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ORIGINAL PAPER

The AMPA Receptor Subunit GluA1 is Required for CA1 Hippocampal Long-Term Potentiation but is not Essential for Synaptic Transmission

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Abstract AMPA receptors mediate the majority of excitatory glutamatergic transmission in the mammalian brain and are heterotetramers composed of GluA1-4 subunits. Despite genetic studies, the roles of the subunits in synaptic transmission and plasticity remain controversial. To address this issue, we investigated the effects of cell-specific removal of GluA1 in hippocampal CA1 pyramidal neurons using virally-expressed GluA1 shRNA in organotypic slice culture. We show that this shRNA approach produces a rapid, efficient and selective loss of GluA1, and removed > 80% of surface GluA1 from synapses. This loss of GluA1 caused a modest reduction (up to 57%) in synaptic transmission and when applied in neurons from GluA3 knock-out mice, a similar small reduction in transmission occurred. Further, we found that loss of GluA1 caused a redistribution of GluA2 to synapses that may compensate functionally for the absence of GluA1. We found that LTP was absent in neurons lacking GluA1, induced either by pairing or by a theta-burst pairing protocol previously shown to induce LTP in GluA1 knock-out mice. Our findings demonstrate a critical role of GluA1 in CA1 LTP, but no absolute requirement for GluA1 in maintaining synaptic transmission. Further, our results indicate that GluA2 homomers can mediate synaptic transmission and can compensate for loss of GluA1.

Keywords LTP · AMPA receptor trafficking · Synaptic plasticity · Glutamate receptor · Hippocampus · Learning and memory · Cognition

Abbreviations

LTP	Long-term potentiation
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
NMDA	*N*-methyl-D-aspartate
CA1	Cornu ammonis 1

Introduction

Glutamate acting at postsynaptic AMPA receptors accounts for the vast majority of fast excitatory synaptic transmission in the mammalian brain. AMPA receptors are tetramers made up of combinations of the GluA1, 2, 3 and 4 subunits [1, 2]. These subunits are differentially expressed throughout the nervous system and thus different subtypes of AMPA receptors exist that contain different subunit combinations. The differential coupling and phosphorylation of subunits provides a basis for diversity in the trafficking and regulation of AMPA receptors at synapses and is hypothesised as an expression mechanism for forms of long-term synaptic plasticity [1, 3–5], but see [6, 7].

One cell type in which the roles of subunits in synaptic function and long-term synaptic plasticity have been

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extensively studied is the hippocampal CA1 pyramidal neuron. A prominent hypothesis is that GluA1-containing AMPARs are selectively incorporated into synapses during LTP and then subsequently exchanged for GluA2/GluA3 heteromers to maintain synaptic strength [8]. However, GluA3 is expressed at low levels in CA1 pyramidal neurons making this hypothesis controversial [1, 9, 10]. An alternative view is that AMPA receptor trafficking is regulated by their interaction with TARPs, in CA1 hippocampus predominantly γ8, and that this mechanism acts independently of AMPA receptor subunit composition to regulate receptor trafficking and plasticity expression [3, 7, 11, 12].

A more fundamental question concerns the subunits that make up the majority of AMPA receptors in CA1 pyramidal neurons. Biochemical studies show robust expression of GluA1 and GluA2 protein, and evidence that GluA1/2 heteromers form a major population of receptors in CA1 [13]. Studies using knock-out mice show a small and/or a variable effect of AMPA receptor subunit loss on synaptic transmission in CA1 pyramidal neurons [14–20]. Genetic loss of GluA1 causes a deficit in LTP under some circumstances, but other studies show that LTP can be induced in GluA1 knock-out mice using a non-standard induction protocol [20–23]. Recent work using floxed AMPA receptor subunit mice and viral expression of Cre recombinase to produce cell specific knock out, suggests that GluA1 is required for the majority of synaptic AMPA receptors as well as for LTP, and a role for GluA1 in maintaining an extrasynaptic reserve pool of AMPA receptors necessary for LTP expression [6, 24].

Thus, there remains considerable controversy about the roles of specific AMPA receptor subunits in synaptic transmission and plasticity. To address this we used the most rapid genetic loss of function approach currently available, shRNA knock down, and focussed on the role of GluA1. We present evidence that GluA1 in CA1 pyramidal neurons is required for LTP, but is not absolutely necessary for maintenance of synaptic transmission. Further, our work indicates that in global genetic knock-out mice lacking GluA1, compensatory mechanisms exist that preserve LTP and synaptic transmission.

Materials and Methods

Animals and Antibodies

The use and care of animals used in this study were conducted according to the guidelines of the NIH Animal Research Advisory Committee. GluA4 antibody was kindly provided by the late Robert J. Wenthold (National Institute of Health, Bethesda, MD). The following antibodies are commercially available: GluA1 monoclonal (RH95), GluA1 polyclonal, GluA2, GluN2A, GluA3, and PSD-95 antibodies (Millipore Corporation), VAMP-2 antibody (Synaptic Systems), α–tubulin antibody (Sigma), GFP polyclonal antibody (Invitrogen).

Surface Biotinylation and PSD Fractionation

Primary hippocampal neurons were biotinylated with membrane impermeable EZ-Link Sulfo-NHS-SS-biotin as previously described [25]. Neurons were homogenized in hypotonic buffer (10 mM Tris–HCl, pH 8.0, 10 mM KCl, 0.1 mM EDTA) and passed through a 23 gauge needle twenty times. Sucrose was then added to a final concentration of 0.32 M and the nuclear pellet was removed by centrifugation at 800×*g* for 5 min. The supernatant was collected and centrifuged at 10,000×*g* for 20 min to obtain a crude synaptosome P2 pellet. The pellet was re-suspended in TE buffer (50 mM Tris–HCl, pH 8.0, 2 mM EDTA) and solubilized with 1% Triton X-100 for 15 min on ice. The lysate was centrifuged at 33,000×*g* for 30 min, the resulting supernatant being the Triton X-100-soluble fraction, and the pellet was further solubilized with 1% SDS in TE buffer. After the insoluble material was removed, the supernatant was harvested as the Triton X-100-insoluble (SDS-soluble) PSD fraction. Surface-expressed receptors from each fraction were isolated as previously described [25].

For PSD fractionation using mouse hippocampi, synaptic plasma membranes (SPM) were harvested by sucrose density gradient centrifugation as previously described [25, 26]. SPM was solubilized in 0.5% Triton X-100 for 15 min on ice and centrifuged at 32,000×*g* for 20 min. The resulting supernatant and pellet constituted the SPM-Triton-X-100-soluble fraction and PSDI fractions, respectively. An aliquot of the re-suspended PSDI was further solublilized in 0.5% Triton X-100 or 3% *N*-lauroyl sarcosyl for 10 min on ice, and then centrifuged at 201,800×*g* for 1 h. The resulting pellets were re-suspended in TE buffer, and used as PSDII and PSDIII, respectively.

