LTP requires a reserve pool of glutamate receptors independent of subunit type

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Long-term potentiation (LTP) of synaptic transmission is thought to be an important cellular mechanism underlying memory formation. A widely accepted model posits that LTP requires the cytoplasmic carboxyl tail (C-tail) of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor subunit GluA1. To find the minimum necessary requirement of the GluA1 C-tail for LTP in mouse CA1 hippocampal pyramidal neurons, we used a single-cell molecular replacement strategy to replace all endogenous AMPA receptors with transfected subunits. In contrast to the prevailing model, we found no requirement of the GluA1 C-tail for LTP. In fact, replacement with the GluA2 subunit showed normal LTP, as did an artificially expressed kainate receptor not normally found at these synapses. The only conditions under which LTP was impaired were those with markedly decreased AMPA receptor surface expression, indicating a requirement for a reserve pool of receptors. These results demonstrate the synapse's remarkable flexibility to potentiate with a variety of glutamate receptor subtypes, requiring a fundamental change in our thinking with regard to the core molecular events underlying synaptic plasticity.

Information storage in the brain is widely accepted to involve the rapid increase in synaptic strength between two neurons that can persist over long periods of time. This phenomenon, known as long-term potentiation (LTP), has been well described at glutamatergic synapses in the hippocampus, a region of the brain that is required for the formation of new memories. At these CA1 synapses, LTP is expressed by the immediate increase in postsynaptic AMPA-type glutamate receptors (AMPARs) after coincident activation of pre- and postsynaptic neurons. However, the exact mechanism of rapid AMPAR insertion during LTP is not fully understood.

AMPARs mediate most fast, excitatory synaptic transmission in the brain. A functional AMPAR is a tetramer of individual subunit proteins, of which there are four unique isoforms, GluA1, GluA2, GluA3 and GluA4 (refs 1, 2). In CA1 pyramidal neurons, which serve as a model for understanding LTP, most receptors exist as GluA1-GluA2 heteromers, with a minor contribution from GluA2-GluA3 receptors^{3,4}. Over the past decade, a large body of research has focused on how individual AMPAR subunits are trafficked. A widely held model posits that GluA1-GluA2 receptors are excluded from synapses unless an LTP stimulus is provided, whereas GluA2-GluA3 receptors traffic to the synapse constitutively. This difference in trafficking behaviour is mediated by the C-tails of the individual subunit proteins⁵⁻⁷. Supporting this model is the finding that LTP is impaired in GluA1 knockout mice8 but is normal in GluA2-GluA3 double knockouts9. On the basis of these findings, a broad consensus has emerged that LTP is mediated by synaptic insertion of GluA1-containing receptors via its C-tail interactions¹⁰⁻¹⁷.

Despite the consensus that GluA1 is required for LTP, no single phosphorylation site or protein–protein interaction in the GluA1 C-tail has been shown to be absolutely necessary. Our goal was to find the minimum requirement of the GluA1 C-tail for LTP, and, if found, use that region to identify crucial protein interactions that mediate synaptic AMPAR potentiation. To accomplish this, we used a single-cell molecular replacement strategy to replace all endogenous AMPARs with transfected subunits^{18,19}. Using this approach, we

systematically mutated the GluA1 C-tail and examined the effects on three stages of AMPAR trafficking in mice: surface expression, synaptic transmission and LTP. Surprisingly, we failed to identify any region in the GluA1 C-tail that was essential either for basal synaptic incorporation or for LTP. In fact, homomeric GluA2 receptors exhibited normal LTP. Most surprisingly, hippocampal synapses in which AMPARs had been replaced with kainate-type glutamate receptors (KARs) also expressed normal LTP. Only manipulations that severely compromised the extrasynaptic surface pool of receptors showed defects in potentiation.

The role of the GluA1 C-tail in surface expression

AMPAR trafficking can be broken down into three distinct steps: surface expression, basal synaptic targeting, and activity-dependent synaptic insertion. Because GluA1 is normally abundantly expressed on the neuronal surface^{3,20}, we first screened for surface expression of various GluA1 C-tail truncations in wild-type neurons using somatic outside-out patches in organotypic slice culture. Because overexpressed receptors form inwardly rectifying homomers⁷, whereas endogenous heteromeric receptors show linear current-voltage (I-V)relationships²¹, we can detect surface expression of the expressed subunits as an increase in surface rectification (Fig. 1a). Overexpression of full-length GluA1 by biolistic transfection into CA1 pyramidal neurons increased the rectification by approximately 40% compared to wild-type controls (Fig. 1b), indicating the presence of surface homomers. In contrast, overexpressing a GluA1 subunit with a full C-tail truncation (ΔC) showed rectification similar to wild-type neurons (Fig. 1b), indicating an impairment in trafficking to the surface. However, a less severe truncation up to amino acid 824 (GluA1(Δ 824)), which removes two serine phosphorylation sites and the PDZ-binding domain, increased rectification to a similar degree as full-length GluA1. Selective excision of the remaining membrane proximal region (Δ MPR), which contains a well-characterized binding site of the protein 4.1N^{22,23}, also significantly increased rectification (Fig. 1b). Combined, these two modified subunits represent complementary

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truncations of the entire C-tail, ruling out a necessary role for any single part of the C-tail for steady-state surface expression.

