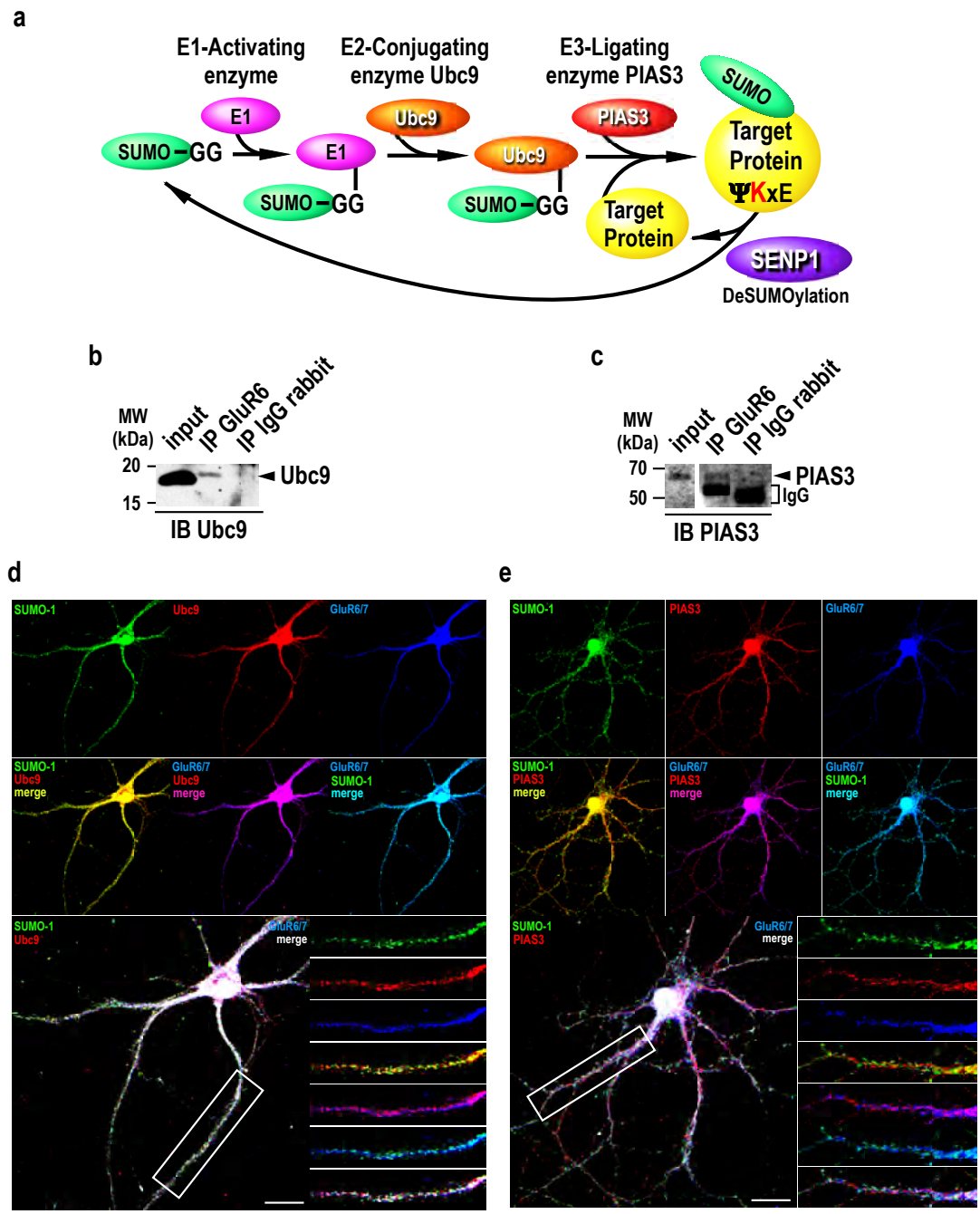
Supplementary figure 1



Supplementary Figure 1: SUMOylation pathway and distributions of E2 and E3 enzymes in cultured hippocampal neurones.

