SUMOylation and phosphorylation of GluK2 regulate kainate receptor trafficking and synaptic plasticity

Sophie E L Chamberlain1,3, Inmaculada M González-González2,3, Kevin A Wilkinson2, Filip A Konopacki2, Sriharsha Kantamneni2, Jeremy M Henley2,4 & Jack R Mellor1,4

Phosphorylation or SUMOylation of the kainate receptor (KAR) subunit GluK2 have both individually been shown to regulate KAR surface expression. However, it is unknown whether phosphorylation and SUMOylation of GluK2 are important for activity-dependent KAR synaptic plasticity. We found that protein kinase C–mediated phosphorylation of GluK2 at serine 868 promotes GluK2 SUMOylation at lysine 886 and that both of these events are necessary for the internalization of GluK2-containing KARs that occurs during long-term depression of KAR-mediated synaptic transmission at rat hippocampal mossy fiber synapses. Conversely, phosphorylation of GluK2 at serine 868 in the absence of SUMOylation led to an increase in KAR surface expression by facilitating receptor recycling between endosomal compartments and the plasma membrane. Our results suggest a role for the dynamic control of synaptic SUMOylation in the regulation of KAR synaptic transmission and plasticity.

RESULTS
Phosphorylation promotes SUMOylation and loss of KARs
Previously, we found SUMOylation of the KAR subunit GluK2 at hippocampal mossy fiber synapses17. The functional consequences of SUMOylation were a removal of KARs from the postsynaptic membrane, leading to a decrease in KAR-mediated synaptic transmission. We reproduced this finding by recording pharmacologically isolated KARs from hippocampal mossy fiber synaptic currents (KAR EPSCs) from CA3 neurons in hippocampal slices and included either SUMO-1 (active SUMO-1, 4.2 μM) or the conjugation-deficient mutant SUMO-1-ΔGG (inactive SUMO-1, 4.2 μM) in the recording pipette. We confirmed that inclusion of SUMO-1 resulted in a rapid decrease in the amplitude of the KAR EPSC to 46.6 ± 5.0% (n = 13, P < 0.001; Fig. 1a) of the initial amplitude obtained within 1 min of rupturing the membrane inside the patch electrode.
Phosphorylation of proteins can either facilitate or inhibit SUMOylation and further suggest that phosphorylation is no longer had any effect (98.2 ± 6.0%, n = 8, P > 0.05; Fig. 1b). In addition, in the presence of chelerythrine (5 µM), active SUMO-1 no longer had any effect (98.2 ± 6.0%, n = 8, P > 0.05; Fig. 1b). These results support the hypothesis that phosphorylation of GluK2 promotes SUMOylation and further suggest that phosphorylation is required for SUMO-dependent internalization of GluK2.

To determine the sites of phosphorylation and SUMOylation of GluK2 that regulate the surface expression of KARs, we expressed recombinant wild-type GluK2 or mutated GluK2 in which either the putative PKC phosphorylation site S868 (refs. 24,25) was altered to either a phosphomimetic aspartate or a non-phosphorylatable (phosphonull) alanine or the SUMOylation site K886 was altered to a non-SUMOylatable (SUMOnull) arginine24. We first characterized kainate responses in HEK cells by expressing wild-type GluK2. This produced stable KAR-mediated response amplitudes to 300-ms applications of 100 µM kainate. Inclusion of inactive SUMO-1 in the patch pipette had no effect on response amplitude (106.3 ± 5.1%, n = 7, P > 0.05; Fig. 2a), but inclusion of active SUMO-1 in the recording pipette induced a rapid depression of response amplitude (52.5 ± 3.6%, n = 6, P < 0.001; Fig. 2a). The speed of depression was faster than that seen in neurons, but the magnitude was similar. The depression of KAR-mediated responses was a direct result of SUMOylation of GluK2, as neither active nor inactive SUMO-1 had any effect on KAR-mediated responses in HEK cells expressing the non-SUMOylatable (SUMOnull) GluK2 mutant K886R17 (inactive SUMO-1, 106.6 ± 8.9%; active SUMO-1, 100.5 ± 12.6%; n = 6 for each, P > 0.05; Fig. 2b).