Virus Preparation

FHUGW lentivirus vector constructs were utilized throughout this study [27] except for Fig. 1a in which the viruses were obtained from a commercial source (America Pharma Source). For GluA1 shRNA expression (#843), a short hairpin targeting the murine GluA1 sequence (GGA ATC CGA AAG ATT GGT T) was cloned following the H1 promoter. For GluA1 rescue studies, eGFP driven by the ubiquitin promoter was replaced with N-terminal eGFP-fused GluA1 which contains silent mutations (GGA ATt CGg AAa ATc GGT T) to avoid a target shRNA knockdown. The lentiviral particles were produced and infected as previously described [25].

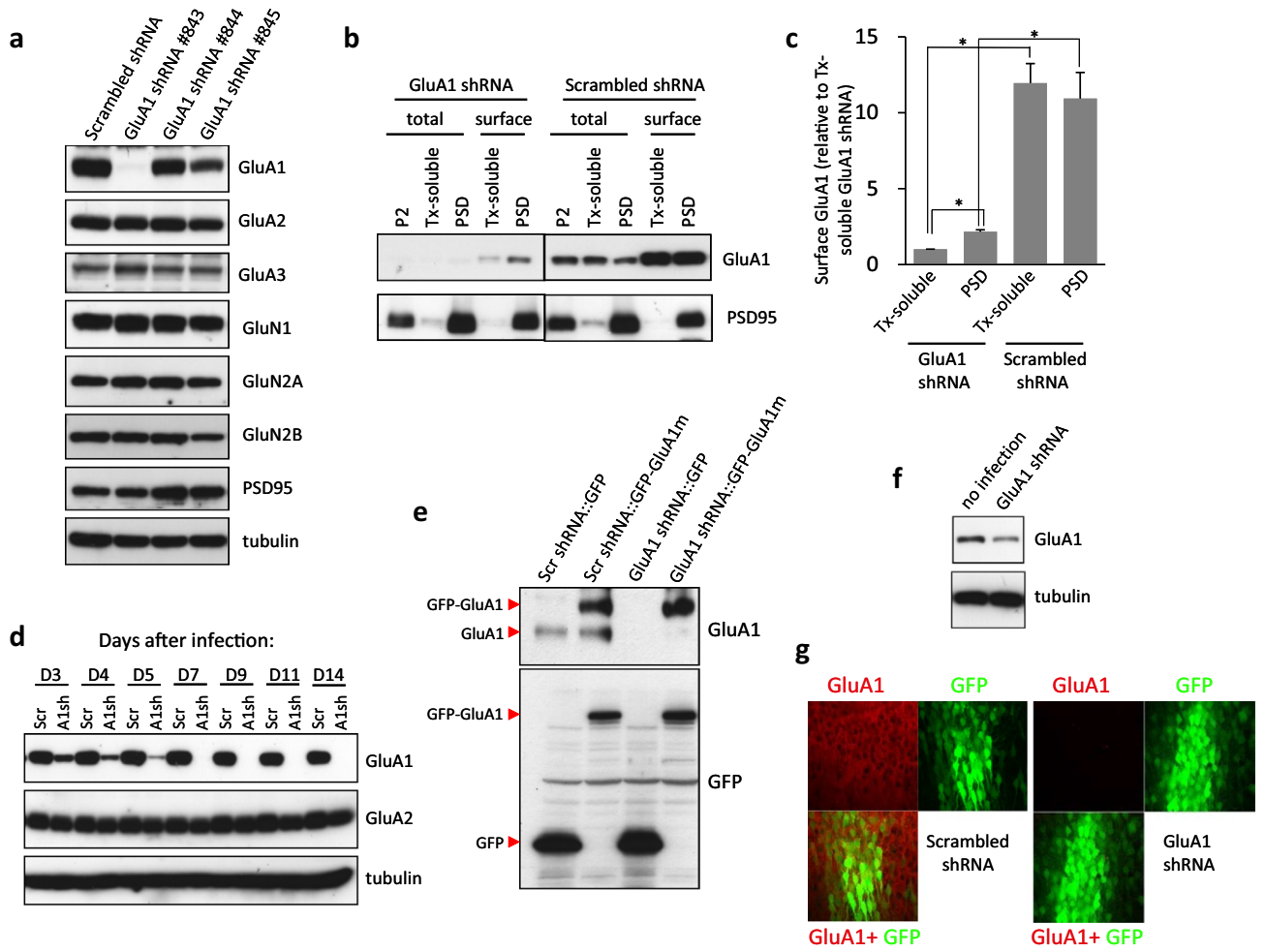

Fig. 1 GluA1 shRNA delivered using lentivirus causes a profound loss of GluA1 in hippocampal neurons. **a** Analysis of effects of three different GluA1 shRNA constructs and a scrambled control on expression of GluA1 and other synaptic proteins in primary cultured hippocampal neurons (infected at DIV7 analyzed at DIV15). **b** Subcellular fractionation from primary hippocampal neurons shows a profound loss of surface GluA1 from the triton X-100 soluble (Tx-soluble) and Tx-insoluble PSD fraction with GluA1 shRNA. **c** Quantification of effects of GluA1 shRNA on surface GluA1 in the Tx-soluble and PSD fraction (expressed relative to surface GluA1 in Tx-soluble fraction from GluA1 shRNA), $*p<0.01$ (n = 3). Bars represent mean ± s.e.m. **d** Time course of the effect of GluA1 shRNA (A1sh) or scrambled shRNA (Scr) on total GluA1 and GluA2 levels in primary hippocampal neurons (infected at DIV7). **e** Rescue of GluA1 expression using co-expression with GFP-GluA1 with silent mutations (GFP-GluA1m) to prevent knock down in primary hippocampal neurons. **f** Knock down of GluA1 by GluA1 shRNA lentivirus in CA1 hippocampus in cultured slice. Slice prepared from P7 rat pup, multiple injections of virus 1 h after slice preparation, analyzed 10 days later. Note that only approximately 50% of neurons are infected using this approach. **g** Immunohistochemistry of GluA1 expression in slices expressing scrambled shRNA or GluA1 shRNA by Lentivirus

Immunocytochemistry

Immunostaining in the cultured slices was performed as previous described [28]. Briefly, cultured slices were rinsed with PBS once and placed in a fix solution (4% PFA/4% sucrose in PBS) and incubated for 2 h at RT. The fix solution was removed, washed, and replaced with 30% sucrose in PBS at 4 °C overnight. The slices were freeze/thawed once and incubated in blocking solution (10% normal goat serum, 2% BSA, 0.25% Triton X-100 in PBS) for 2 h at RT. The blocking solution was removed and the slices were incubated at 4 °C overnight with anti-GluA1 antibody (RH95) diluted (1:250) in fresh blocking solution. The slices were washed four times for 15 min each with gentle shaking. Anti-mouse Alexa Fluor 568-conjugated secondary antibody was incubated for 4 h at RT with gentle shaking. The slices were washed four times for 15 min each with gentle shaking and mounted (ProLong Antifade Kit, Invitrogen). Serial Z-stack images were captured using a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc.) and maximum projection images were created as previously described [25].