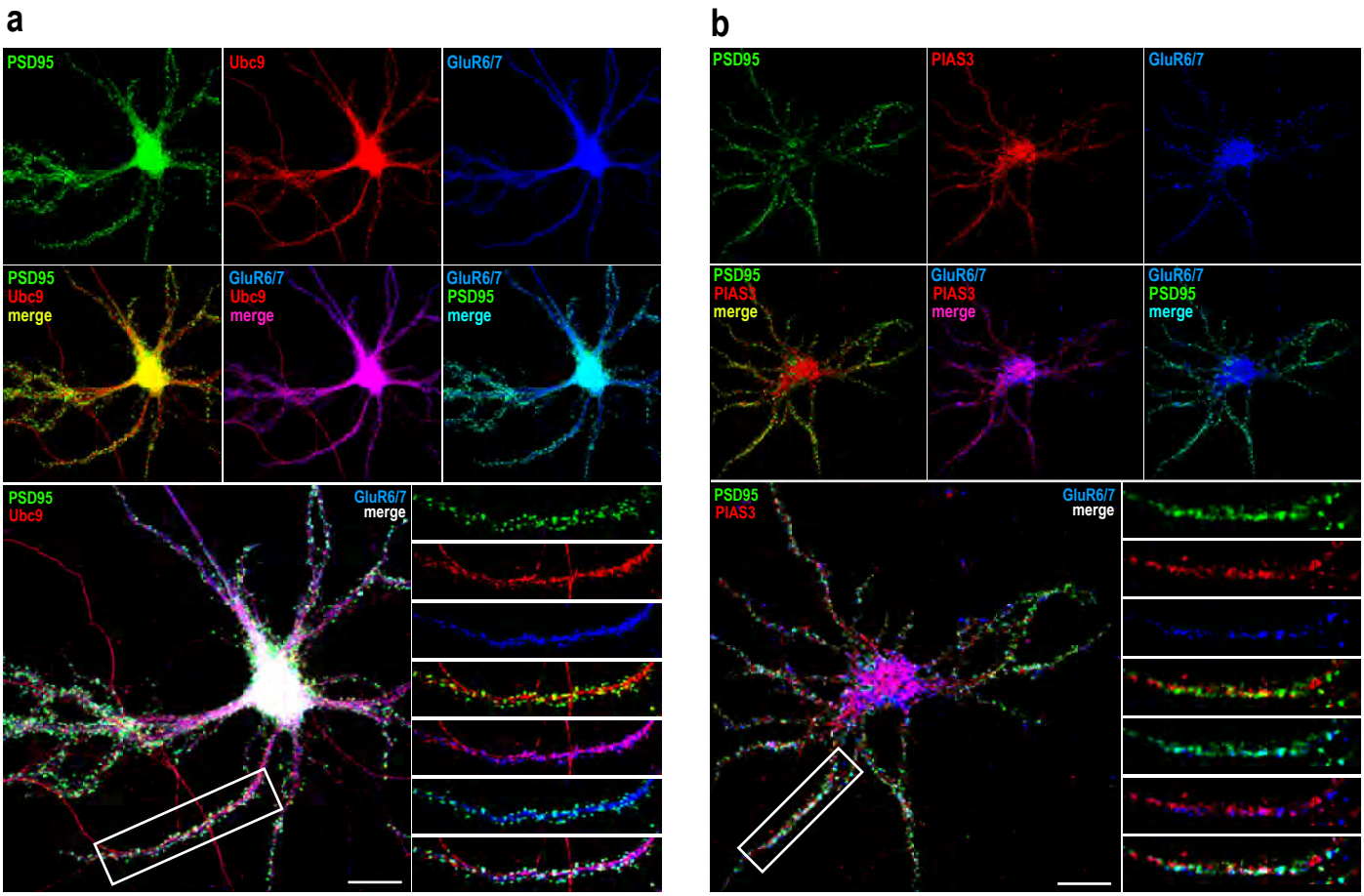
(a) Schematic of the SUMOylation pathway and some of the enzymes involved.

(b) The SUMO E2 enzyme Ubc9 co-immunoprecipitates with GluR6 from cultured hippocampal neuronal detergent extracts.

(c) The SUMO E3 PIAS3 co-immunoprecipitates with GluR6 from cultured hippocampal neuronal detergent extracts.

(d,e) Distribution and colocalisation of SUMO-1, GluR6 and Ubc9 (d) and SUMO-1, GluR6 and PIAS3 (e) in cultured hippocampal neurones. White denotes colocalisation of all three proteins. In all cases the data are representative of at least 3 separate experiments.