We next used the phosphomimetic and non-phosphorylatable mutants of serine 868 to test the role of phosphorylation in SUMO-mediated removal of surface KARs. In HEK cells expressing the S868A (phosphonull) GluK2 mutant, infusion of active SUMO-1 via the recording pipette had no significant effect on the KAR-mediated responses when compared with the inactive control (inactive SUMO-1, 98.2 ± 9.4%; active SUMO-1, 105.0 ± 8.3%; n = 6 for each, P > 0.05; Fig. 2c). However, in HEK cells expressing the S868D (phosphomimetic) GluK2 mutant, infusion of active SUMO-1 depressed KAR-mediated responses (27.8 ± 3.5%, n = 6) compared with infusion of inactive SUMO-1 (142.5 ± 11.2%, n = 6, P < 0.001; Fig. 2d), but not compared with infusion of active SUMO-1 in cells expressing wild-type GluK2 (Fig. 2a). These data suggest that phosphorylation promotes the SUMO-1–dependent removal of synaptic KARs. (a) Inclusion of 4.2 µM SUMO-1 in the patch solution caused a decrease in the KAR current amplitude in HEK cells expressing wild-type GluK2, whereas inclusion of inactive SUMO-1–ΔGG did not. The KAR currents shown are responses to 300-ms applications of 100 µM kainate. Responses are normalized to the first minute. Example traces are taken as the average of the first minute (black) and of 10–15 min (gray). (b) SUMO-1 no longer caused a decrease in the KAR current amplitude evoked from HEK cells expressing K886R GluK2. (c) SUMO-1 failed to decrease the amplitude of KAR currents evoked from HEK cells expressing S868A GluK2. (d) Inclusion of SUMO-1 in the patch solution caused a decrease in the KAR current amplitude in HEK cells expressing S868D GluK2, which was comparable to the decrease seen in wild type. However, inclusion of inactive SUMO-1 revealed an increase in KAR current amplitude. Data are plotted as mean ± s.e.m. Scale bars represent 100 pA and 500 ms.
that phosphorylation of GluK2 at S868 is required for SUMO-mediated removal of KARs from the plasma membrane.

Previously, we found that phosphorylation of S868 can enhance SUMOylation of GluK2 in Cos-7 cells. To confirm this finding, we quantified the amount of SUMOylated GluK2 in HEK cells expressing wild-type GluK2 or the S868A, S868D or K886R mutants. Similar to the situation in neurons, some SUMOylation of wild-type GluK2 was detectable under basal conditions. However, SUMOylation of the S868D phosphomimetic mutant was increased compared with wild type (Supplementary Fig. 1), suggesting that phosphorylation of S868 enhances SUMOylation of GluK2.

**Phosphorylation of GluK2 increases KAR EPSC amplitude**

Infusion of inactive SUMO-1 into HEK cells expressing the phosphomimetic S868D mutant of GluK2 led to an increase in the amplitude of the KAR-mediated current when compared with wild type (S868D, 142.5 ± 11.2%; wild type, 106.3 ± 5.1%; P < 0.05; Fig. 2d). These data suggest that phosphorylation of S868 combined with receptor activation may increase surface expression of GluK2, which would directly oppose the increased removal of GluK2 by SUMOylation. Consistent with this interpretation, PMA (1 µM) caused an increase in the amplitude of the KAR EPSCs recorded from CA3 neurons (139.3 ± 12.2%, n = 7, P < 0.05; Fig. 3a). Furthermore, the PKC inhibitor chelerythrine (5 µM) caused a decrease in KAR EPSCs (68.5 ± 8.0%, n = 8, P < 0.01; Fig. 3b). PKC inhibition by infusion of the PKC inhibitory peptide PKC19–36 also caused a decrease in KAR EPSC, confirming the role of PKC inhibition (57.4 ± 12.4%, n = 5, P < 0.05; Supplementary Fig. 2a).

To investigate this further, we recorded from PMA-treated HEK cells expressing GluK2. Perfusion of PMA (1 µM) onto HEK cells expressing wild-type GluK2 led to a significant increase in the amplitude of the KAR current to 170.3 ± 23.6% (n = 6, P < 0.05; Fig. 3c). Perfusion of PMA (1 µM) onto HEK cells expressing the non-SUMOylatable K886R GluK2 mutant also led to an increase in KAR-mediated response amplitude to 178.3 ± 38.3% (n = 6, P < 0.05; Fig. 3d). Notably, although the increase was not significant after 10 min of PMA application in HEKs expressing wild-type GluK2 (146.0 ± 20.3%, n = 6, P > 0.05; Fig. 3e), it was significant after 10 min of PMA application in HEKs expressing K886R (184.0 ± 40.6%, n = 6, P < 0.05; Fig. 3f), suggesting that the increase is faster in HEK cells expressing the non-SUMOylatable K886R GluK2 mutant. We also tested the effects of PMA (1 µM) perfusion on HEK cells expressing the S868A non-phosphorylatable mutant. In this case, PMA no longer led to an increase in amplitude of the KAR current, but instead resulted in a small decrease (77.5 ± 8.4%, n = 5, P > 0.05; Fig. 3f). These data indicate that, in the absence of SUMOylation, PKC-mediated phosphorylation of GluK2 at S868 leads to an increase in GluK2 surface expression.

**GluK2 phosphorylation regulates KAR recycling**

Our results implicate PKC in both increased and decreased GluK2 surface expression. We reasoned that PKC phosphorylation might be involved in both the exo- and endocytosis components of GluK2 trafficking. We first addressed whether the PKC-dependent increase in the amplitude of the KAR EPSC is a result of changes in the rate of delivery and surface expression of de novo synthesized GluK2 in neurons. We used Sindbis virus to express super eclipctic pHluorin–tagged GluK2 (SEP-GluK2)26 in neurons and monitored the dynamics of SEP-GluK2 membrane insertion using antibody to GFP. In these experiments, live neurons were first incubated with unlabeled antibody to GFP for 5 min to occupy and block the GFP epitope on all existing surface-expressed SEP-GluK2. Following washing, to remove all unbound, unlabeled antibody to GFP, the amount of newly inserted SEP-GluK2 was assessed at 0, 10 or 20 min using antibody to GFP directly coupled to Alexa594. We found that the amount of SEP-GluK2 inserted into the plasma membrane was unaffected after a 10- or 20-min treatment with PMA or chelerythrine compared with control (Fig. 4a).