Because competition with endogenous receptors may have hindered GluA1(Δ C) trafficking, we next studied surface expression in the absence of native AMPARs. To accomplish this, we used mice in which the genes coding for GluA1, GluA2 and GluA3 were flanked by loxP sites ($Gria1^{fl/fl}$ $Gria2^{fl/fl}$ $Gria3^{fl/fl}$; hereafter referred to as $Gria1-3^{fl/fl}$). A previous study has shown that expression of Cre into Gria1-3^{fl/fl} neurons results in a complete absence of AMPARs within 12-15 days³, providing an effective AMPAR-null background onto which mutant GluA1 subunits can be expressed. We confirmed that Cre expression eliminated all glutamate-evoked current from somatic outside-out patches of Gria1-3^{fl/fl} CA1 neurons, which can be rescued to control amplitudes by co-expression with full-length GluA1 (Fig. 1c, d), indicating full rescue of surface expression. Consistent with overexpression, molecular replacement with GluA1(Δ C) showed significantly decreased glutamate-evoked currents (Fig. 1c, d). This trafficking defect was not due to decreased association with TARPs (that is, auxiliary subunits important for AMPAR trafficking^{24,25}), as both full-length and GluA1(Δ C) subunits had KA/Glu ratios similar to control (Supplementary Fig. 1a). Also, both GluA1 and GluA1(ΔC) replacement subunits showed strong inward rectification, confirming the absence of endogenous receptors (Supplementary Fig. 1b). Because both GluA1(Δ MPR) and GluA1(Δ 824) showed normal surface trafficking, the GluA1(Δ C) subunit may be impaired owing to its severe truncation so close to the transmembrane region, which may inhibit proper protein folding.

Synaptic transmission doesn't require the GluA1 C-tail

Given the decreased surface expression caused by complete truncation of the GluA1 C-tail, we next examined whether it would also

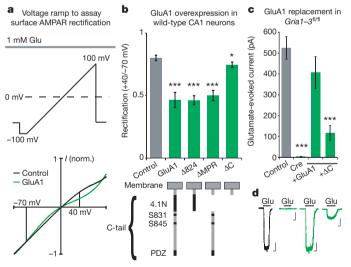


Figure 1 | The role of the GluA1 C-tail in surface trafficking. a, Experimental protocol and example trace showing voltage ramps applied to outside-out patches of control (black) and GluA1-overexpressing (green) CA1 neurons. Rectification was measured as the normalized glutamate-evoked current at +40 mV over -70 mV. **b**, Full-length GluA1, GluA1(Δ 824) and GluA1(Δ MPR) significantly increased rectification of surface currents compared to control. Overexpression of GluA1(Δ C) slightly increased rectification (control, *n* = 47; GluA1, n = 10, P < 0.001; GluA1(Δ 824), n = 13, P < 0.001; GluA1(Δ MPR), n = 18, P < 0.001; GluA1(Δ C), n = 8, P < 0.05). c, Cre expression eliminates glutamate-evoked currents in Gria1-3^{fl/fl} CA1 neuron outside-out patches, which is rescued to control levels by co-expression with full-length GluA1, but not GluA1(Δ C) (control, n = 28; Cre, n = 9, P < 0.001; GluA1, n = 11, P > 0.05; GluA1(Δ C), n = 15, P < 0.001). **d**, Example traces of glutamate-evoked current from (left to right) Gria1-3^{fl/fl} control neurons, Cre-expressing neurons, GluA1, and GluA1(Δ C) replacement neurons. Scale bars: 1 s, 100 pA. Error bars represent mean \pm s.e.m. **P* < 0.05; ****P* < 0.001 in **b**, **c**.

impair basal synaptic targeting. Similar to surface currents, we assessed baseline synaptic transmission by transfecting *Gria1*–3^{fl/fl} organotypic slice cultures with Cre and a replacement GluA1 subunit. After 17 days, we recorded evoked AMPAR excitatory postsynaptic currents (EPSCs) simultaneously from control and neighbouring GluA1-replacement CA1 neurons. Similar to previously described results¹⁹, full-length GluA1 rescued AMPAR EPSC amplitudes to ~68% of control cells, while leaving NMDAR EPSCs unchanged (Fig. 2a, c). However, these results contrast with previous studies showing that GluA1 only traffics to synapses after an LTP stimulus^{5.7}. For experimental exploration of this discrepancy, please refer to