Slice Culture Preparation and Viral Infection

For all synaptic physiology lentivirus shRNA expression experiments, hippocampal slice cultures 7–10 days in vitro were used, as previously described [29], to allow the lentivirus-mediated shRNA to effectively knock down GluA1 expression. Hippocampal slices (350 µm thick) were prepared from rats or mice aged P6-7 using a McIllwain tissue chopper. Following 1 h recovery after slicing, lentivirus was pressure ejected into part of the CA1 cell body layer and slices were explanted onto a membrane (Millicell-CM, 0.4 µm pore size) placed in 1 ml of MEM (GIBCO No. 61100-061) containing 2.5 mM glutamine, 30 mM Hepes, 5 mM $NaHCO_3$, 30 mM D-glucose, 0.5 mM L-ascorbate, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 1 µg insulin, and 20% horse serum. Slices were incubated at 35 °C for 7–10 days before use.

Electrophysiology

Whole-cell patch-clamp recordings were made from CA1 pyramidal neurons in slice cultures using standard techniques [29, 30]. Infected neurons were identified as those expressing GFP, and near-by non-infected neurons were used for in-slice controls. The extracellular solution during recordings was as follows (mM): 125 NaCl, 3.25 KCl, 1.25 $NaHPO_4$, 25 $NaHCO_3$, 4 $CaCl_2$, 4 $MgSO_4$, 10–15 glucose, 0.1 picrotoxin, 0.004 bicuculline and 4 µM 2-chloroadenosine saturated with 95% O_2/5% CO_2. The whole-cell recording solution was (mM): 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 1–5 QX-314, 0.1 spermine, pH 7.2, 285–290 mOsm. EPSCs were evoked by electrical stimulation of axons in stratum radiatum at a frequency of 0.25 Hz at a holding potential of −70 mV. For whole-cell recordings from CA1 pyramidal neurons in mouse cultured slices the same solutions as for rat slices were used except that Na-phosphocreatine (0.6 mM) was included in the intracellular solution. For analysis of the I–V relationship of EPSCs, 25 µM D-APV was added to the external solution. For the recordings of miniature EPSCs, 1 µM TTX was added to the external solution and the miniature EPSCs were recorded at a holding potential of −70 mV for 15 min. Only neurons expressing high levels of GFP were recorded from indicating a high level of shRNA expression. Data were collected using a Multiclamp 700B amplifier (Axon Instruments), filtered at 5 KHz and digitised at 10 KHz. EPSC amplitude, DC current, input resistance and series resistance were continuously monitored on-line. Recordings were terminated if series resistance deviated by more than 20%. For LTP experiments EPSCs at a holding potential of −70 mV were evoked at 0.25 Hz at two independent inputs by two stimulation electrodes placed in the stratum radiatum on the same side of the recording site, one proximal and the other distal to the pyramidal cell body layer. LTP was induced in one input, the other was the control pathway (the LTP and control inputs were randomly assigned prior to applying the induction protocol). The LTP pairing protocol was: voltage clamp, pairing 3 Hz stimulation with a holding potential of 0 mV for 3 min [31]. The theta-burst pairing LTP induction protocol was: current clamp, burst of 5 EPSPs and 5 co-incident postsynaptic APs at 100 Hz given 5 times at 5 Hz, all repeated three times at an interval of 10 s [22]. For the theta burst pairing LTP experiments, a current-clamp intracellular solution was used in which KMe SO_4 replaced $CsMeSO_4$ and QX314 was omitted.

Electrophysiology Analysis

AMPA EPSC rectification was estimated as previously described [29, 30]; briefly, ten averaged EPSCs (each an average of three consecutive single EPSCs) were collected at −70 mV, interleaved with ten averaged EPSCs (each an average of three consecutive single EPSCs) collected at 0 mv and ten averaged EPSCs (each an average of three consecutive single EPSCs) collected at +40 mV. The rectification index is expressed as EPSC−70/EPSC+40. In the viral expression experiments, the amplitude of the AMPA EPSC at −70 mV in infected cells was compared to that in neighbouring un-infected cells in the same slices with the same stimulus position and intensity, as previously described [29, 30]. Miniature EPSCs were analysed off-line using the Mini-Analysis software (Synaptosoft, Decatur, USA) and were detected using a threshold of twice the RMS noise (typically 4–6 pA) and subsequently verified visually [32]. For mEPSC amplitude a single mean value was obtained for each cell and then averaged across all cells with the same experimental manipulation. Statistical significance was assessed using an unpaired t-test vs scrambled shRNA control. For mEPSC frequency, event interval cumulative distribution was determined for all events pooled across all cells of the same manipulation and compared to scrambled control using a Kolmogorov–Smirnov (K–S) test. For determination if LTP was induced, the average EPSC amplitude at 25–30 min after induction was compared to the average amplitude during the baseline for the same pathway. Statistical analyses was performed using the Student's *t* test (paired or unpaired as appropriate) or K–S test, $P < 0.05$ was considered significant. In the figures $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

Results

Lentivirally-Delivered shRNA Causes Efficient Removal of GluA1 from CA1 Pyramidal Neurons

To knock down GluA1 in CA1 pyramidal neurons, we evaluated three shRNAs generated against GluA1 to identify one that was specific and most efficient. These shRNAs were encoded in lentiviral vectors together with GFP as a marker for infection. Using lentviral expression in cultured hippocampal neurons, which at the titre we used infects greater than 90% of neurons, we identified an shRNA, #843, which very efficiently knocks down GluA1 protein expression after 8 days of expression, but did not affect levels of a number of other synaptic proteins including NMDA receptor subunits and PSD95 (Fig. 1a). We next quantified the loss of GluA1 at synapses by using cell surface protein labelling combined with subcellular fractionation of cultured hippocampal neurons virally expressing shRNA #843. We found on average in three independent experiments that the GluA1 shRNA reduced surface GluA1 expression in the Triton X-100 soluble fraction (extrasynaptic) by 91% and in the Triton X-100 insoluble PSD fraction (synaptic) by 80% compared to scrambled shRNA (Fig. 1b, c). Next, we measured the time course of the loss of GluA1 and found that total GluA1 levels had started to decline 3 days after infection and by 7 days the loss of GluA1 appeared to be maximal (Fig. 1d). An important control for shRNA-mediated knock down studies is the ability to rescue function by expressing a mutant shRNA-resistant form of the protein of interest [33]. Therefore, we generated a virally-expressed rescue construct containing shRNA #843 and GFP-GluA1 mutated (GFP-GluA1m) at the shRNA recognition sequence to render it resistant to #843 shRNA knock down. The rescue construct efficiently knocked down endogenous GluA1 and expressed GFP-GluA1 in cultured hippocampal neurons (Fig. 1e). We also analysed the effects on GluA1 expression on a number of control rescue constructs containing GFP alone, and the equivalent constructs expressing scrambled shRNA (Fig. 1e). Taken together, this characterisation shows that using lentivirally-delivered shRNA, we can specifically, rapidly and profoundly knock down endogenous GluA1 at synapses in neurons, and rescue with an shRNA-resistant form of the subunit.