As expected, inhibition of protein synthesis with cycloheximide prevented any increase in the amount of GluK2 inserted into the plasma membrane. These results suggest that PKC activation does not affect the rate of insertion of de novo GluK2 in neurons.

Given that inserting newly synthesized receptors does not account for the increased surface expression of GluK2, we investigated whether there was increased recycling to the plasma membrane from early endosomes27 by functional colocalization analysis with the transferrin receptor (TIR). In the presence of transferrin, the TIR is internalized into early endosomes and then constitutively sorted to recycling endosomes.28 As expected, after 5 min of pulse and 15 min of chase in the presence
of either chelerythrine or vehicle, Alexa594-conjugated transferrin was localized predominantly in intracellular endosomes with a moderate colocalization with GluK2 (Fig. 4b). In the presence of the PKC inhibitor chelerythrine, GluK2 showed limited colocalization with TfR (Pearson’s coefficient = 0.38 ± 0.01, n = 28) and this level of colocalization was increased in cells treated with PMA (Pearson’s coefficient = 0.64 ± 0.01, n = 31). This suggests that PKC activation increases the proportion of GluK2 sorted into recycling pathways. Intermediate levels of colocalization between GluK2 and TfR were observed under control conditions (Pearson’s coefficient = 0.55 ± 0.04, n = 25); however, this value was highly variable, likely a result of differences in basal PKC activity.

Receptor recycling consists of both activity-dependent and activity-independent components. In the presence of tetrodotoxin, which blocks the activity-dependent component of GluK2 recycling, the colocalization of GluK2 with TfR was substantially decreased (Pearson’s coefficient = 0.44 ± 0.03, n = 19). Notably, this level of colocalization in the presence of tetrodotoxin was unaffected by the addition of chelerythrine (Pearson’s coefficient = 0.38 ± 0.09, n = 31, P > 0.05), suggesting that chelerythrine acts on the activity-dependent component of GluK2 recycling, leaving basal recycling unaffected. Thus, these results suggest that PKC phosphorylation regulates activity-dependent recycling of GluK2.

**Figure 4** PKC activation promotes KAR localization in recycling pathways. (a) PKC activation does not alter the amount of exocytosis of KARs in hippocampal neurons when receptors already at the surface are excluded. A schematic of the experimental procedure to exclude all initially surface-expressed KARs from analysis is shown. Representative images show antibody to GFP binding to surface-expressed SEP-GluK2 at intervals of 0, 10 and 20 min. Scale bars represent 10 µm. Application of PMA (1 µM) or chelerythrine (5 µM) did not change the amount of KAR insertion into the plasma membrane in comparison with control. Inhibition of protein synthesis with cycloheximide prevented KAR insertion. Data are quantified as the change in fluorescence divided by the fluorescence at time 0 (ΔF/F0). (b) Colocalization of GluK2 with TfR was increased by application of PMA, but not chelerythrine. Representative images show increased GluK2 and TfR colocalization along the dendrites of hippocampal neurons following treatment with PMA. Scale bars represent 2 µm. Arrowheads indicate puncta with high colocalization. (c) Colocalization of wild-type GluK2 with EEA1 was increased by application of PMA (1 µM), but this was prevented by expression of the K886R, S868A or S868D GluK2 mutants. Representative images show increased GluK2 and EEA1 colocalization along the dendrites of hippocampal neurons following treatment with PMA. Scale bars represent 1 µm. Data are plotted as mean ± s.e.m. *P < 0.05, ***P < 0.001.
An increase in receptor recycling leads to greater numbers of receptors in early endosomal compartments. Thus, PKC phosphorylation of GluK2 is expected to increase the amount of GluK2 associated with early endosomes. Consistent with our hypothesis, addition of PMA to cultured neurons transduced with wild-type GluK2 led to an increase in GluK2 colocalization with the early endosomal marker EEA1 (Pearson’s coefficient: control, 0.42 ± 0.02; PMA treated, 0.53 ± 0.04; n = 12–15, P = 0.004; Fig. 4c). In contrast, PMA had no effect on the colocalization of EEA1 with S868A, S868D or K886R GluK2 mutants. Notably, in the absence of PMA, the K886R GluK2 mutant showed an increased colocalization in comparison with wild type (P < 0.001), whereas the phosphomimetic S868D GluK2 mutant showed a small decrease (P < 0.05) and the non-phosphorylatable S868A GluK2 mutant showed no change (P > 0.05). Notably, the pattern of GluK2 expression was not markedly altered by GluK2 mutations when colocalized with PSD95 staining (Pearson’s coefficient: wild type, 0.57 ± 0.02, n = 10; S868A, 0.59 ± 0.03, n = 10; S868D, 0.55 ± 0.08, n = 10; K886R, 0.56 ± 0.02, n = 15) and the pattern of EEA1 expression was not affected by application of PMA (Fig. 4c), although chronic chelerythrine treatment did change EEA1 staining to a less punctate distribution (Supplementary Fig. 3), preventing analysis of mutant GluK2 colocalization with EEA1 under these conditions. These data support the conclusion that PKC phosphorylation of S868 increases recycling of GluK2 between the plasma membrane and early endosomal compartments.