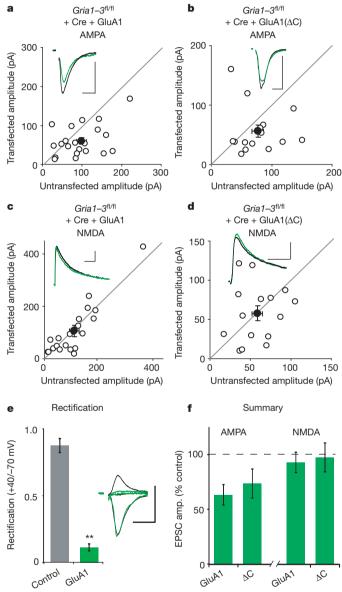


Figure 2 | **GluA1**(**ΔC**) has normal synaptic targeting. Paired whole-cell recordings from control and Cre plus GluA1 or Cre plus GluA1(ΔC)-expressing CA1 neurons in *Gria1–3*^{1/d} organotypic slice cultures. **a**, **c**, Full-length GluA1 rescued synaptic AMPAR EPSCs to 68% of control cells (n = 13, P > 0.05), whereas NMDA EPSCs remained unchanged between control and transfected cells (P > 0.05). **b**, **d**, Replacement with GluA1(ΔC) results in 73% rescue of AMPA EPSCs without a change in the NMDA EPSC (n = 15, both P > 0.05). **e**, Replacement with GluA1 showed inwardly rectifying EPSCs (n = 8, P < 0.01). **f**, Summary graph of AMPA and NMDA EPSC rescue between GluA1 and GluA1(ΔC). Example traces show average EPSCs for paired control (black) and replacement (green) neurons. Scale bars: 20 ms (AMPA), 100 ms (NMDA), 50 pA. Error bars represent mean ± s.e.m. **P < 0.01 in **e**.

Supplementary Fig. 2. We also observed no change in paired-pulse ratio, indicating that GluA1 molecular replacement did not affect presynaptic release probability (Supplementary Fig. 3a). Synaptic EPSCs from GluA1-replacement CA1 neurons were strongly inwardly rectifying compared to control, confirming the absence of endogenous receptors (Fig. 2e and Supplementary Fig. 3b). Replacement with GluA1(Δ C) rescued AMPAR EPSCs to the same degree as full-length GluA1, and also had no effect on the NMDA EPSCs (Fig. 2b, d). Replacement with GluA1(Δ 824) produced similar results (Supplementary Fig. 3c–e). This demonstrates that despite having markedly decreased somatic expression owing to its severe truncation, GluA1(Δ C) manages to rescue basal synaptic transmission effectively.

GluA1 C-tail domains are not required for LTP

To assess how GluA1 C-tail truncations affect LTP, we transfected Cre and GluA1 into the hippocampus of embryonic day ~E15.5 Gria1-3^{fl/fl} mouse embryos by electroporation. Like biolistic transfection, this results in sparse expression of transfected cells. Electroporation of Cre alone resulted in complete absence of an AMPAR EPSC by post-natal day 10 (P10) with no effect on NMDAR EPSCs, and no AMPAR EPSCs appeared after an LTP stimulus (Supplementary Fig. 4a-c). In P17-20 acute hippocampal slices, we induced LTP after recording stable (3-5 min) baseline AMPAR EPSCs simultaneously from control and GluA1-replacement neurons. We found that replacement with full-length GluA1 exhibited normal LTP (Fig. 3a), confirming that the GluA1 subunit is sufficient. To avoid the confounding effect of decreased surface expression seen by $GluA1(\Delta C)$, we next assessed the competence of GluA1(Δ 824) and GluA1(Δ MPR) subunits, which represent overlapping truncations of the entire C-tail. Both truncated subunits expressed LTP comparable to control (Fig. 3b, c), as did neurons replaced with a truncated GluA1(Δ 824) subunit with S816A and S818A mutations (GluA1(Δ 824-AA)) to specifically prevent 4.1N binding²⁶ (Supplementary Fig. 5a, b). We also found that expressing $GluA1(\Delta C)$ with GluA2 to produce more natural GluA1-GluA2 heteromers was able to rescue the surface trafficking defect of GluA1(Δ C) (Supplementary Fig. 6a–d), and show synaptic responses similar to controls (Supplementary Fig. 6e-h). Finally, LTP was fully rescued by replacement with GluA1(ΔC)-GluA2 (Fig. 3d). Combined, these data show that the GluA1 C-tail is not required for LTP.