We also evaluated the effects of GluA1 knock down in hippocampal slices in culture. Slices were prepared from P7 rat pups and on the same day, 1 h later, lentivirus containing the GluA1 shRNA #843 was injected into the extracellular space in a region of CA1. To maximize infection as much as possible, multiple injections in the same region were performed. After 10 days of expression, Western blot analysis and immunocytochemistry showed a significant reduction in GluA1 protein in CA1 pyramidal neurons (Fig. 1f, g). The smaller reduction of GluA1 in slices compared to culture observed using Western blot analysis is likely due to the less efficient expression of virus in the slice and the difficulty of restricting the excision of tissue for analysis to that only expressing virus. Indeed, this is confirmed by the immunocytochemistry, which shows a very profound loss of GluA1 in infected (GFP positive) neurons.

Loss of GluA1 Causes a Partial Reduction in AMPA Receptor-Mediated Synaptic Transmission in CA1 Pyramidal Neurons

Having characterised the lentiviral knock down approach we investigated the effects of loss of GluA1 on synaptic transmission in cultured hippocampal slices as previously described [25, 29]. Hippocampal slices were prepared from P7 rat pups and virus injected 1 h after slice preparation. Following 7–10 days of viral expression whole-cell patch-clamp recordings were performed from CA1 pyramidal neurons expressing the construct (identified by GFP expression) or from neighbouring uninfected neurons (Fig. 2a–c). In a first set of experiments we assayed effects on basal AMPA receptor-mediated synaptic transmission by recording the amplitude and frequency of AMPA receptor-mediated miniature EPSCs (mEPSCs) in the presence of 1 uM TTX and picrotoxin. Loss of GluA1 caused a depression in mEPSC amplitude of 24% compared to uninfected control neurons in the same slices or to neurons expressing scrambled shRNA in separate controls experiments (Fig. 2b, c). Consistent with previous reports [14, 19], but see [24], there was no effect of loss of GluA1 on EPSC decay kinetics (not shown). We also found that the reduction in mEPSC amplitude was not observed when cells were infected with the GluA1 shRNA::GFP-GluA1 mutant rescue construct (Fig. 2b, c), demonstrating that the decrease in mEPSC amplitude with GluA1 knock down is due to loss of GluA1 and not an off target effect. There was no statistically significant change in mEPSC frequency observed with GluA1 knock down or GluA1 shRNA::GFP-GluA1 compared to scrambled shRNA control (scrambled shRNA: 0.37 Hz, GluA1 shRNA: 0.48 Hz, GluA1 shRNA::GFP-GluA1: 0.43 Hz). We next investigated effects on evoked transmission. The size of the EPSC evoked in infected and uninfected neurons in the same slice in response to the same stimulus intensity and location was compared. GluA1 knock down caused a 57% reduction in EPSC amplitude compared to in slice un-infected control neurons (Fig. 2d). Taken together, these findings show that loss of GluA1 causes a reduction in basal AMPA receptor-mediated transmission of up to 57% suggesting that at least approximately half of synaptic receptors require GluA1 subunits in CA1 pyramidal neurons.

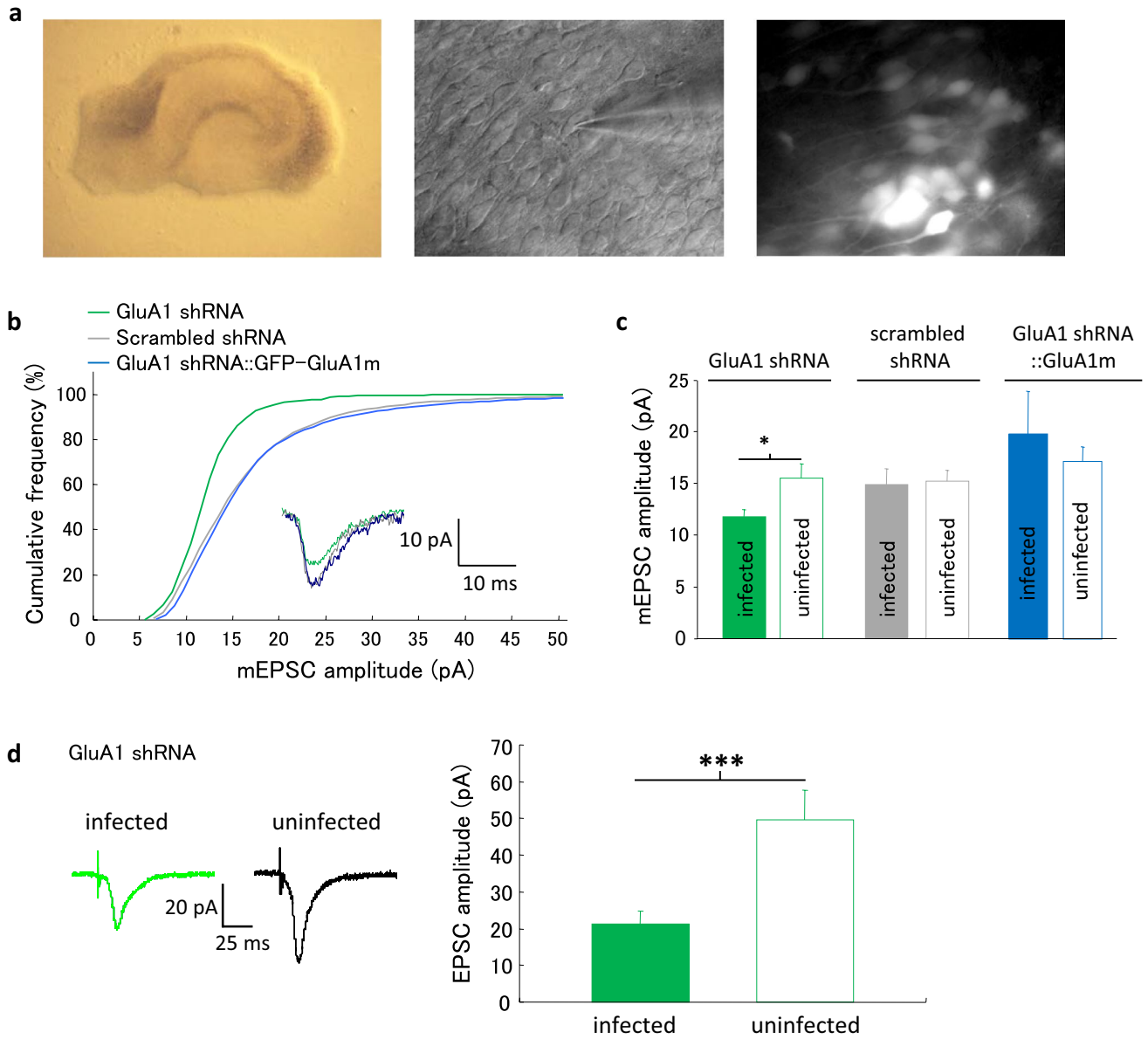

Fig. 2 Loss of GluA1 causes a partial reduction in AMPAR-mediated synaptic transmission. **a** Hippocampal slice culture; low power image (left), high power DIC image of CA1 region with patch electrode (middle); epifluorescence image of CA1 pyramidal neurons expressing virus (different slice from middle panel). **b** Cumulative frequency histogram of AMPA mEPSC amplitude for GluA1 shRNA, scr shRNA and GluA1 shRNA::GFP-GluA1mutant expressing cells (single examples; inset; averaged traces from these experiments). **c** Mean mEPSC amplitude for the three different viruses and in slice un-infected controls (GluA1 shRNA n = 9; scr shRNA n = 9; GluA1 shRNA::GFP-GluA1mutant n = 10). **d** Evoked EPSC amplitude (same stimulus intensity and position) for GluA1 shRNA infected and un-infected cells in the same slices (n = 7)