To address the influence of PKC activation on GluK2 recycling, we compared recycling in neurons incubated in PMA or chelerythrine. Using live-cell antibody feeding, we quantified the recycling of SEP-GluK2, again using SEP as an extracellular epitope for antibody to GFP. Neurons were incubated with Alexa594 conjugated antibody to GFP together with PMA or chelerythrine for 10 min to allow internalization of surface-labeled receptors. The Alexa594-coupled antibody to GFP was then stripped from any remaining surface receptors and the reappearance (recycling) of Alexa594-tagged SEP-GluK2 at the plasma membrane after 10 or 20 min was measured (Fig. 5a). The intracellular signal from internalized Alexa594-tagged SEP-GluK2 was diffuse and largely below detection threshold and was subtracted for analysis (see Online Methods). The amount of Alexa594-tagged SEP-GluK2 that was previously surface expressed, internalized and returned back to the surface was increased at 10 and 20 min in cells treated with PMA. In contrast, GluK2 recycling was undetectable in cells treated with chelerythrine (Fig. 5a). These data indicate that PKC activation increases the rate of GluK2 recycling in neurons.

We further confirmed the effect of PKC phosphorylation of GluK2 on recycling using primaquine, an inhibitor of exocytosis from early endosomes30. Application of primaquine to slices produced a dose-dependent decrease in KAR EPSC amplitude. Primaquine (100 µM) decreased KAR EPSC amplitude to 6.0 ± 1.1% after 10 min, whereas 1 µM primaquine decreased KAR EPSC amplitude to 57.0 ± 8.5%, which stabilized after 60 min (Supplementary Fig. 4). Application of PMA following 1 h pre-incubation with 1 µM primaquine failed to elicit an increase in KAR EPSC (106.8 ± 6.5%, n = 7, P > 0.05; Fig. 5b) in contrast with control conditions (data from Fig. 3a is reproduced for comparison), indicating that exocytosis of GluK2 during receptor recycling is necessary for increased surface expression of KARs after activation of PKC.

To determine whether PMA exerts its effects directly via phosphorylation of GluK2, we used live-cell antibody feeding experiments to quantify PKC-dependent recycling in HEK cells expressing either wild-type GluK2 or the non-phosphorylatable S868A GluK2 mutant. We initially confirmed that GluK2 is recycled in HEK cells by comparing the colocalization of GluK2 and TfR (Supplementary Fig. 5)17. In HEK cells expressing wild-type GluK2, application of PMA increased the amount of recycled (reinserted) GluK2 at 10 and 20 min. In contrast, GluK2 recycling was undetectable in control cells or in cells treated with chelerythrine (Fig. 6a). However, in HEK cells expressing the S868A GluK2 mutant, there was no detectable reinsertion in the presence of either PMA or chelerythrine (Fig. 6b), indicating that phosphorylation of this specific residue on GluK2 is required for the enhancement of GluK2 recycling by PKC.

**Figure 5** PKC activation enhances KAR recycling in neurons. (a) PMA, but not chelerythrine, increased the amount of KAR recycling. Schematic illustrates the experimental procedure for selectively analyzing surface reinsertion of KARs. Representative images show an increase in surface GluK2 in hippocampal neurons after antibody stripping in the presence of PMA, but not chelerythrine. Scale bars represent 10 µm. *P < 0.05. (b) Incubation of hippocampal slices in primaquine (1 µM) to inhibit exocytosis from early endosomes prevented the increase in KAR EPSC caused by application of PMA (1 µM). Responses for each cell are normalized to the first 10 min. Example traces are taken as the average between 5–10 min (black) and 20–25 min (gray). Scale bars represent 20 pA and 50 ms. Data are plotted as mean ± s.e.m.
Figure 6 Phosphorylation of S868 on GluK2 enhances KAR recycling in HEK cells. (a) PMA, but not chelerythrine, increased the amount of KAR recycling compared with control in HEK cells expressing wild-type GluK2. Representative images show an increase in surface GluK2 on multiple HEK cells after antibody stripping in the presence of PMA, but not chelerythrine. Scale bars represent 1 μm. Data are plotted as mean ± s.e.m. *P < 0.05.

n = 9; P > 0.05; Fig. 7a). Similar to previous findings, KAR LTD was not dependent on changes in postsynaptic [Ca²⁺], as it was insensitive to inclusion of BAPTA (10 mM) in the patch solution (63.9 ± 3.4%, n = 7, P < 0.05; Fig. 7b), but was sensitive to blockade of mGluR5 receptors by bath perfusion with 30 μM 2-methyl-6-(phenylethyl)pyridine (MPEP, 94.3 ± 12.5%, n = 8, P > 0.05; Fig. 7c). We also confirmed that PKC phosphorylation is required for KAR LTD, as inclusion of the PKC inhibitor peptide PKC19–36 (4.8 μM) in the recording pipette, but not the inactive (Glu27)PKC19–36 peptide (4.8 μM), blocked KAR LTD ((Glu27)PKC19–36, 44.9 ± 2.9%, n = 5; PKC19–36, 44.9 ± 2.9%; n = 5; P < 0.05; Fig. 7d). This was confirmed using chelerythrine (5 μM), which also completely blocked KAR LTD (Supplementary Fig. 2b).