GluA2 is sufficient for LTP

Given that no individual portion of the GluA1 C-tail was necessary for LTP, we hypothesized that expression of an alternative AMPAR subunit might also rescue LTP. GluA2 is another such subunit with limited C-tail homology to GluA1 (ref. 15) that is normally highly expressed in CA1 neurons, but is ineffective at forming homomers and trafficking to the cell surface^{3,20}. This is attributable to Q/R RNA editing in the pore of the receptor, which severely limits channel permeability and may make formation of homomers energetically unfavourable²⁷. Expression of unedited GluA2 with an R586Q mutation (GluA2(Q)) resulted in abundant appearance of homomers on the neuronal surface, as observed by increased rectification (Fig. 4a). Like GluA1(ΔC), GluA2((Q) ΔC) also showed impaired surface expression (Supplementary Fig. 7d). Similarly, both full-length GluA2(Q) (Fig. 4b) and GluA2((Q) Δ C) (Supplementary Fig. 7b) trafficked to the synapse, arguing against any necessary role for the GluA2 C-tail in synaptic targeting, in agreement with previous experiments²¹. NMDA EPSCs and paired-pulse ratio remain unchanged in these replacement neurons, and complete replacement of endogenous receptors was confirmed by synaptic rectification (Supplementary Fig. 7a, b). Moreover, LTP in $Gria1-3^{fl/fl}$ neurons that expressed only GluA2(Q) was indistinguishable from control cells (Fig. 4c), despite lacking any of the intracellular phosphorylation sites and proteinprotein binding sites of GluA1. Similarly intact synaptic targeting and LTP was seen in a GluA2(Q) truncation that lacks most of its C-tail and known protein-interaction sites (Supplementary Fig. 7c–e).

LTP requires a reserve pool of AMPARs

Previous studies have shown that LTP is impaired in mice with constitutive deletion of GluA1 (ref. 8), but not GluA2 or GluA3 (ref. 9), demonstrating that GluA1 is both necessary and sufficient for LTP. These findings seem to contradict our data showing that GluA2(Q) homomers readily express LTP. We therefore re-examined the requirement for GluA1 in single-cell conditional knockouts and

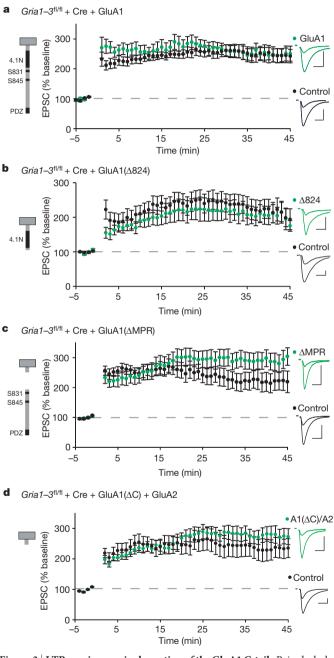


Figure 3 | **LTP requires no single portion of the GluA1 C-tail.** Paired wholecell recordings from control CA1 neurons and neighbouring Cre plus GluA1expressing neurons in P17–20 *Gria1*–3^{fl/fl} acute slices. **a**–**d**, LTP is similar to control in GluA1 (**a**), GluA(Δ 824) (**b**), GluA1(Δ MPR) (**c**) and GluA1(Δ C) plus GluA2 (**d**) replacement neurons (GluA1, *n* = 11; GluA1(Δ 824), *n* = 11; GluA1(Δ MPR), *n* = 20; GluA1(Δ C) plus GluA2, *n* = 11; all *P* > 0.05). Example traces show EPSCs before and 45 min after LTP induction in paired control (black) and GluA1-replacement neurons (green). Scale bars: 20 ms, 100 pA. Error bars represent mean ± s.e.m.

found that conditional deletion of GluA1 alone did indeed impair LTP (Fig. 5a). Furthermore, deletion of GluA2 or GluA3 separately (Supplementary Fig. 8a, b) or in combination (Fig. 5b) had no effect. How can these data be reconciled with our previous experiments? One profound difference between deleting GluA1 and deleting GluA2 and/or GluA3 is that in the former condition there is an absence of extrasynaptic receptors^{3,8,20}, whereas in the latter condition this pool remains entirely intact³. Also, unlike endogenous GluA2, our replacement GluA2(Q) showed abundant surface expression. We reasoned that perhaps it is the depletion of this pool that accounts for the loss of LTP in the GluA1 knockout. To test this possibility, we again used the extreme C-tail truncations of both GluA1 and GluA2(Q), in which surface expression is impaired but synaptic targeting is maintained (Fig. 1b, c and Supplementary Fig. 7d). Indeed, LTP was substantially impaired in both GluA1(Δ C) and GluA2((Q) Δ C) replacement neurons (Fig. 5c, d). These findings suggest that the minimum requirement for LTP is a reserve pool of extrasynaptic AMPARs, regardless of the subunit type.