Removal of GluA1 from CA1 Pyramidal Neurons Lacking GluA3 Produces a Very Similar Reduction in AMPA Receptor-Mediated Transmission to Loss of GluA1 Alone

The GluA1 knock down data suggest that a significant proportion of synaptic AMPA receptors lack GluA1. This is consistent with the proposal that GluA2/3 heteromers mediate a significant fraction of synaptic transmission in CA1 pyramidal neurons [8, 13], but inconsistent with the low levels of GluA3 mRNA observed in this cell type [9, 10], the lack of impact of GluA3 knock out on synaptic transmission in CA1 [17] and the profound loss of transmission in cells in which GluA1 is deleted in individual cells genetically [24]. To further investigate this issue, we studied the effects of GluA1 knock-down in slices prepared

from GluA3 knock-out mice. We first confirmed the absence of GluA3 in hippocampus from the GluA3 knock-out and found no change in the levels of GluA1, 2 (Fig. 3a) or in GluA4 (not shown). We next investigated whether there are any compensatory changes in expression levels or in the subcellular distribution of AMPA receptors and other synaptic proteins. Other than loss of GluA3 in the knock out, no changes in expression levels or subcellular distribution were observed (Fig. 3b). Using patch-clamp recordings from infected and neighbouring uninfected CA1 pyramidal neurons we found that GluA1 knock down in neurons lacking GluA3 produced a trend to a reduction in mEPSC amplitude (20% reduction; Fig. 3c) and no change in mEPSC frequency (not shown). Thus, the effect of loss of GluA1 in the absence of GluA3 was very similar to that observed with loss of GluA1 alone. These findings indicate that GluA3-containing receptors do not make up any significant fraction of synaptic AMPA receptors in CA1 pyramidal neurons.

Compensatory Increase of GluA2 at Synapses in Neurons Lacking GluA1

The modest effect of GluA1 knock down and the lack of any additional effect of the combined absence of GluA1 and GluA3 on synaptic transmission indicate that the other AMPA receptor subunits, GluA2 or GluA4, may mediate synaptic transmission in the absence of GluA1. GluA4 is not expressed in CA1 pyramidal neurons [15, 24, 34]; therefore, the AMPA receptor-mediated EPSC observed in the absence of GluA1 and GluA3 is presumably mediated by GluA2 homomers. Initial studies on recombinant systems indicated that edited GluA2 homomers possess a very low single channel conductance and thus produce almost no detectible current on activation [35]. However, more recently it has been found that when edited GluA2 homomers co-assemble with TARPs, a single channel conductance similar to GluA1/GluA2 heteromers is observed [36]. In the present study to

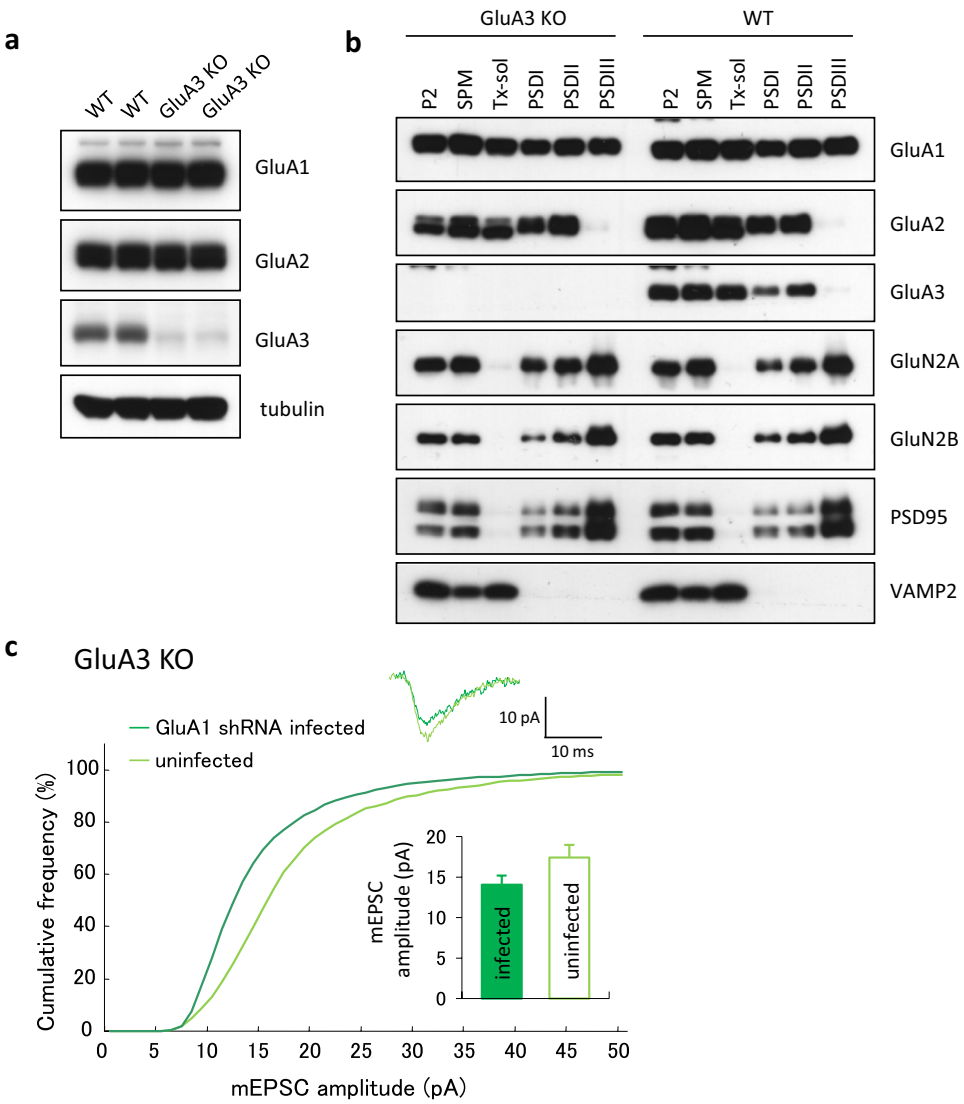

Fig. 3 Partial loss of AMPAR-mediated synaptic transmission in hippocampal CA1 pyramidal neurons lacking GluA1 and GluA3. **a** Analysis of AMPA receptor subunit expression in hippocampus from 2 week old wild type and GluA3 KO mice. **b** Analysis of expression of synaptic proteins in subcellular fractions from 2 week old hippocampus of GluA3 KO and wild type mice. **c** AMPA mEPSC amplitude in GluA3 KO neurons expressing GluA1 shRNA (n = 12)