Having established and validated conditions for robust KAR LTD, we tested the effect of protein SUMOylation on LTD expression. Inclusion of inactive SUMO-1–ΔGG had no effect on LTD compared with control conditions (44.5 ± 6.8% after 30 min, n = 7, P < 0.001; Fig. 8a). However, infusion of the active SUMO-1 peptide caused a decrease in KAR EPSC to 58.1 ± 8.6% (n = 5, P < 0.01), after which LTD was completely occluded (100.0 ± 6.3% after 30 min, n = 8, P < 0.01 compared with inactive; Fig. 8a). In addition, we tested whether inhibiting SUMOylation blocked the expression of LTD. Inclusion of the active catalytic domain of the SUMO-specific isopeptidase SENP-1 caused an increase in KAR EPSC to 155.4 ± 10.3% (n = 6, P < 0.01) after which KAR LTD was completely blocked (93.8 ± 8.1% after 30 min, n = 8, P > 0.05; Fig. 8b). However, infusion of an inactive point mutant of SENP-1 (SENP C603S) in the patch solution had no effect on LTD (59.6 ± 9.0% after 30 min, n = 6, P < 0.05 compared with active SENP-1; Fig. 8b).

To define whether GluK2 SUMOylation and phosphorylation are necessary for KAR LTD, we virally expressed yellow fluorescent protein (YFP)-GluK2 constructs in CA3 neurons of acute hippocampal slices cultured for 24 h to allow expression of wild-type, K886R or S868A GluK2 in CA3 neurons. Viral expression of GluK2 did not

Figure 7 KAR LTD at mossy fiber synapses is dependent on activation of mGluR5 and PKC. (a) A 1-Hz stimulation for 5 min (300 pulses) induced LTD of KAR EPSCs at mossy fiber–CA3 synapses. Responses are normalized to the first 10 min. Example traces are taken as the average of the first 10 min (black) and 30–35 min after LTD (gray). The black bar represents the period of LTD induction. (b) Inclusion of BAPTA (10 mM) in the patch solution had no effect on KAR LTD. (c) Incubation in MPEP (30 μM) to block mGluR5 abolished KAR LTD. (d) Inclusion of the PKC inhibitory peptide PKC19–36, but not the inactive (Glu27)PKC19–36, in the patch solution completely abolished KAR LTD. Data are plotted as mean ± s.e.m. Scale bars represent 50 pA and 50 ms.
change KAR EPSC amplitude when comparing transduced and non-transduced neurons (non-transduced, 62.5 ± 11.7 pA, n = 15; wild type, 55.1 ± 10.0 pA, n = 5; K886R, 45.5 ± 17.1 pA, n = 5; S868A, 55.7 ± 9.4 pA, n = 5; P > 0.05 for all comparisons with ANOVA). Infusion of the active SUMO-1 peptide into CA3 neurons expressing wild-type GluK2 depressed KAR EPSCs in a similar fashion to non-transduced CA3 neurons (wild type, 49.2 ± 4.4%; non-transduced, 49.4 ± 7.1%; n = 5; Fig. 8c). In addition, KAR LTD was indistinguishable between wild-type GluK2 transduced and non-transduced neurons (wild type, 42.2 ± 12.6%; non-transduced, 32.1 ± 7.6%; n = 5; Fig. 8d). In contrast, infusion of the active SUMO-1 peptide into neurons transduced with either non-SUMOylatable K886R GluK2 or non-enzymatically phosphorylatable S868A GluK2 did not depress KAR EPSCs (K886R, 16.3 ± 7.3%; S868A, 7.7 ± 12.7%; n = 5) and KAR LTD was completely blocked (K886R, 11.5 ± 10.9%; S868A, increased 3.7 ± 21.8) (Fig. 8e–h). Taken together, these results indicate that SUMOylation of GluK2 at K886 and phosphorylation at S868 are required for KAR LTD.

**DISCUSSION**

Our results indicate that PKC phosphorylation of GluK2 is necessary for SUMOylation-dependent internalization of KARs from the synaptic membrane. Thus, we provide evidence for the dynamic interaction of two forms of post-translational protein modification controlling the subcellular localization of KARs. Furthermore, we found that this is the mechanism for the removal of KARs from the postsynaptic membrane during activity-dependent LTD.

The observations that the phosphomimetic GluK2 mutant shows an increase in KAR EPSC under baseline stimulation conditions (Fig. 2d) and that PMA causes an increase in KAR responses in HEK cells expressing wild-type GluK2 or the non-SUMOylatable GluK2 mutant (Fig. 3c,d), but not the non-phosphorylatable GluK2 mutant (Fig. 3f), suggest that phosphorylation of S868 is also involved in non-SUMOylation-dependent trafficking of KARs. This is supported by the increase in KAR recycling between the plasma membrane and intracellular compartments after phosphorylation of GluK2 at S868 (Figs. 4–6). We propose that increased recycling leads to an increase in surface expressed KARs. Notably, either pre-incubation in PMA (Fig. 1b) or expression of the phosphomimetic S868D GluK2 mutant (Fig. 2d) produced a greater decrease in KAR response amplitude when SUMO-1 was applied compared with control conditions. This suggests that PKC phosphorylation in the absence of SUMOylation promotes recycling and increased surface insertion of KARs, and can also promote enhanced endocytosis through SUMOylation of GluK2 (ref. 17). Thus, we propose that SUMOylation is the molecular switch that determines whether KAR surface expression is enhanced or decreased.