GluK1 is sufficient for mediating LTP

Having failed to identify any specific domains in the C-tails that are important for LTP, we wondered whether other domains in the AMPAR are required. In search of a null condition to conduct domain-swapping experiments, we turned to KARs, a separate class of fast, ionotropic glutamate receptor which differs in fundamental ways from AMPARs. KARs bind to different auxiliary subunits and have no sequence homology in their C-tails²⁸. We therefore set out to replace all endogenous AMPARs with KARs at CA1 synapses. CA1 pyramidal neurons do not express synaptic KARs, as shown by the

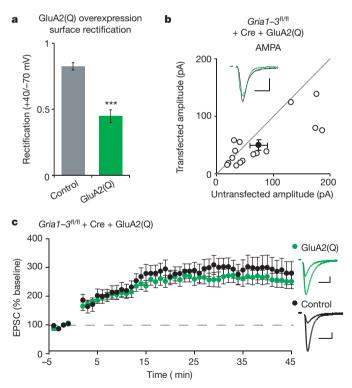


Figure 4 | **GluA2(Q) is sufficient to express LTP. a**, Overexpression of GluA2(Q) caused significantly increased surface rectification compared to control (control, n = 8; GluA2(Q), n = 14, P < 0.001). **b**, Paired whole-cell recordings between control and Cre plus GluA2(Q)-expressing *Gria1*–3^{fl/fl} CA1 neurons show rescue of AMPA EPSCs (GluA2(Q), n = 16, P < 0.05). Average AMPA EPSC example traces are shown for paired control (black) and GluA2-replacement neurons (green). **c**, Expression of Cre plus GluA2(Q) shows LTP similar to control (n = 14, P > 0.05, minute 45). Example traces show average AMPA EPSCs before and 45 min after LTP induction. Scale bars: 20 ms and 50 pA. Error bars represent mean \pm s.e.m. ***P < 0.001 in **a**.

absence of synaptic currents in the presence of the AMPAR-selective antagonist GYKI (4-(8-methyl-9*H*-1.3-dioxol[4,5-*h*][2,3]benzodiazepine-5-yl)-benzamine dihydrochloride) (Supplementary Fig. 9a). However, co-expression of the KAR subunit GluK1 with the auxiliary

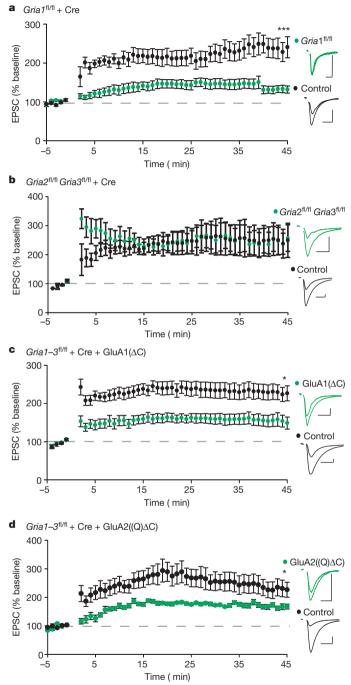


Figure 5 | Lack of surface expression corresponds with loss of LTP in GluA1 conditional knockouts, and GluA1(Δ C) and GluA2((Q) Δ C) replacement neurons. a, Conditional GluA1 knockout cells (*Gria1*^{fl/fl} plus Cre) demonstrate impaired LTP compared to control (n = 13, P < 0.001, 45 min). b, GluA2 and GluA3 knockout cells (*Gria2*^{fl/fl} *Gria3*^{fl/fl} plus Cre) demonstrate comparable LTP to control (n = 6, P > 0.05, 45 min). c, d, Molecular replacement with either GluA1(Δ C) or GluA2((Q) Δ C) results in reduced expression of LTP (GluA1(Δ C), n = 16, P < 0.05; GluA2((Q) Δ C), n = 10, P < 0.05, both at 45 min). Example traces show averaged AMPA EPSCs before and 45 min after induction of LTP in paired experimental neurons (green) and control cells (black). Scale bars: 20 ms, 50 pA. Error bars represent mean \pm s.e.m. ***P < 0.001 in a; *P < 0.05 in c, d.

subunit Neto2^{29,30} in wild-type CA1 neurons generated a GYKIresistant current that was blocked by NBQX (2,3-dioxo-6-nitro-1,2,3, 4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide), an antagonist that blocks both KARs and AMPARs (Supplementary Fig. 9a). This indicates that overexpressed KARs are capable of being targeted to the synapse and contribute to EPSCs. To examine KAR currents in isolation, we coexpressed Cre with GluK1 and Neto2 in Gria1-3^{fl/fl} CA1 neurons. In this case, we recorded a population of pure KARs on the surface that desensitize to glutamate even in the presence of cyclothiazide and are completely blocked by ACET, a highly specific GluK1 antagonist³¹ (Fig. 6a). Furthermore, in this AMPAR-null background, these neurons exhibit EPSCs that are entirely blocked by ACET, whereas the EPSCs in neighbouring control neurons are unaffected (Fig. 6b), further demonstrating that exogenously expressed KARs are capable of being targeted to synapses. As with AMPAR replacement, NMDA EPSCs were unaffected (Supplementary Fig. 9b). Finally, we tested whether neurons expressing only KARs could express LTP. Unexpectedly, we found that the KAR EPSCs showed potentiation indistinguishable from that recorded simultaneously from neighbouring control neurons (Fig. 6c). To ensure that the EPSC in the KAR-expressing neuron was mediated entirely by KARs, we applied ACET at the end of the experiments and found that it abolished the EPSC, but had no effect on neighbouring control neurons (Fig. 6c). We also wanted to confirm that LTP mediated by KARs, which are also Ca²⁺-permeable, was not induced by a fundamentally different mechanism than wild-type LTP. We therefore tried inducing LTP in the presence of the NMDAR antagonist AP5 (D(-)-2-amino-5phosphonovaleric acid), and saw no significant potentiation (Supplementary Fig. 9d, e). These experiments demonstrate that even neurons completely lacking AMPARs can undergo LTP, as long as they are provided with an alternative fast, ionotropic glutamate receptor.