Supplementary figure 2



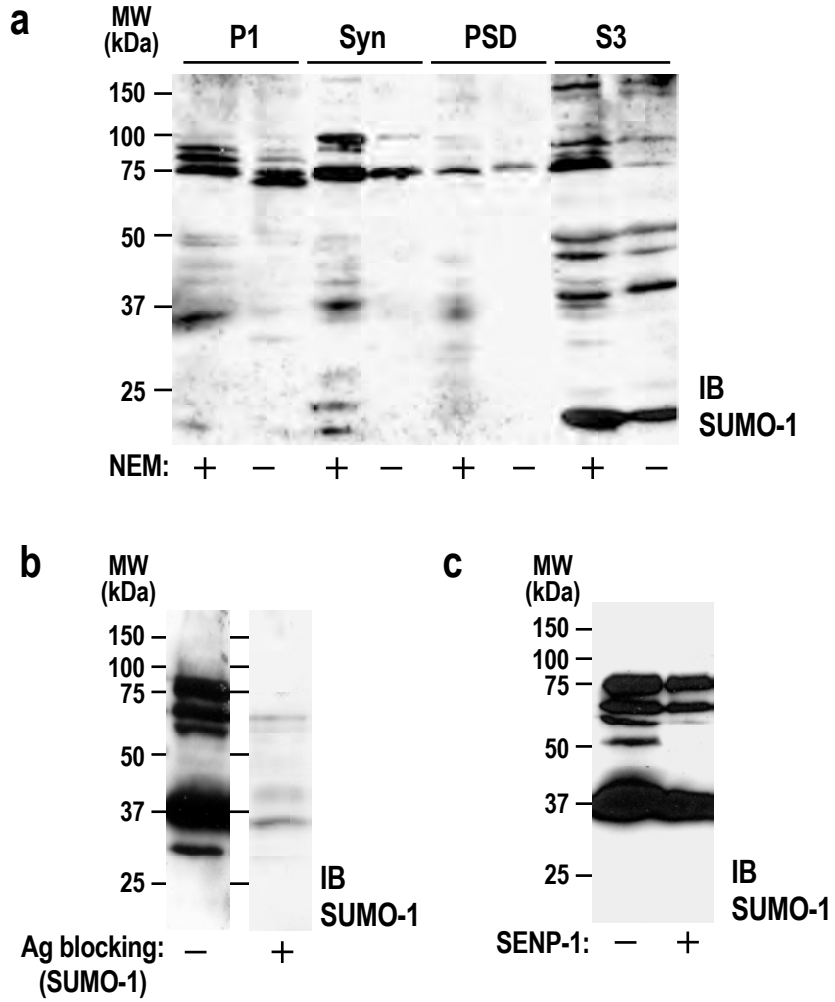
Supplementary Figure 2: Ubc9 and PIAS3 are present at synapses.

(a) Immunocytochemical distributions and colocalisation of Ubc9 and GluR6 with the synaptic marker PSD95 in cultured hippocampal neurones (DIV 21). White denotes co-localisation of all three proteins.

(b) Immunocytochemical distributions and colocalisation of PIAS3 and GluR6 with the synaptic marker PSD95 in cultured hippocampal neurones (DIV 21). White denotes colocalisation of all three proteins.

For all panels the data are representative of at least 3 separate experiments.

Supplementary figure 3



Supplementary Figure 3: Effects of NEM on SUMO-1 conjugated proteins and immunoblot antigen controls.

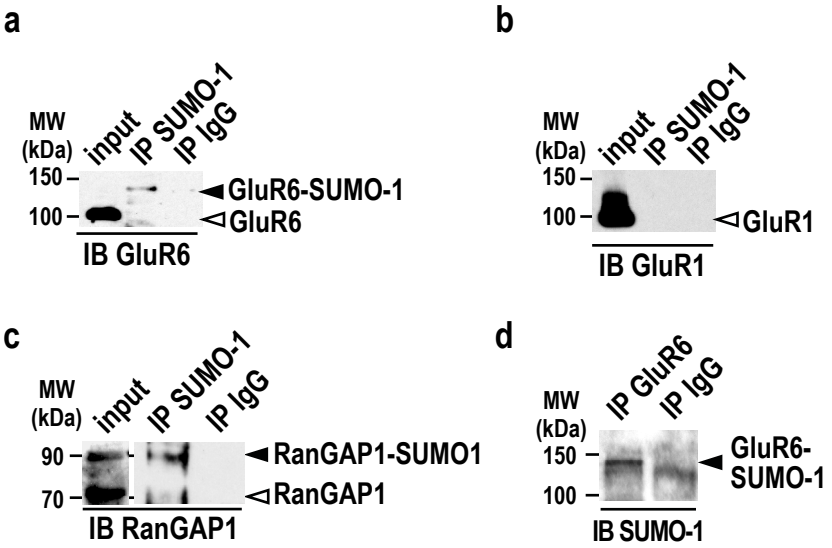
(a) Levels of detectable SUMO-1 conjugated proteins are decreased in the nuclear (P1), synaptic (SYN), postsynaptic density (PSD) and cytosolic (S3) fractions in the absence of the cysteine protease inhibitor NEM. Note the blots were loaded and exposed for optimal resolution of discrete protein bands in the + NEM conditions.

(b) Preabsorption with recombinant SUMO-1 (antigen, Ag) verifies that the anti-SUMO antibody specifically recognises SUMO-1-conjugated proteins in the P1 fraction.