We found that phosphorylation of GluK2 at S868 by PKC has dual, competing effects on KAR localization. Phosphorylation of GluK2 increased recycling and the proportion of KARs on the plasma membrane and promoted SUMOylation, which led to a loss of surface KARs. This is highlighted by the observation that PMA increased KAR EPSC amplitude faster in cells expressing the SUMONull K886R GluK2 mutant than in cells expressing wild-type GluK2 (Fig. 3c,d).
In addition, PMA caused an increase in KAR EPSC amplitude in CA3 neurons, whereas chelerythrine led to a decrease (Fig. 3a,b), strongly suggesting that the increase in receptor insertion outweighs the increase in SUMOylation-dependent internalization under these conditions. Notably, the equilibrium between these two events may help to explain why, in different recording conditions, different studies have shown opposing effects of PKC inhibitors at mossy fiber CA3 synapses.10,14 Phosphorylation of S846 by PKC has been shown to increase KAR endocytosis25, even though it does not promote SUMOylation of K886 (ref. 24). Our data suggest that the phosphorylation of S868 and subsequent SUMOylation of K886 override the role of S846 phosphorylation. Furthermore, phosphorylation of the S868 residue on GluK2 has also been shown to increase KAR retention in the endoplasmic reticulum25, providing evidence for an additional role of S868 phosphorylation in controlling KAR localization.

We found that SUMOylation is critical for KAR LTD at mossy fiber–CA3 synapses. The degree of depression seen after LTD (Figs. 7 and 8) was comparable to the proportion of KARs internalized after infusion of SUMO-1 into the cell (Fig. 1a). This suggests that the inclusion of SUMO-1 in the patch solution occludes LTD via prior removal of SUMO-1–sensitive KARs (Fig. 8a). Conversely, infusion of SENP blocked LTD by removing SUMO-1 from GluK2 before KARs could be internalized (Fig. 8b). The dominant-negative effect of transduced GluK2 (Fig. 8) implies that expressed mutant GluK2 supplants endogenous GluK2 at mossy fiber synapses. Notably, KAR LTD at mossy fiber–CA3 synapses has also been shown to require the destabilization of surface KARs containing the GluK5 subunit SNAP-25 (ref. 14). Our results do not directly address the role of GluK5, but, taken together, suggest that KARs removed from the synaptic membrane during LTD contain both GluK2 and GluK5 and that endocytosis during LTD requires dual synergistic processes initiated by PKC phosphorylation that lead to both the binding of PICK1 and SNAP-25 to GluK5 and the SUMOylation of GluK2.

In conclusion, our results demonstrate a role for SUMOylation in synaptic plasticity of KARs at mossy fiber synapses in the hippocampus, suggesting that this form of post-translational protein modification may be involved in other forms of synaptic plasticity. SUMO proteins and the enzymes required for SUMO conjugation are found throughout dendrites and are concentrated at spines17, and accumulating evidence suggests that many synaptic proteins other than KARs are also SUMOylated12. Thus, post-translational protein modifications, such as SUMOylation, phosphorylation and ubiquitination, may dynamically interact to coordinate neurotransmitter receptor localization and function.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS
We thank A. Randall for providing electrophysiological facilities for experiments on cultured preparations, and S. Martin and J. Hanley for comments on previous versions of the manuscript. This work was funded by Biotechnology and Biological Sciences Research Council (S.E.L.C., J.R.M. and J.M.H.), the European Research Council (J.A.W. and J.M.H.), Medical Research Council (F.A.K., S.K. and J.M.H.) and the Wellcome Trust (J.R.M., J.M.H.). I.M.G.-G. is a European Molecular Biology Organization Fellow.

AUTHOR CONTRIBUTIONS
S.E.L.C. performed the electrophysiology experiments and analyzed the results. J.M.G.-G. performed the imaging experiments and analyzed the results. K.A.W. and F.A.K. performed the biochemical experiments and analyzed the results. S.K. prepared SUMO and SENP proteins for electrophysiological experiments. J.M.H. and J.R.M. supervised the project. S.E.L.C., I.M.G.-G., J.M.H. and J.R.M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nn.3089. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
ONLINE METHODS