investigate the possibility that GluA2 can compensate for loss of GluA1 at synapses, we quantified the levels of GluA2 in the PSD in cultured hippocampal neurons expressing the GluA1 shRNA (using the same approach as described for Fig. 1b, c). In cells in which GluA1 had been knocked down, surface and total GluA2 levels in the PSD fraction were approximately double those observed in cells expressing scrambled shRNA (Fig. 4a, b). This compensation is notable for its rapidity: the GluA1 shRNA was expressed for 7–10 days and causes a loss of GluA1 starting after 5 days of expression (see Fig. 1d); therefore, GluA2 compensates within a few days of loss of GluA1. There is evidence that edited GluA2 homomers exhibit an outward rectification [37, 38], but see [24]. We therefore measured the rectification properties of pharmacologically-isolated evoked AMPA receptor-mediated EPSCs. AMPA EPSCs in GluA1-lacking neurons exhibited an outward rectification; whereas, neurons expressing scrambled shRNA exhibited linear or slightly inwardly rectifying I–V relationships (Fig. 4c, d). Thus, our data show that GluA2 levels increase at synapses in neurons lacking GluA1 and indicate that GluA2 homomers, presumably co-assembled with TARPs, produce functional AMPA receptors capable of mediating synaptic transmission that can compensate for the lack of GluA1.

GluA1 is Required for LTP in CA1 Pyramidal Neurons

The requirement for GluA1 in the expression of hippocampal LTP is controversial: studies using recombinant GluA1 expression indicate that GluA1 is recruited to synapses

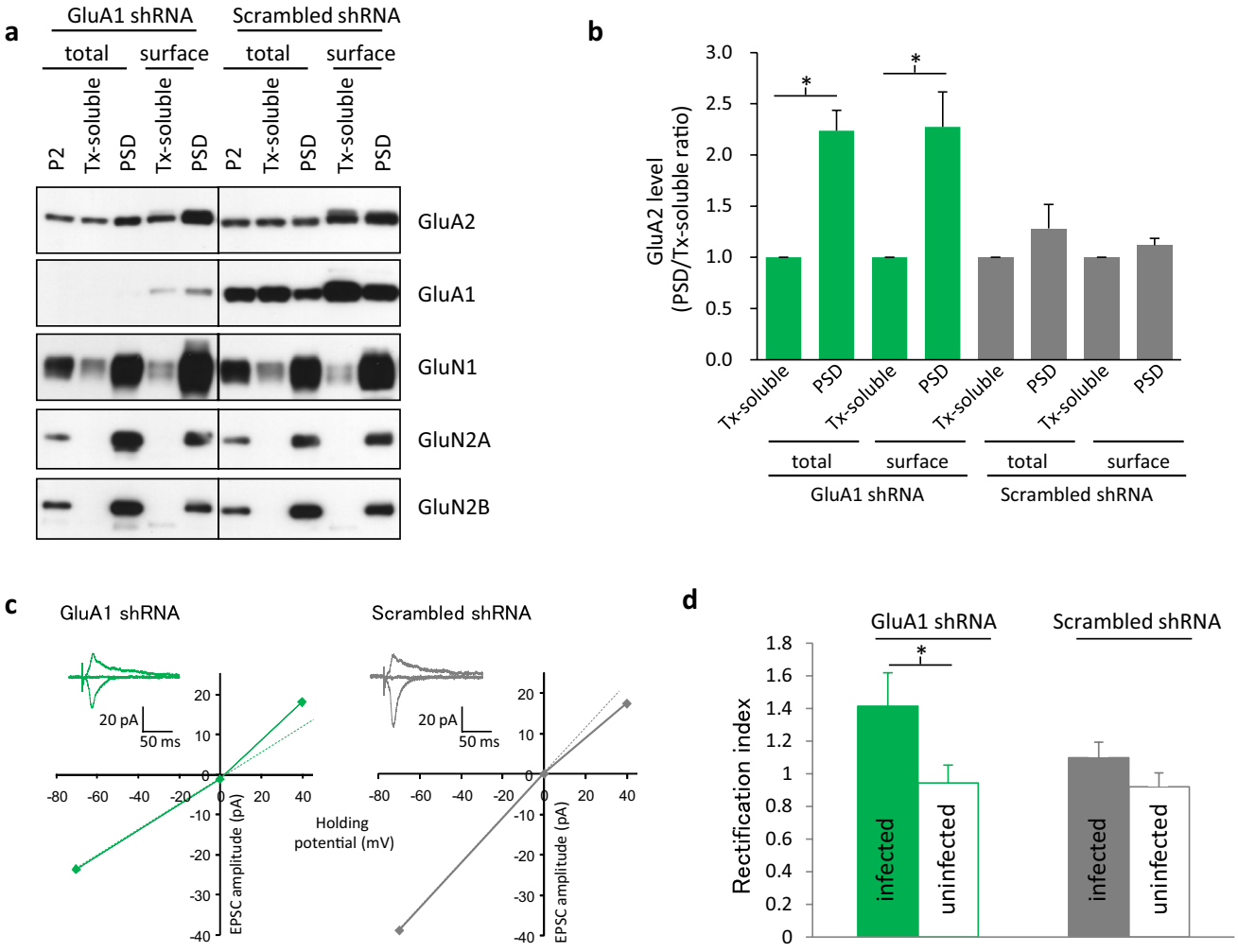

Fig. 4 A doubling of GluA2 at synapses and outwardly rectifying AMPA EPSCs in neurons in which GluA1 has been acutely knocked down. **a** Surface biotinlyation analysis of surface GluA2 in PSD from cultured neurons in which GluA1 has been knocked down using GluA1 shRNA virus (n = 4). **b** Quantitation of GluA2 levels in Triton-X soluble and insoluble (PSD) fractions for total and surface labelled protein in GluA1 knock down or scrambled shRNA controls (n = 4). Bars represent mean ± s.e.m. **c** Example I–V plots for pharmacologically-isolated AMPA EPSCs in CA1 pyramidal neurons expressing GluA1 shRNA or scrambled shRNA. **d** Mean data for rectification index for experiments as in **c** (n = 9)

during LTP [5, 39]. Initial work on the GluA1 knock out supported the view that GluA1 is required for LTP expression [19], however, subsequent work shows that LTP can be induced in hippocampal slices from the GluA1 knock-out earlier in development or in adult using different types of induction protocols [15, 20–23]. Nevertheless, more recent work using Cre-dependent single cell deletion of GluA1, showed a loss of LTP in the absence of GluA1 [6]. One possibility to explain these disparate results is compensation in the global GluA1 knock-out that preserves LTP. We took advantage of the relatively rapid and cell autonomous loss of GluA1 afforded by the shRNA approach to test the requirement for GluA1 in LTP. Using two-pathway LTP experiments, we found that a pairing induction protocol failed to induce LTP in neurons expressing GluA1 shRNA (Fig. 5a). In interleaved control experiments, the pairing protocol induced robust LTP in neurons expressing the scrambled shRNA (Fig. 5b). We next tested whether LTP induced by a theta-burst pairing protocol requires GluA1. This protocol was shown previously to successfully induce LTP in CA1 pyramidal neurons in slices from the GluA1 knock out [21–23]. However, we found that LTP induced by this protocol was absent in neurons expressing the GluA1 shRNA (Fig. 5c). In cells expressing the scrambled shRNA, the theta burst pairing protocol produced potentiation in the LTP pathway (measured at 25–30 min post induction) but no lasting potentiation in the control pathway, despite a transient potentiation that was observed (Fig. 5d). The transient potentiation of the control pathway is consistent with previous work using this particular induction protocol [22, 40].