(c) Incubation of the P1 fraction with SENP-1 reduces the levels of some detectable SUMO-1 conjugated proteins. Note, consistent with previous report [9] some SUMOylated proteins are not sensitive to cysteine proteases.

All blots are representative of at least 3 separate experiments.

Supplementary figure 4



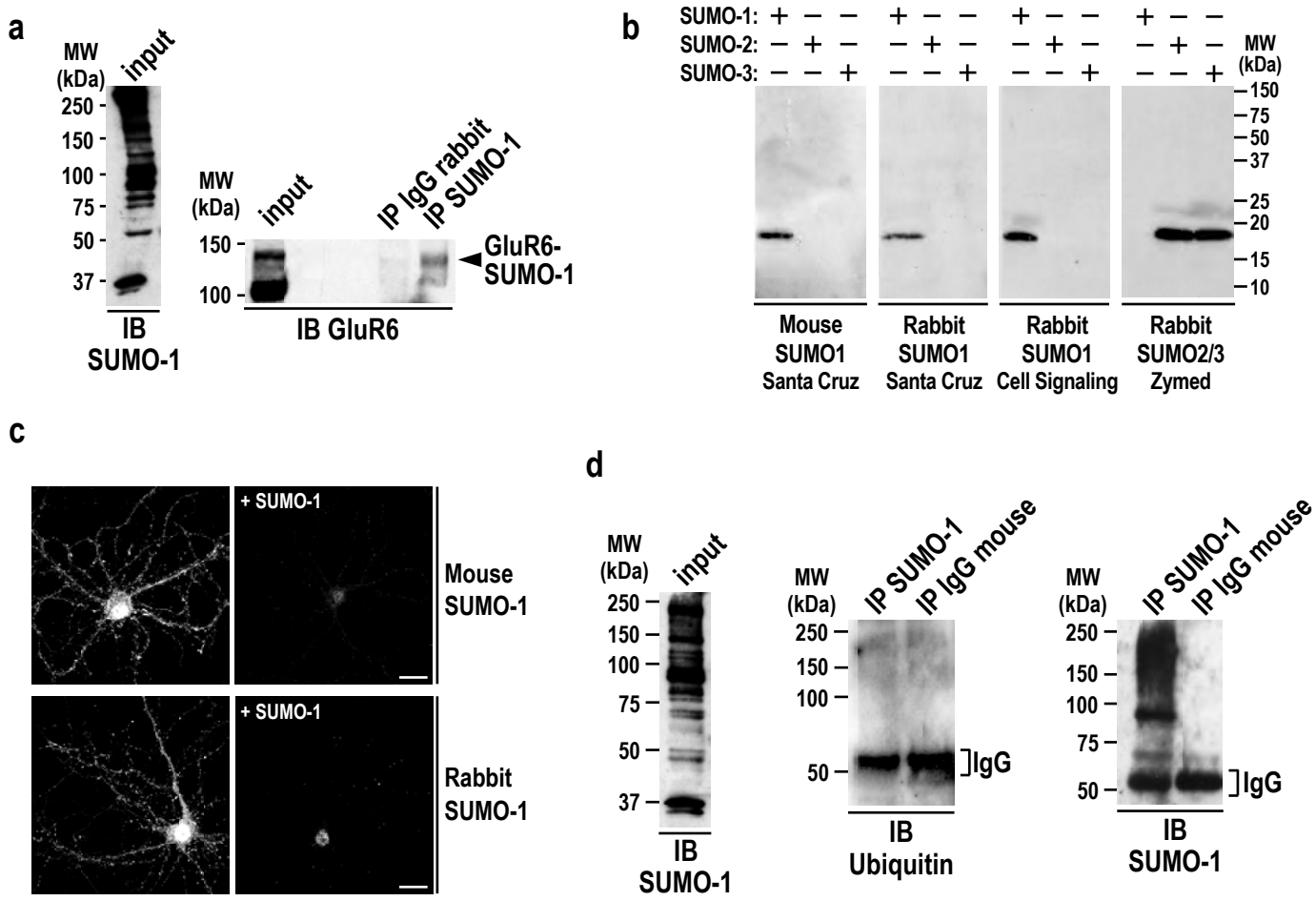
Supplementary Figure 4: Positive and negative controls for SUMO-1 Immunoprecipitation.

Control co-immunoprecipitation experiments confirming that SUMO-1 immunoprecipitate contains GluR6 (a) and RanGAP1 (c) but does not contain the AMPAR subunit GluR1 (b).

(d) Immunoprecipitation with anti-GluR6 antibody and blotted with anti-SUMO-1 antibody. Note the lower diffuse band in the IgG lane is non-specific immunoreactivity.