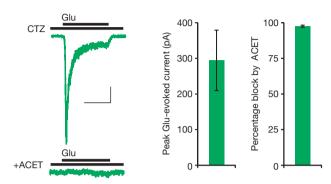
Discussion

Using a single-cell molecular replacement approach that gave us complete control over the complement of expressed AMPA receptors, we found no requirement for the GluA1 C-tail for basal synaptic transmission or for LTP. In fact, we found no requirement for the GluA1 subunit generally, as both GluA2(Q), another AMPAR subunit, and GluK1, an entirely separate class of glutamate receptor, exhibited normal levels of LTP. Previous studies that have implicated the GluA1 C-tail in LTP demonstrated phenotypes with a largely normal initial stage of potentiation, followed by a gradual decrease in EPSC amplitude towards baseline^{5,6,28,32}. The most compelling of these studies demonstrates impaired LTP in mice with phospho-null knock-in mutations of two key phosphorylation sites, and complete absence of long-term depression (LTD)³². Another study with phospho-mimetic knock-in mutations also demonstrated a decreased threshold for LTP induction³³. Given these findings, we cannot rule out the possibility that the C-tails have some modulatory effect on synaptic plasticity. In the present experiments, however, we saw immediately impaired potentiation in GluA1 conditional knockout cells and cells with GluA1(Δ C) and GluA2(Δ C) replacement, which more closely mimics the absence of LTP seen with pharmacological blockade of NMDA receptors. With all three of these manipulations, there was a profound decrease in the pool of extrasynaptic receptors, indicating that the main requirement for LTP is an adequate reserve pool of glutamate receptors. Another equally plausible model is that AMPARcontaining recycling endosomes are required for LTP, and conditions that deplete the surface receptor pool also deplete this pool³⁴ (see Supplementary Discussion).

Fundamentally, our results suggest that synapses can accumulate a broad variety of receptors after LTP, shifting the focus of LTP expression from the receptor subunits to the synapse itself and specifically the postsynaptic density (PSD). Our data suggest a model in which AMPARs freely diffuse on the neuronal surface, and are trapped at the PSD for use in synaptic transmission³⁵. LTP, then, can be understood as an immediate increase in the ability of the PSD to trap receptors

that relies on a reserve pool of freely diffusing surface receptors. This model is consistent with evidence from two-photon glutamate uncaging experiments, which show an immediate increase in the volume of postsynaptic spines after LTP induction^{36–38}, suggesting significant alterations to the synapse and PSD. Despite this shift of focus, research

a GluK1 surface replacement in Gria1-3^{fl/fl}



b GluK1 synaptic replacement in Gria1–3^{fl/fl}

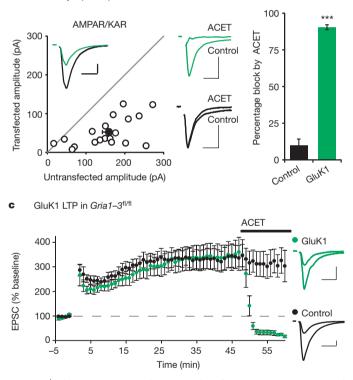


Figure 6 GluK1 expresses on the neuronal surface, targets to synapses and mediates LTP. a, Co-expression of Cre, GluK1 and Neto2 in Gria1-3^{fl/fl} neurons results in robust glutamate-evoked currents from somatic outside-out patches (n = 10). The current desensitizes in the presence of $100 \,\mu\text{M}$ cyclothiazide (CTZ), and is completely blocked by 1 µM ACET. b, Paired recordings from Cre/GluK1/Neto2-expressing and neighbouring control CA1 neurons resulted in a 33% rescue of synaptic EPSCs (n = 20, P < 0.001). Example trace (inset) shows paired control (black) and GluK1-replacement (green) EPSCs. 1 µM ACET completely blocks the GluK1 replacement EPSCs (green example traces, upper middle), with no block of control cell EPSCs (black example traces, lower middle) (n = 14, P < 0.001). c, Paired whole-cell recording from control and Cre/GluK1/Neto2-expressing Gria1-3fl/fl CA1 neurons shows similar levels of LTP (n = 12, P > 0.05, minute 45). 1 µM ACET completely blocks the GluK1-replacement EPSC, but not control (n = 11, P < 0.001, minute 60). Example traces show average EPSCs before and 45 min after LTP induction in control (black) and GluK1-replacement neurons (green). Scale bars: 1 s (a), 20 ms (b, c) and 50 pA (a-c). Error bars represent mean \pm s.e.m. ****P* < 0.001 in **b**.

on AMPARs and their auxiliary subunits, such as TARPs, remain important for identifying LTP-related PSD proteins. In the absence of a role for the GluA1 C-tail, the question remains exactly which specific interactions cluster AMPARs at the synapse both basally and during plasticity. Identification of these interactions may be crucial to understanding the synaptic modifications that underlie learning in the brain.