Electrophysiological recordings in hippocampal slices. Transverse hippocampal slices (400–500 µm thick) were prepared from postnatal day 14 Wistar rats. All experiments were performed in accordance with Home Office guidelines as directed by the Home Office Licensing Team at the University of Bristol. Slices were cut in ice-cold artificial cerebrospinal fluid (aCSF) containing 119 mM NaCl, 10 mM glucose, 26 mM NaHCO₃, 2.5 mM KCl, 1 mM NaH₂PO₄, 0.5 mM CaCl₂, 5 mM MgSO₄, saturated with 95% O₂ and 5% CO₂. Slices were transferred directly after preparation to tissue culture inserts (Millicell-CM, 0.4 µm pore size) and bathed in sterile MEM (Gibco) containing 5 mM NaHCO₃, 30 mM HEPES, 1 mM glucose, 1 mM CaCl₂, 2 mM MgSO₄, 13 mM glucose and 20% horse serum (wt/vol), adjusted to pH 7.28 with NaOH and 320 mMOS. Slices were incubated at 35 °C and 5% CO₂ for 1 h before being pressure injected at multiple sites within CA3 with Sindbis virus, which resulted in 10–20 successfully transduced neurons in each slice. Slices were then returned to the incubator for 20–24 h before recording for electrophysiological recordings, cultured and acute slices were immersed in recording aCSF at 35 °C containing 117 mM NaCl, 5 mM KCl, 30 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂ and 0.3 mM NaGTP, 0.2 mM EGTA, 0.1 mM bestatin and 0.1 mM leupeptin, at pH 7.4 and 280 mOsm. Before whole-cell configurations were obtained, cells were returned to complete media. Electrophysiology was performed using Clampfit software (Axon Instruments). Experiments were performed 2000 (Invitrogen) according to the manufacturers’ instructions and 3 h post-transfection, cells were returned to complete media. Electrophysiological recordings in HEK cells. HEK cells were cultured in DMEM (Gibco) containing 10% fetal calf serum (wt/vol), 2 mM glutamine and 2% penicillin/streptomycin. Cells were split 24 h before transfection onto 25-mm coverslips coated with 5 µg ml⁻¹ poly-l-lysine in 35-mm dishes. Cells were transfected in plain DMEM with 2 µg of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions and 3 h post-transfection, cells were returned to complete media. Electrophysiology was performed 48 h after transfection. Recordings were made using an Axopatch 200B amplifier and pClamp 10.2 software (Axon Instruments). Borosilicate patch electrodes had series resistances of 2–6 MΩ. Extracellular solution contained 135 mM NaCl, 5 mM KCl, 30 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES at pH 7.3 and 305 mMOS. Intracellular solutions contained 117 mM CsMeSO₄, 8 mM NaCl, 10 mM HEPES, 5 mM QX-314Cl, 4 mM MgATP, 0.3 mM NaGTP, 0.2 mM EGTA, 0.1 mM bestatin and 0.1 mM leupeptin at pH 7.4 and 280 mMOS. Before whole-cell configurations were obtained, cells were lifted from the coverslip into a laminar stream of extracellular solution containing conconavalin A (0.3 µg ml⁻¹) to block receptor desensitization. Kainate (100 µM) was applied by multi-barrel fast perfusion system controlled by Clampex software (Axon Instruments). Analysis was performed off-line using Clampfit software (Axon Instruments). Experiments were performed at 20–24 °C.

Receptor exocytosis experiments. Dissociated hippocampal neuronal cultures were pretreated with PMA, chelerythrine or vehicle for 15 min at 37 °C before live-cell imaging experiments in the continued presence of the drugs. At the beginning of the experiment, GFP-reactive sites on the cell surface of neurons expressing SEP-GluK2 were blocked by pretreatment with an excess of unlabeled rabbit polyclonal antibody to GFP (rabbit IgG fraction A11122, Invitrogen, I:2000) at 20–24 °C for 5 min (rather than at 37 °C, to limit receptor endocytosis). Neurons were immediately placed on a microscope stage heated to 37 °C and incubated with diluted rabbit antibody to GFP directly coupled to Alexa594 (1:500) for 2 min to visualize the plasma membrane. Neurons were quickly washed twice and the fluorescence at time zero was acquired. Cells were incubated either a further 10 or 20 min in the presence of PMA, chelerythrine or vehicle and then reprobed with Alexa594-conjugated antibody to GFP (I:2000), rapidly washed twice and imaged to determine the SEP-GluK2 inserted during the 10- or 20-min incubation interval.

Receptor recycling experiments. SEP-GluK2 recycling was quantified by live-cell antibody feeding in the presence of PMA or chelerythrine. Cells were incubated in PMA or chelerythrine for 10 min with Alexa594-conjugated antibody to GFP (I:2000) at 37 °C to allow the endocytosis of the labeled surface receptors. Cells were washed twice in aCSF and the total surface SEP-GluK2 was determined by confocal microscopy. Cells were then washed with stripping buffer (recording buffer, pH 3.2, plus 50 mM MesNa) for 30 s. Because MesNa is membrane impermeant, it removes antibody to GFP from surface-expressed SEP-GluK2, but not from SEP-GluK2 that has been internalized. Immediately after stripping, the cells were imaged (t = 0) and then incubated at 37 °C for 10 min and 20 min (t = 10 and t = 20) to allow recycling to occur in the presence of either PMA or chelerythrine.

The red fluorescence corresponding to GFP-labeled SEP-GluK2 returning to the plasma membrane was recorded at 0, 10 and 20 min from the same cell as a series of z stacks (0.25-µm spacing between single confocal slices) using a Zeiss LSM 510 confocal laser-scanning station with an oil-immersion 63× 1.4 NA objective (Zeiss). Alexa594 was visualized using a 543-nm HeNe laser line and a 585-nm long-pass filter, whereas SEP was excited with the 488-nm laser line from an argon-krypton laser and the emitted light was detected using a 505–550-nm bandpass filter. Data are quantified as the change in fluorescence divided by the fluorescence at time 0 (ΔF/F₀).