The data are representative of at least 3 separate experiments for each blot.

Supplementary figure 5



Supplementary Figure 5: Anti-SUMO-1 antibody specificity controls.

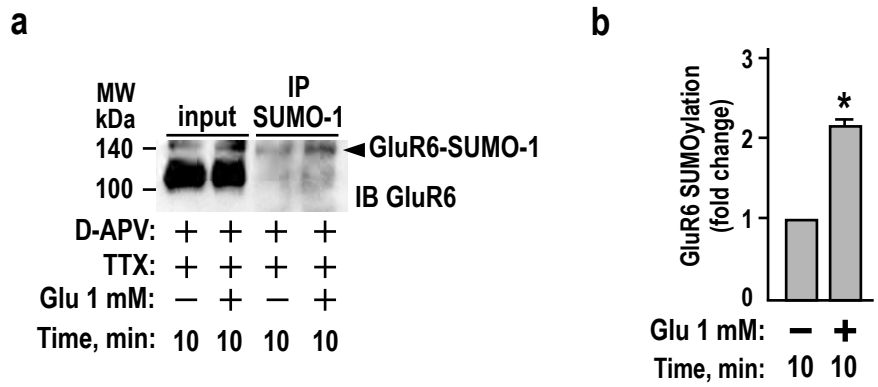
(a) Left panel: Immunoblot with polyclonal rabbit anti-SUMO-1 antibody from Cell Signaling demonstrating multiple SUMO-1 conjugated proteins in cultured hippocampal neurones (21 DIV) in the presence of 20 mM NEM. Right panel: SUMO-1 GluR6 is specifically immunoprecipitated by rabbit anti-SUMO-1 antibody and not by control rabbit IgG.

(b) Specificity of anti-SUMO-1 monoclonal and polyclonal antibodies against recombinant SUMO-1, SUMO-2 and SUMO-3.

(c) Preabsorption with recombinant SUMO-1 blocks immunocytochemical detection of endogenous SUMO-1 and SUMOylated proteins in permeabilised cultured hippocampal neurones by both mouse monoclonal and rabbit polyclonal anti-SUMO-1 antibodies.

(d) SUMO-1 immunoprecipitate does not contain ubiquitinated protein.
The data are representative of at least 3 separate experiments for each blot.

Supplementary figure 6

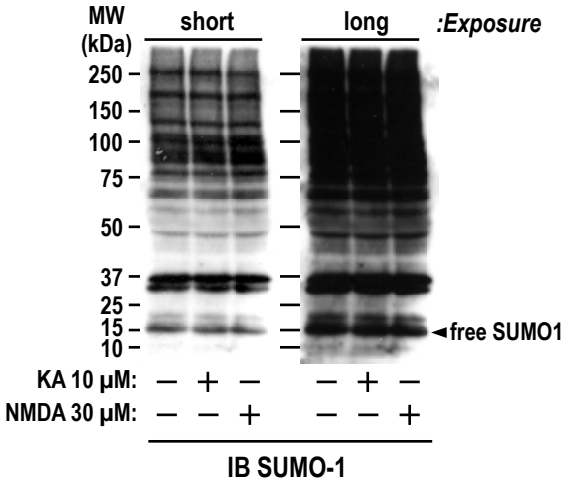


Supplementary Figure 6: Glutamate evokes GluR6 SUMOylation.

(a) Application of the endogenous agonist glutamate (1 mM, 10 min) evokes SUMOylation of GluR6.

(b) Quantification of the data represented in (a), n = 3 separate experiments. * $p < 0.05$

