itself, or to other L27 domain containing proteins such as CASK, Lin2 and Mint-1 (Lee *et al.* 2002a; Leonoudakis *et al.* 2004). These proteins are important for anchoring at the cytoskeleton (Lee *et al.* 2002a; Leonoudakis *et al.* 2004). Recent studies have shown that the β-isoform of SAP97 resides at extra synaptic locations on the dendritic surface, and that it is important for maintaining GluA1-containing AMPARs in a reserve pool at the dendrite that are cycled to the membrane following induction of LTP (Schluter *et al.* 2006; Waites *et al.* 2009). Thus, the dominant negative effect of Flag-myoVI-DN on SAP97 and AMPARs with respect to membrane localisation may be caused by inhibition of the binding of the N terminus of SAP97 to L27 containing proteins associated with the cytoskeleton, preventing stabilisation. In this context, it has been shown that phosphorylation of serine 39 within the L27 domain of the N terminus (S_{39}; Fig. 1a) is required for the delivery of both SAP97 and GluA1 to the dendritic membrane (Mauceri *et al.* 2004), and that mutation of this residue results in a diffuse staining pattern of these two proteins. Thus, Flag-myoVI-DN may bind to the N terminus of SAP97, masking the serine 39 residue (Fig. 1a), so preventing Ca^{2+}/calmodulin-dependent protein kinase II-mediated phosphorylation, and disrupting SAP97 and GluA1 localisation at the membrane. If expression of Flag-myoVI-DN blocked the function of the N terminus of SAP97 by inhibiting any of these mechanisms, it would result in a decrease in the number of GluA1 at the membrane as observed here. Given that targeting of GluA1 and GluA2 containing AMPARs is driven by GluA1 (Hayashi *et al.* 2000), this would also explain the parallel decrease in the levels of GluA2.

It is plausible that myosin VI and/or SAP97 regulate the trafficking of neurotransmitter receptors other than AMPARs. For example, Ca^{2+}/calmodulin-dependent protein kinase II-dependent differential phosphorylation of SAP97-Ser29/Ser232 appears to be involved in the trafficking and plasma membrane insertion of GluN2A subunit-containing NMDARs (Mauceri *et al.* 2007). However, it has also been shown that myosin VI and NMDARs do not form a complex despite the presence of SAP97 (Osterweil *et al.* 2005). This is consistent with the observation that Flag-myoVI-DN expression does not reduce NMDAR surface expression (Fig. 5). Therefore, it is unlikely that Flag-myoVI-DN expression-related changes in synaptic activity observed in this study are caused by the lack of NMDARs on the cell surface. The less punctuate distribution of GluN1 in Flag-myoVI-DN transfected neurons reported in this study is consistent with the observed reduction in the number of synapses and suggests an increase in the extra-synaptic NMDAR population.

Concluding remarks

We have identified a molecular tool that interferes with the synaptic targeting of AMPARs under basal and activity-dependent conditions. In particular, it blocks the NMDAR-dependent delivery of AMPARs to 'silent' synapses. This tool interferes with the motor protein, myosin VI and the membrane-associated guanylate kinase, SAP97. These proteins form a complex with the GluA1 subunit of the AMPAR. These data therefore support the idea that myosin VI and SAP97 have key roles in the trafficking of AMPARs at synapses.

Acknowledgements

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