Finally, to determine that the loss of LTP we observed was due to lack of GluA1 rather than an off-target effect of the shRNA, we used the GluA1 shRNA::GFP-GluA1 mutant rescue construct. In neurons virally expressing this rescue construct, robust LTP was induced using the pairing protocol that was indistinguishable from LTP induced in interleaved scrambled shRNA control experiments (Fig. 5e, f). These findings, therefore, demonstrate a requirement for GluA1 in CA1 LTP induced by pairing or the theta-burst pairing protocols. Further, they suggest that the capability of CA1 pyramidal neurons from global GluA1 knock-out mice to

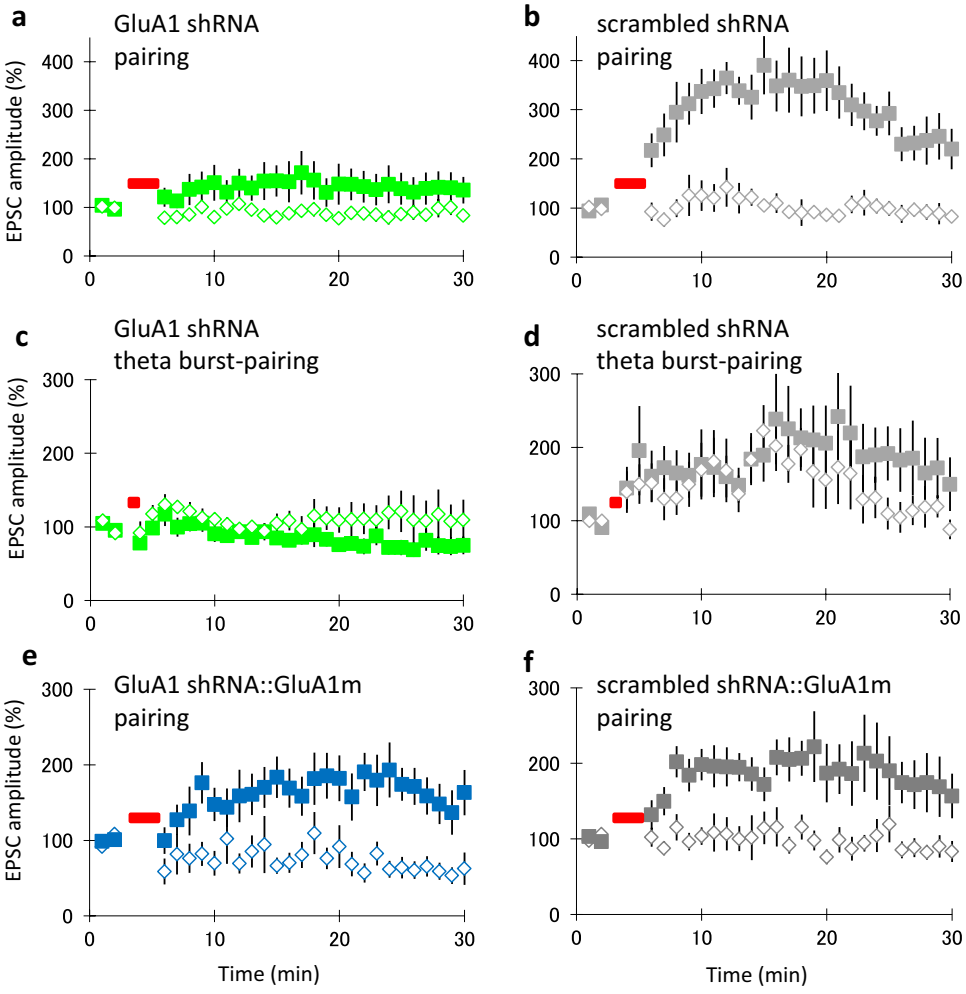

Fig. 5 GluA1 is required for multiple forms of hippocampal LTP. **a** LTP induced by a pairing protocol is absent in neurons expressing GluA1 shRNA (n = 8; two-pathway experiments, green symbols represent LTP path, pink control path). **b** Scrambled shRNA does not prevent pairing induced LTP (n = 8). **c** LTP induced by a theta-burst pairing protocol is absent in neurons expressing GluA1 shRNA (n = 10; two-pathway experiments, green symbols represent LTP path, pink control path). **d** Scrambled shRNA does not prevent theta-burst pairing induced LTP (n = 10). **e** The GluA1 shRNA deficit in pairing-induced LTP is rescued by GluA1 expression (n = 8). **f** Scrambled shRNA control for the rescue experiment (n = 8). (Color figure online)

express LTP is the result of compensation to global germ line removal of GluA1.

Discussion

Here using virally-delivered shRNA to knock down GluA1, we show a role for GluA1 in synaptic transmission, but not a requirement. In contrast, we find that GluA1 is required for CA1 LTP. Additionally, we find no evidence for GluA3 involvement in synaptic AMPA receptors, but a redistribution of GluA2 homomers to synapses in the absence of GluA1, indicating that GluA2 homomers can functionally compensate for GluA1-containing AMPA receptors at synapses.

The Subunit Composition of Synaptic AMPA Receptors in CA1 Pyramidal Neurons

The subunit composition of AMPA receptors at synapses in CA1 pyramidal neurons is a matter of considerable controversy. Genetic knock-out of GluA1, GluA2 or GluA3 produces variable effects on the size of the synaptic AMPA receptor-mediated response [14–19]. A prominent hypothesis is that GluA1/2 receptors are selectively recruited to synapses during LTP and then subsequently exchanged for GluA2/3 receptors [5]. However, the very low levels of expression of GluA3 in CA1 pyramidal neurons [9, 10] and the lack of effect of the GluA3 knock out on synaptic transmission and LTP [17, 24] does not support this model. Our data further provide evidence against the presence of a major population of GluA2/3, because knock down of GluA1 either in wild type or in neurons lacking GluA3 produced indistinguishable, modest effects of synaptic transmission. Therefore, it seems unlikely that GluA2/3 receptors exist in any large amount in CA1 pyramidal neurons and therefore could not fulfil the hypothesised role in the stabilisation/maintenance of synaptic strength in LTP.