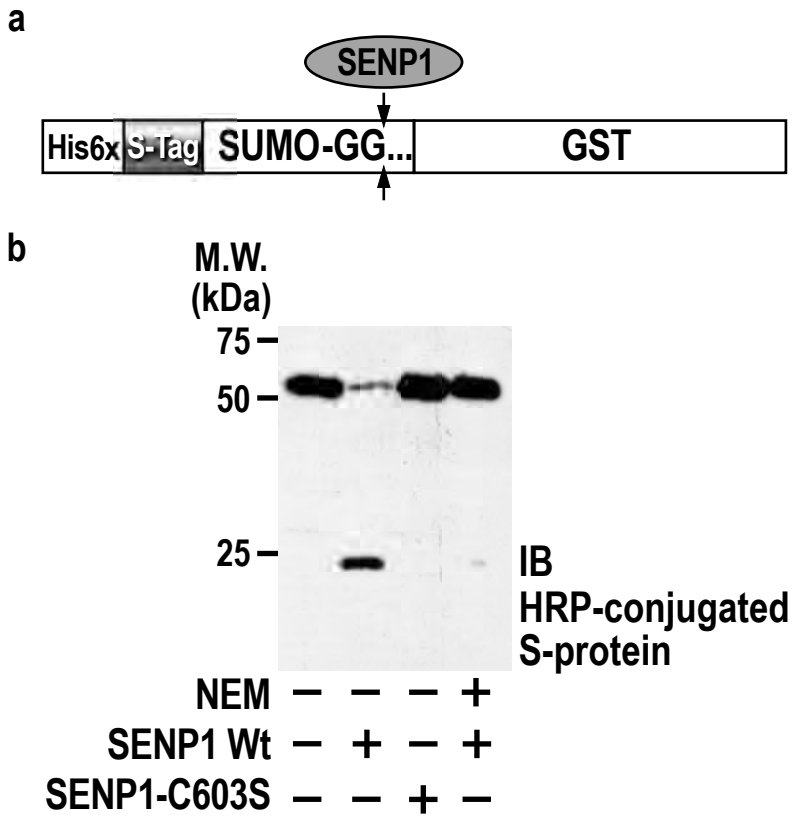
Supplementary figure 7



Supplementary Figure 7: Effect of KA and NMDA application on total protein SUMOylation.

Multiple SUMO-1 conjugated proteins are present in cultured hippocampal neurones (21 DIV). The hippocampal neurones were stimulated for 10 min with kainate (10 μM) or NMDA (30 μM) and lysed in the presence of 20 mM NEM. Application of either kainate or NMDA does not markedly alter the levels of total protein SUMOylation although there are some subtle differences.

