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Age-dependent requirement of AKAP150-anchored PKA and GluR2-lacking AMPA receptors in LTP

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Association of PKA with the AMPA receptor GluR1 subunit via the A kinase anchor protein AKAP150 is crucial for GluR1 phosphorylation. Mutating the AKAP150 gene to specifically prevent PKA binding reduced PKA within postsynaptic densities (>70%). It abolished hippocampal LTP in 7–12 but not 4-week-old mice. Inhibitors of PKA and of GluR2-lacking AMPA receptors blocked single tetanus LTP in hippocampal slices of 8 but not 4-week-old WT mice. Inhibitors of GluR2-lacking AMPA receptors also prevented LTP in 2 but not 3-week-old mice. Other studies demonstrate that GluR1 homomeric AMPA receptors are the main GluR2-lacking AMPA receptors in adult hippocampus and require PKA for their functional postsynaptic expression during potentiation. AKAP150-anchored PKA might thus critically contribute to LTP in adult hippocampus in part by phosphorylating GluR1 to foster postsynaptic accumulation of homomeric GluR1 AMPA receptors during initial LTP in 8-week-old mice.
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Introduction

LTP is thought to underlie learning and memory (Martin *et al*, 2000; Bredt and Nicoll, 2003; Collingridge *et al*, 2004; Malenka and Bear, 2004; Whitlock *et al*, 2006). PKA is critical for the transcription-dependent late phase of LTP (Frey *et al*, 1993; Nayak *et al*, 1998; Winder *et al*, 1998) at the hippocampal CA3-CA1 synapse but its role in early LTP is less clear. Regulation of various ion channels, including AMPA receptors (AMPARs), by PKA depends on anchoring of this kinase by specific AKAPs (Rosenmund *et al*, 1994; Wong and Scott, 2004). AKAP150, also called AKAP5, is the major AKAP that recruits PKA to postsynaptic sites (Gomez *et al*, 2002; Wong and Scott, 2004). Our earlier work shows that the postsynaptic scaffolding protein SAP97 directly binds to the AMPAR GluR1 subunit (Leonard *et al*, 1998) and AKAP150, thereby linking AKAP150 and the associated PKA to GluR1 (Tavalin *et al*, 2002; Wong and Scott, 2004). Association of PKA via AKAP150 and SAP97 with GluR1 is crucial for phosphorylation of GluR1 at S845 by PKA (Tavalin *et al*, 2002). This phosphorylation stimulates postsynaptic accumulation of GluR1-containing AMPARs during LTP (Esteban *et al*, 2003; Oh *et al*, 2006).

About 8% of the AMPARs in 5- to 6-week-old rats are GluR1 homomeric receptors (Wenthold *et al*, 1996). In contrast to the majority of AMPARs, which contain GluR2, these AMPARs are Ca^{2+} permeable and inwardly rectifying due to an intracellular pore block by spermine under depolarized (outside negative) conditions (Bowie and Mayer, 1995; Kamboj *et al*, 1995; Koh *et al*, 1995). These AMPARs are temporarily functionally expressed to increase postsynaptic AMPAR responses during various forms of synaptic plasticity (Thiagarajan *et al*, 2005; Bellone and Luscher, 2006; McCormack *et al*, 2006; Sutton *et al*, 2006). In 2-week-old mice, the first 10–15 min of LTP are mediated by postsynaptic incorporation of rectifying, GluR2-lacking AMPARs, which are later replaced by GluR2-containing AMPARs (Plant *et al*, 2006; see also Figure 8 and Discussion, but see Adesnik and Nicoll, 2007). At this young age LTP is partially dependent on PKA (Wikstrom *et al*, 2003). GluR4 is expressed in the hippocampus in young rodents (Jensen *et al*, 2003), albeit at least in more adult mice at a rather low level (Wenthold *et al*, 1996), and, like GluR1, accumulates at postsynaptic sites upon activation of PKA (Esteban *et al*, 2003). It is conceivable that PKA fosters accumulation of GluR4 or GluR1 homomeric AMPARs during early LTP at this age.

To test whether PKA anchoring is required for synaptic plasticity, we truncated the C-terminus of AKAP150 by 36 residues by introducing a stop codon in the mouse genome at the respective position (D36 mice). These 36 residues bind to and anchor the native dimers of type II regulatory R subunits of PKA, with each RII subunit binding one catalytic C subunit (Wong and Scott, 2004). RII subunit binding to various AKAPs mediates most of the targeted PKA localization, whereas RI subunits are anchored to a much lesser degree. There are two isoforms of type II R (RIIα and RIIβ) and two isoforms of C (Cα and Cβ) present in mice. The D36 mutation does not disrupt the overall expression of AKAP150 and leaves intact the identified binding sites for F-actin, calmodulin, cadherin, PKC, calcineurin, and phosphatidylinositol-4,5-bisphosphate (Gomez *et al*, 2002; Gorski *et al*, 2005).

We find that hippocampal LTP induced by a single tetanus is normal in 4 but absent in 7- to 12-week-old D36 mice. We show that inhibitors of PKA and of GluR2-lacking AMPARs prevent LTP in hippocampal slices from 8 but not 4-week-old WT mice. We conclude that AKAP150-anchored PKA critically contributes to LTP in the adult hippocampus, likely at least in part via phosphorylation of GluR1 (Lee *et al*, 2003) to

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induce the functional availability of GluR1 homomeric AMPARs at postsynaptic sites.