Single cell Cre-mediated postnatal deletion of GluA1-4 (in transgenic mice in which the genes have been floxed) has also been used to address the roles of AMPA receptor subunits in synaptic transmission in CA1 pyramidal cells [24]. This approach potentially avoids some of the confounding factors of redundancy and compensation encountered with global germ-line removal of AMPA receptor subunits. This study provided evidence that GluA1/2 heteromers mediate the vast majority of synaptic AMPA receptor transmission with only a very minor role for GluA2/3 heteromers. One striking disparity between this work and our present study is the size of the effect of loss of GluA1. Cre-mediated deletion of GluA1 was found to cause > 90% reduction in the size of the EPSC, whereas, our more acute shRNA-mediated loss of GluA1 caused a 24% reduction in mEPSC amplitude and a 57% loss of evoked EPSC amplitude. It is important to consider whether the differences between our study and that of the previous work using genetic deletion of GluA1 can be explained by an incomplete knock down of GluA1 in the present study. We think this is unlikely for two reasons: (1) In studies using germ-line removal of GluA1 a modest reduction at most in AMPAR-mediated synaptic transmission in CA1 pyramidal cells is reported (e.g. [19, 20, 23]), and (2) we show using biochemical analysis of surface expressed GluA1 in the PSD that > 80% loss of synaptic GluA1 is achieved by our shRNA. Thus, our data strongly indicate that the more modest effect on synaptic transmission that we observe compared to the study of Lu and co-workers is due to the more rapid removal of GluA1 achieved with our virally-delivered shRNA compared to the Cre-mediated excision of the gene and subsequent loss of expression. Indeed, we show biochemically that GluA1 is lost within 7 days of viral infection and at this time point we observe the modest reduction in synaptic transmission. In contrast, Lu et al. show that it takes 14 days for the effects of Cre-mediated GRIA1 excision on synaptic transmission to reach steady state, either in vivo or in slice culture. The precise temporal relationship of this loss of synaptic function to loss of GluA1 protein is unknown in the Lu et al. study because no analyses of GluA1 protein levels were reported. Therefore, one possibility to explain the much greater loss of synaptic transmission in that study is that loss of GluA1 results in a more slowly developing depression of synaptic transmission due to an absence of an on-going activity-dependent synaptic strengthening that otherwise maintains synaptic strength. This possibility is further supported by the finding that cell specific deletion of GluA1 causes a loss of LTP (present study, [6]).

Despite the potential advantages of our knock down approach for studying the roles of individual AMPA receptor subunits, a confounding issue of possible compensation of function by other subunits remains. Indeed we observe a twofold increase in GluA2 levels at synapses in neurons in which GluA1 was knocked down. This finding suggests that the loss of GluA1 can be at least partially functionally compensated for by GluA2 homomers and indicates that even with this relatively rapid loss-of-function manipulation, it is hard to precisely estimate the contribution of GluA1 to synaptic transmission. The functional compensation by GluA2 suggests that the up to 57% loss of AMPA EPSC amplitude with GluA1 removal is an underestimate of the true contribution of GluA1 to synaptic transmission under normal physiological conditions.

Our finding that GluA1 knock down in CA1 pyramidal neurons from GluA3 KO mice results in only a small reduction the AMPAR EPSC further strongly supports the idea that GluA2 homomers can functionally compensate for loss of GluA1 at CA1 synapses. Since we achieve > 80%

reduction in synaptic GluA1 with our shRNA it is very unlikely that synaptic transmission is being supported by residual GluA1 subunits. Furthermore, the EPSCs present in the absence of GluA1 and GluA3 do not show inward rectification indicating that they are not mediated by unedited GluA2. Original work on GluA2 showed that the great majority of GluA2 is edited at the Q/R site and that this produces GluA2 homomers with a fS single channel conductance, at least ten fold lower than the single channel conductance for other AMPA receptor subunit combinations [35]. Thus, it had been assumed that edited GluA2 homomers are not functionally relevant. However, this view has been challenged more recently by the finding that edited GluA2 homomers co-assembled with TARP exhibit pS single channel conductance, similar to other subunit compositions [36]. Our present work now provides evidence that native edited GluA2 homomers, presumably associated with TARP, are indeed functional and can support synaptic transmission. Thus, the role of GluA2 homomers in neuronal function may need to be re-evaluated. Finally, it is worth noting that the rapid and aggressive compensation by GluA2 for loss of GluA1 highlights the ability of neurons and neural circuits to rapidly adapt to maintain critical functionality further illustrating the need to interpret genetic loss- or gain-of-function studies with caution [41].

The Requirement for GluA1 in LTP in CA1 Pyramidal Neurons

Previous work examining the role of GluA1 in CA1 hippocampal LTP provided conflicting results concerning the requirement for GluA1 in LTP expression. Over-expressed recombinant GluA1 is actively recruited to synapses during LTP to mediate the increase in postsynaptic AMPA receptors [8, 39, 42, 43]. However, GluA1 knock-out mice can express CA1 LTP under certain conditions: during development [15] and at more mature stages in response to a theta-burst pairing induction protocol [20–23]. In contrast cell specific deletion of GluA1, either using Cre-mediated excision of floxed alleles [6] or shRNA (present study), shows that removal of GluA1 leads to a loss of LTP induced either by pairing (both studies) or by the theta-burst pairing protocol (present study). Previous work shows that loss of GluA1 either by global knock out or in a cell automonous manner using floxed alleles does not lead to a reduction in synaptic NMDA receptor function [15, 19, 24]. Thus, the loss of LTP in GluA1 lacking neurons is unlikely to be due to a loss of NMDA receptor function, but rather indicates that GluA1 is required for LTP expression. For the loss of LTP induced by pairing, note that in the present study we use slices at a similar developmental stage to those in which LTP could be induced in the GluA1 knock-out. Therefore, our data suggests that the ability to express LTP in CA1 hippocampus in the GluA1 knock out represents a compensation that preserves LTP, a critical mechanism for brain development and survival. Our data suggest that GluA1 is an absolute requirement for CA1 LTP in wild type brain.

In the recent study of Granger et al. [6], a new hypothesis was put forward for the role of GluA1 in LTP expression, in which GluA1 maintains an extrasynaptic pool of AMPA receptors that are required for LTP expression. This hypothesis is consistent with our present work; however, the hypothesis is inconsistent with a previous study [44] in which hypomorphic rescue of GluA1 expression in CA1 pyramidal neurons from GluA1 knock out mice resulted in no rescue of the extrasynaptic pool of AMPA receptors, yet LTP was restored. Future studies will be necessary to resolve this interesting issue. Granger and colleagues also show that LTP can be rescued in neurons lacking GluA1 by other AMPA receptor subunits and indeed by kainate receptor subunits [6, 7]. These intriguing findings point to a lack of specificity of the trafficking mechanism that recruits glutamate receptor subunits to synapses to achieve the increase in synaptic strength during LTP expression. However, our data and that of Granger et al., firmly points to the absolute requirement of GluA1 for the expression of LTP in wild-type CA1 pyramidal neurons.

Conclusion

In summary our data support the idea that GluA1 plays a requisite role in LTP in CA1 pyramidal neurons, but that it is not absolutely required for basal synaptic transmission. We find no evidence for the presence of GluA3-containing AMPA receptors in CA1 pyramidal neurons, but do observe a rapid functional compensation by GluA2 homomers for loss of GluA1 from synapses. This latter finding demonstrates the remarkable adaptive ability of neurons and circuits and urges caution for interpreting genetic loss or gain of function studies.

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