Supplementary figure 8

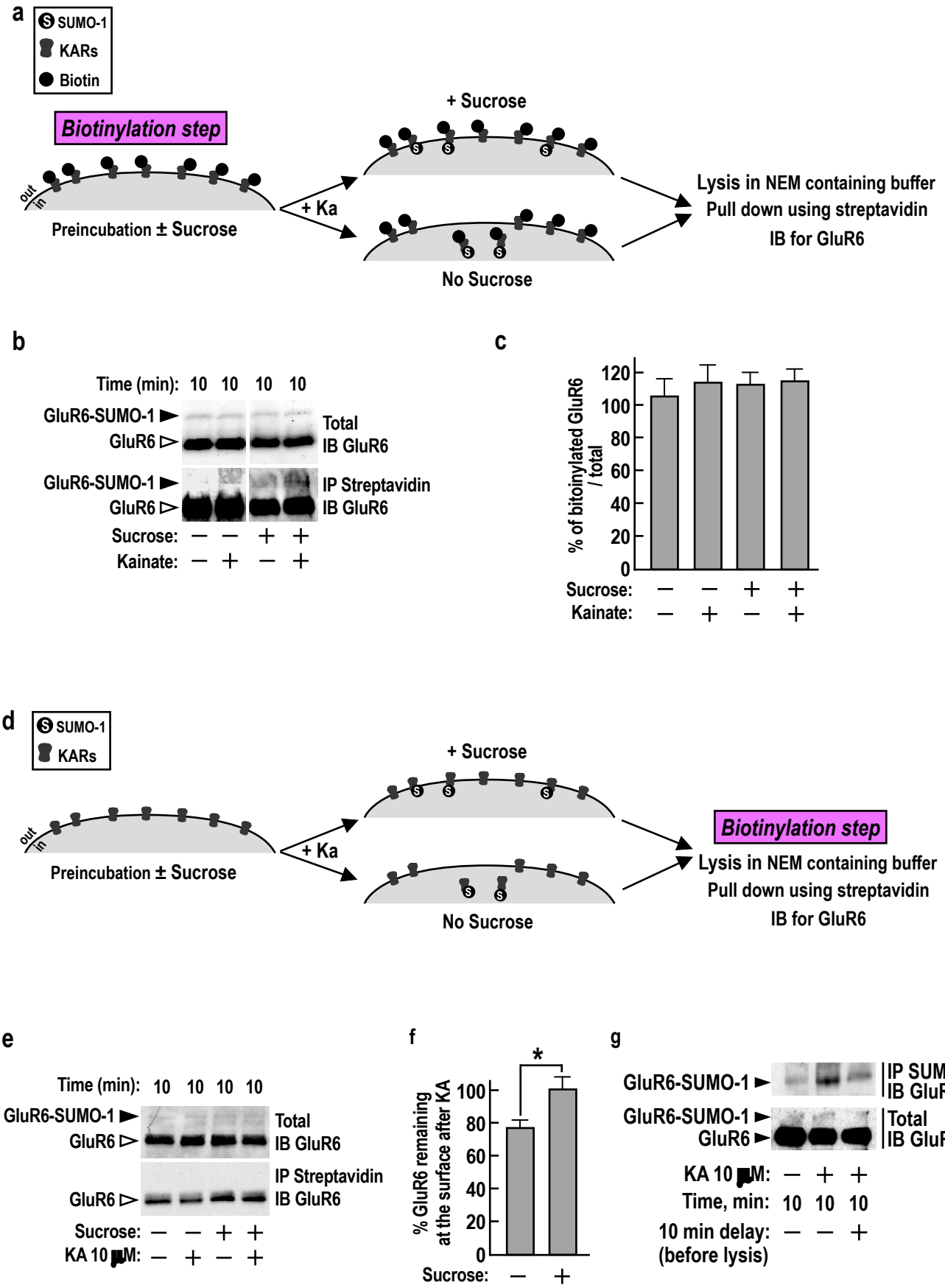


Supplementary Figure 8: Construct for in vitro assay for SENP-1 activity.

(a) Schematic structure of His6x-S-tag-SUMO1-GST substrate and the SENP-1 cleavage site.

(b) Demonstration of the specific action of recombinant SENP-1 and inhibition in the presence of NEM. His6x-S-tag-SUMO1-GST substrate (1 μM) was incubated with 75 nM of WT or mutant recombinant SENP-1 at 30^{o}C for 2 hours. After separation on a 15% SDS-PAGE gel proteins were transferred onto PVDF membrane and probed with HRP conjugated S-protein.

Supplementary figure 9



Supplementary figure 9 : SUMOylation of GluR6 occurs at the plasma membrane.

(a) Schematic representation of experiment shown in b. Live cultured hippocampal neurones (21-23 DIV) were preincubated for 30 min at 37°C in Earle‘s buffer with or without 0.45M sucrose to block endocytosis. Surface proteins were then biotinylated on ice for 10 min with the membrane impermeant Sulfo-NHS-LC-Biotin (0.3 mg/ml) in PBS. Following extensive washes in Earle’s Buffer, neurones were incubated in Earle‘s buffer with or without 0.45 M sucrose in the presence or absence of 10 µM kainate for 10 min at 37°C. After immediate lysis in the presence of 20 mM NEM, solubilized proteins were precipitated using streptavidin beads, separated on SDS-PAGE and then immunoblotted for GluR6.

(b) The SUMOylated form of biotinylated GluR6 is also visible upon stimulation with kainate in the presence of the internalisation blocker sucrose indicating that agonist induced GluR6 SUMOylation can occur at the plasma membrane.

(c) The stability of GluR6 is not effected during the time course of the experiment. Histograms show means ± s.e.m. obtained from b and demonstrate that there is no significant degradation of GluR6. Data are representative of 4 independent experiments. To ensure the signal shown in (b) did not result from internalised SUMOylated GluR6 we performed the following experiment to show that hyperosmolar sucrose treatment prevents kainate induced GluR6 endocytosis.

(d) Schematic representation of the experiment shown in e. Live cultured hippocampal neurones (21-23 DIV) were preincubated for 30 min at 37°C in Earle’s buffer with or without 0.45 M sucrose to block endocytosis. Neurones were then incubated in the same buffer with or without 10 µM kainate for 10 min at 37°C. Surface proteins were then biotinylated on ice for 10 min with the membrane impermeant Sulfo-NHS-LC-Biotin (0.3 mg/ml) in PBS. After lysis in the presence of 20 mM NEM, surface solubilized proteins were precipitated using streptavidin beads, resolved on SDS-PAGE and finally immunoblotted for GluR6 to measure the amount of GluR6 remaining at the surface after kainate stimulation.