METHODS SUMMARY

Electrophysiology and neuronal transfection. Whole-cell and outside-out patch recordings were performed as previously described³. Slice cultures were prepared on P6–8 as previously described³⁹ and recorded on day *in vitro* (DIV) 9–24 depending on the experiment. Acute slices for LTP experiments were prepared between P17–23. All slices were maintained during recording in artificial cerebral spinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26.2 NaHCO₃ and 11 glucose. For acute slices, 2.5 mM CaCl₂ and 1.3 mM MgSO₄ were added to the aCSF, and 4 mM CaCl₂ and MgSO₄ were added for organotypic slice cultures. The internal whole-cell recording solution contained (in mM): 135 CsMeSO₄, 8 NaCl, 10 HEPES, 0.3 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP and 0.1 spermine. Osmolarity was adjusted to 290–295 mOsm, and pH buffered at 7.3–7.4. Synaptic responses were evoked by stimulating with a monopolar glass electrode filled with aCSF in stratum radiatum of CA1. Biolistic transfections and ~E15.5 electroporations were carried out as previously described⁴⁰⁻⁴².

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

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.C. carried out electroporations and maintained *Gria1–3^{fl/fl}* mice. Y.S. collected GluK1 overexpression data. W.L. was involved in study design and cloned several constructs. A.J.G. designed the study, collected and analysed data, and wrote the paper. R.A.N. conceived the study, contributed to the design of experiments and wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.A.N. (nicoll@cmp.uscf.edu).

METHODS

Mouse genetics. Animals were housed according to the IACUC guidelines at the University of California, San Francisco. *Gria1–3*^{fl/fl} mice were generated and genotyped as previously described³.

Experimental constructs. Flip-isoform GluA1, GluA2(Q) and Cre:mCherry were cloned into the pFUGW expression plasmid by PCR and In-Fusion HD Cloning System (Invitrogen). pFUGW-GluA1 and GluA2(Q) co-expressed with GFP behind an internal ribosomal entry site (IRES). GluA1 and GluA2(Q) truncations were generated by overlapping extension PCR. GluA1(Δ C) ended in amino acid 812, with the last four amino acids being EFCY. GluA1(Δ S2) ended in amino acid 824, with the sequence MKGF. GluA(Δ S24-AA) contained the C-tail sequence EFCYKSRAEAKRMKGF. GluA1(Δ MPR) had the following amino acids excised from the C-tail: KSRSESKRMKGFC, with the rest of the C-tail intact. GluA2((Q) Δ C) also truncated to amino acids EFCY, and GluA2((Q) Δ S47) ended in amino acids MKGF. GluK1 and Neto2 were cloned into the pCAGGs expression plasmid with GFP and mCherry, respectively, co-expressed behind an IRES.

Neuronal transfection. Sparse biolistic transfections of organotypic slice cultures were performed as previously described^{3,40}. Briefly, 80 μ g total of mixed plasmid DNA was coated on 1 μ M-diameter gold particles in 0.5 mM spermidine, precipitated with 0.1 mM CaCl₂, and washed four times in pure ethanol. The gold particles were coated onto PVC tubing, dried using ultra-pure N₂ gas, and stored at 4 °C in desiccant. DNA-coated gold particles were delivered with a Helios Gene Gun (BioRad). Cre expression was confirmed by mCherry epifluorescence, and replacement AMPA/KAR subunits confirmed by GFP epifluorescence.

For *in utero* electroporations, ~E15.5 pregnant *Gria1*–3^{fl/fl} mice were anaesthetized with 2.5% isoflurane in O₂ and injected with buprenorphine for analgesic. Embryos within the uterus were temporarily removed from the abdomen and injected with 2 µl of mixed plasmid DNA into the left ventricle via a bevelled micropipette. pFUGW-Cre:mCherry was typically diluted to approximately 0.5 µg µl⁻¹ in 2–3 µg µl⁻¹ of the replacement pFUGW AMPAR or pCAGGS GluK1 plasmid. Each embryo was electroporated with 5 × 50 ms, 35 V pulses. The positive electrode was placed in the lower right hemisphere and the negative electrode placed in the upper left hemisphere⁴¹. After electroporation, the embryos were sutured into the abdomen, and killed on P17–20 for LTP recording. For further detail on electroporation, please see ref. 42.