The efficiency of the stripping was normalized in each cell as the ratio of fluorescence pre-stripping to post-stripping. If the efficiency of stripping was not greater than 40%, then the analysis did not continue. Differences in expression were normalized to the mean of the fluorescence at time 0 and the rate of receptor reappearance at the cell surface in individual cells was then determined. To ensure that the red fluorescence from the area of interest came only from surface-expressed KARs, all SEP-GluK2 experiments included a brief (10 s) pH 6 wash at the end of the experiment to quench surface SEP-GluK2 fluorescence. Any areas of interest that retained SEP fluorescence during the pH 6 wash were excluded from the analysis of the red Alexa594 fluorescence (Supplementary Fig. 6). In HEK cells, the same control was performed by incubating the cells with Cy5-conjugated antibody to rabbit (goat antibody to rabbit A10523, Invitrogen, I:500) at the end of the experiment for 2 min at 20–24 °C (Supplementary Fig. 7). Statistical analysis of differences between experimental groups was performed using one-way ANOVA followed by post hoc Tukey test calculated using SigmaStat software. Data are presented as mean ± s.d.

GluK2 colocalization analysis. HEK cells grown on coverslips were washed 48 h after transfection and pre-incubated with serum-free DMEM for 2 h. For transferrin assays in neurons, the neurons were pre-incubated with B27-free Neurobasal for 1 h. HEK293 cells or neurons were then incubated with 10 µg ml⁻¹ of Alexa594-conjugated transferrin (Molecular Probes) in serum/B27-free medium for 5 min at 20–24 °C. Cells were then washed with phosphate-buffered saline (PBS) twice and shifted to 37 °C for 20 min to label recycling endosomes in the presence of PMA, chelerythrine or vehicle. Following incubation, the cells were washed with PBS twice and fixed in 4% paraformaldehyde (wt/vol). For the HEA1 GluK2 colocalization experiment, 24 h after infection, the neurons were incubated with PMA, chelerythrine or vehicle for 30 min. For HEA1 or PDS95 GluK2 colocalization, neurons were then washed with PBS, fixed in 4% 0.4...
paraformaldehyde and stained with specific primary antibodies to EEA1 (BD Biosciences 610457, 1:400) or PSD95 (Millipore 7E3-1B8 MAB1598, 1:2000).

Three-dimensional volumes of z stacks (0.25-µm spacing between single confocal slices) were analyzed using ImageJ. The degree of colocalization was assessed in whole cell volumes and subvolumes by calculating the Pearson’s correlation coefficient in the region of interest using a semi-automated algorithm embedded in the JaCoP plugin33 of the ImageJ software. The colocalization plugin also performed a two-step analysis to calculate the Pearson’s correlation coefficient for the original data and for a large set (approximately 500) of images randomized with a grain size determined by the point spread function of the microscope objective. If the Pearson’s correlation coefficient of the original image was not greater than 95% of the randomized images, then the samples were not used. In addition, cells displaying saturated or low, near-threshold signals were discarded, and user bias in setting analysis parameters was avoided by using an automated thresholding procedure33. Histograms presenting the mean correlation coefficient (derived from 19 to 31 cells assessed per treatment condition) are shown with s.d. bars in all figures. Statistical significance for differences between paired combinations of images was calculated using the two-tailed Student’s t test.

GluK2 SUMOylation in HEK cells. HEK cells in 35-mm dishes were transfected with 1 µg of plasmid DNA encoding YFP-myc-GluK2 wild type, S868D, S868A or K886R along with FLAG–SUMO-1 and FLAG–Ubc9. Cells were washed twice with PBS 36 h post-transfection and lysed in 500 µl of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 (vol/vol), 0.1% SDS (wt/vol), 20 mM NEM and protease inhibitors (Roche)). Lysates were then sonicated briefly and GluK2 solubilized by rotation for 1 h at 4 °C. Lysates were then cleared by centrifugation at 16,000 g for 15 min. We then added 600 µl of lysis buffer lacking NEM to 200 µl of cleared lysate. GluK2 was immunoprecipitated by addition of 1 µg of sheep antibody to myc (made in-house) and left rotating at 4 °C overnight. To each immunoprecipitate, 20 µl (bed volume) of protein G sepharose (Sigma) pre-washed in lysis buffer (lacking NEM) was added, and immunoprecipitates incubated for a further 2 h at 4 °C. After extensive washing in lysis buffer lacking NEM, beads were boiled in 50 µl of 2× Laemmli buffer. Immunoprecipitates and corresponding inputs were then subjected to SDS-PAGE and western blotting for FLAG (M2 monoclonal antibody, Sigma) or myc (9E10, Santa Cruz), respectively. Blots were analyzed using ImageJ by normalizing the band representing SUMOylated GluK2 to total GluK2. Statistical significance was determined using two-tailed Student’s t test.

33. Bolte, S. & Cordelieres, F.P. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213–232 (2006).