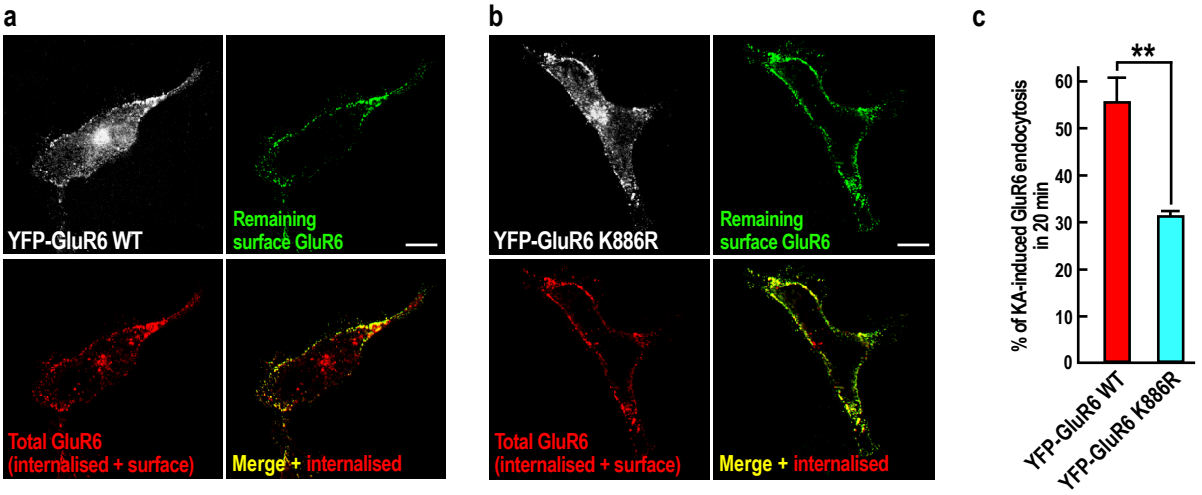
(e) Sucrose treatment completely prevents GluR6 endocytosis induced by the agonist. However, no band corresponding to the SUMOylated form of GluR6 was detected under these experimental conditions.

(f) Quantification of kainate-induced loss of GluR6 at the surface as shown in e. Histogram shows mean ± s.e.m. of three independent experiments. *$p<0.01$.

g) Neurones (21-23 DIV) were stimulated for 10 min at 37°C in Earle‘s buffer with or without 10 µM kainate and then incubated or not for 10 min at 4°C in PBS under the biotinylated conditions described above prior to lysis in the presence of 20 mM NEM. SUMOylated proteins were isolated by immunoprecipitation with a specific anti-SUMO-1 antibody, resolved on SDS-PAGE and then probed with an anti- GluR6 antibody. The 10 min treatment in PBS (right lane) at 4°C results in the loss of GluR6 SUMOylation induced by kainate indicating that the SUMO conjugation to GluR6a is highly labile and explaining why the SUMOylated form of GluR6 was not detectable in the experimental conditions described in e.

All experiments performed in the presence of 2 µM TTX and 50 µM D-APV.

Supplementary figure 10

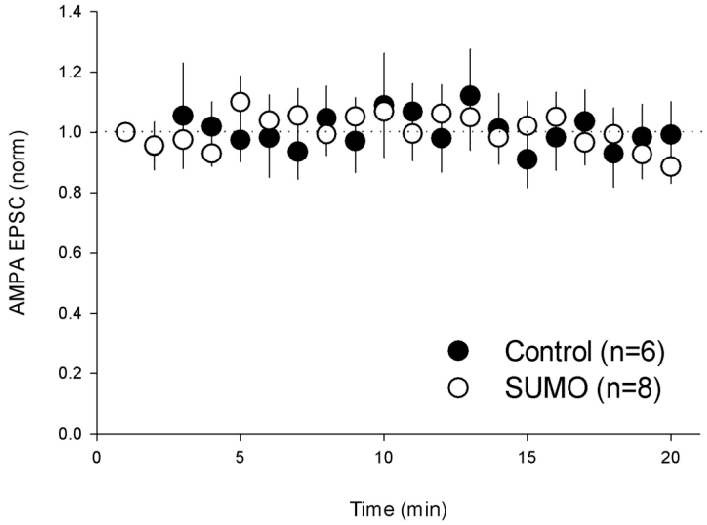


Supplementary Figure 10: K886 residue in GluR6a is essential for kainate-induced endocytosis.

YFP-GluR6a (a) and the non-SUMOylatable point mutant YFP-GluR6-K886R (b) were transiently transfected into COS-7 cells and surface receptors were labelled in living cells using an Alexa Fluor 647 anti-GFP antibody for 10 min. After extensive washes in Earle's buffer, cells were incubated at 37°C for 20 min in Earle's buffer containing 100 µM kainate prior to fixation with PAF 4% for 5 min. The receptors remaining at the surface were then labelled with an Alexa Fluor 555 anti-GFP antibody. The cells were then washed and further fixed in PAF for 15 min before mounting in Mowiol and confocal visualization.

(c) Quantification of YFP-GluR6a WT (55 ± 6.42%) and YFP-GluR6-K886R (31.5 ± 1.10%) endocytosis induced by 100 µM kainate at 20 min. Data were obtained from at least six cells for each condition and the results are given as percentage ± s.e.m. **p<0.01 compared with YFP-GluR6-K886R (t-test).

Supplementary figure 11



Supplementary Figure 11: SUMO-1 does not affect AMPAR EPSCs.

AMPAR EPSCs recorded in CA1 pyramidal neurones while stimulating Schaffer collateral axons show no effect of SUMO-1 (4.2 μM) in the patch pipette.