Electrophysiology. Voltage-clamp recordings were taken from CA1 pyramidal neurons in either acute hippocampal slices or organotypic slice cultures. For acute slices, 300 μ M transverse slices were cut using a Microslicer DTK-Zero1 (Ted Pella) in chilled high sucrose cutting solution containing (in mM): 2.5 KCl, 7 MgSO₄, 1.25 NaH₂PO₄, 25 NaHCO₃, 7 glucose, 210 sucrose, 1.3 ascorbic acid, 3 sodium pyruvate. The slices were then incubated for 30 min at 34 °C in artificial cerebral spinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26.2 NaHCO₃ and 11 glucose. For acute slices, 2.5 mM CaCl₂ and 1.3 mM MgSO₄ were added to the aCSF, and for organotypic slice cultures 4 mM CaCl₂ and MgSO₄ were added. The aCSF was bubbled with 95% O₂ and 5% CO₂ to maintain pH, and the acute slices allowed to recover at room temperature for 45 min to 1 h. Cultured slices were prepared as previously described³⁹, and recorded between 7–24 days *in vitro* (DIV) depending on the experiment. During recording, slices were transferred to a perfusion stage on an Olympus BX51WI upright microscope

and perfused at 2.5 ml min⁻¹ with aCSF containing 0.1 mM picrotoxin for acute slices experiments, and 0.01 mM gabazine, and 2–5 μ M 2–Cl⁻ adenosine for organotypic slice cultures. Synaptic responses were evoked by stimulating with a monopolar glass electrode filled with aCSF in stratum radiatum of CA1. To ensure stable recording, membrane holding current, input resistance, and pipette series resistance were monitored throughout recording. Data were gathered through a MultiClamp 700B amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 10 kHz.

Whole-cell synaptic recordings and LTP. Simultaneous dual whole-cell recordings were made between GFP and/or mCherry positive experimental cells as identified by epifluorescence, and neighbouring non-transfected control cells. Internal recording solution contained (in mM): 135 CsMeSO₄, 8 NaCl, 10 HEPES, 0.3 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP, and 0.1 spermine. Osmolarity was adjusted to 290-295 mOsm, and pH buffered at 7.3-7.4. AMPAR- and KAR-mediated responses were isolated by voltage-clamping the cell at -70 mV, whereas NMDA responses were recorded at +40 mV, with amplitudes taken 100 ms after stimulation to avoid contamination by AMPA receptor current. Paired-pulse ratios of AMPAR EPSCs were taken by stimulating twice at a 40-ms interval. To examine AMPA receptor rectification, 0.1 mM AP5 (D(-)-2-amino-5-phosphonovaleric acid) was washed in to block NMDA receptors. LTP was induced by stimulating at 2 Hz for 90 s while clamping the cell at 0 mV, after recording a stable 3-5 min baseline, but not more than 6 min after breaking into the cell. To minimize run-up of baseline responses during LTP, slices were stimulated for ~ 10 min before breaking in, and both cells held cell-attached for 2-5 min before breaking into the whole cell. Before breaking in, stimulation intensity was calibrated just below the threshold required to elicit an action potential from the wild-type control neuron. Rectification was calculated as the ratio of the slopes of the lines connecting AMPA EPSC amplitude from 0 to +40 mV and from -70 mV to 0 mV. This calculation can be taken as follows: $RI = 7(I_{40} - I_0)/4(I_0 - I_{70})$ where I_x represent EPSC amplitude at x mV.

Outside-out patches. Outside-out patches were taken from CA1 cells by obtaining whole-cell access to CA1 pyramidal neurons at -70 mV with a 4-5 M Ω patch pipette, then slowly pulling the pipette away from the soma until a high-resistance seal reformed. HEPES-aCSF containing (in mM) 150 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 1 MgCl₂, 2 CaCl₂, 0.1 AP5, 0.1 picrotoxin, 0.1 cyclothiazide and 0.5 μ M TTX was then perfused over the tip of the pipette. Glutamate and kainate currents were evoked by perfusion of HEPES-ACSF containing 1 mM L-glutamic acid and 1 mM kainic acid, respectively. A ValveLink 8 (AutoMate Scientific Inc.) was used for fast perfusion of control, glutamate and kainate containing HEPES-ACSF. During outside-out patch experiments, experimental cells were interleaved with non-transfected control cells. Rectification was calculated as in synaptic experiments.

Statistics. For all experiments involving unpaired data, including all outside-out patch data, a Mann–Whitney *U*-test with Bonferonni correction for multiple comparisons was used. For all experiments using paired whole-cell data, including all synaptic replacement and synaptic overexpression, a two-tailed Wilcoxon signed-rank test was used. LTP data were gathered as pairs of control and experimental neurons, but occasionally during experiments one of the cells would be lost. Comparisons were therefore made using the Mann–Whitney *U*-test, and the reported *n* values represent the number of cells at the end of each experiment. Data analysis was carried out in Igor Pro (Wavemetrics), Excel (Microsoft), and R (The R Project for Statistical Computing, http://www.r-project.org/).