Results

Production and genotyping of D36 mice

We engineered a stop codon into the mouse AKAP150 gene to truncate the last 36 residues, which constitute the PKA binding site (see Supplementary data). For genotyping, DNA was isolated from tail clippings. The 3′ coding region of AKAP150 was amplified by PCR and treated with *Xba*I to detect whether one or both alleles contained the *Xba*I restriction site. This site was only present in the newly created stop codon in the D36 allele, which yielded cleavage products of 208 and 119 bp (Figure 1). Homozygous WT mice exhibited only the uncleaved 327 bp band, whereas all three DNA fragments were detectable in heterozygous mice (Figure 1).

Postsynaptic targeting of PKA by AKAP150

To quantify the level of PKA at postsynaptic sites, we enriched postsynaptic densities (PSDs) from D36 and litter-matched WT mice by subcellular fractionation using differential and sucrose gradient centrifugations (Carlin *et al*, 1980). Immunoblotting of the different fractions illustrates that AKAP150 was prominent in the synaptosomal fraction, which mainly contained pre and postsynaptic membranes (Figure 2A, top; 'Syn'). Extraction of this fraction with Triton X-100 effectively solubilized presynaptic membranes but not the core of PSDs ('Tx-1') (Carlin *et al*, 1980). PSDs were further purified by sucrose gradient centrifugation. A second extraction with Triton X-100 and subsequent ultracentrifugation yielded final PSD fractions (Carlin *et al*, 1980). The steep enrichment of the Triton X-100-resistant specific PSD marker PSD-95 and the complete loss of the presynaptic marker synaptophysin documented the high quality of these PSD preparations (Figure 2A, bottom). The presence of equal amounts of PSD-95 in PSDs from D36 and WT mice served as a second loading control in addition to our protein assay

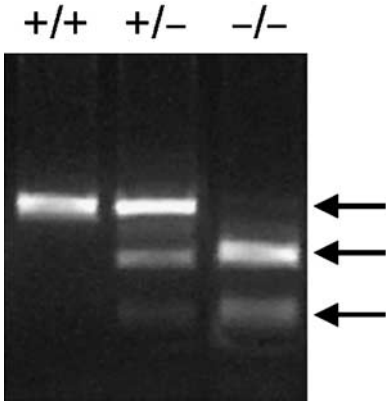

Figure 1 Genotyping of AKAP150 D36 mice. The AKAP150 C-terminal coding sequence was amplified by PCR from tail DNA. The resulting 327 bp product (+/+) was treated with *Xba*I to detect the restriction site that had been introduced by the creation of the stop codon that truncated the last 36 amino acids of AKAP150. The D36 but not the WT allele gave rise to a 208 bp fragment and a 119 bp fragment (−/−). Arrows indicate from top 327, 208, and 119 bp bands.

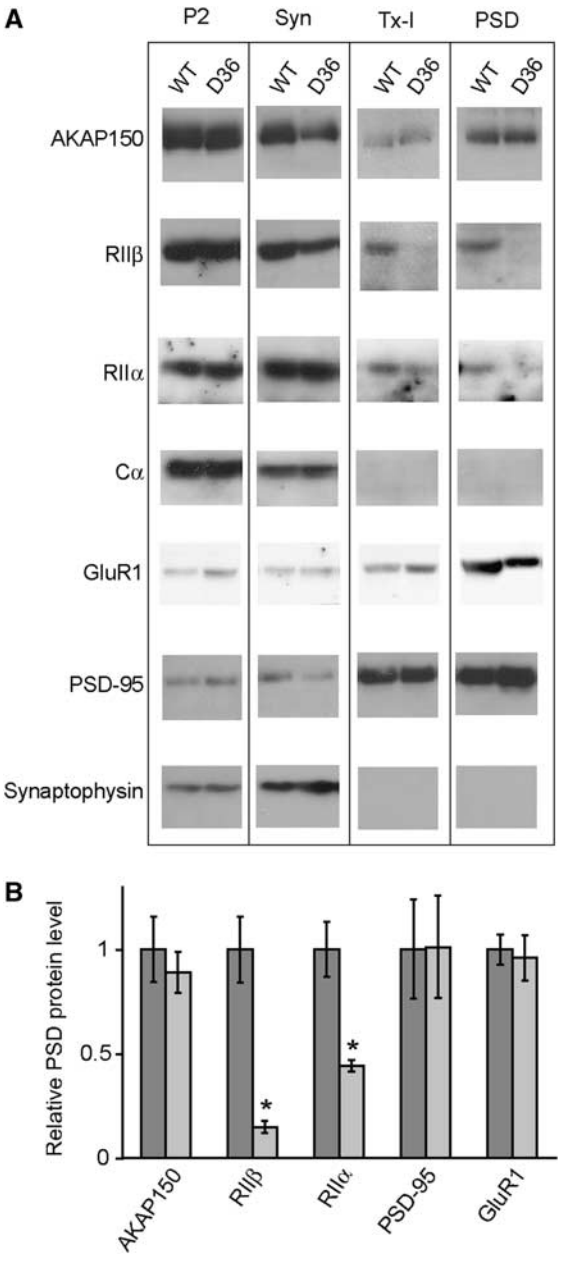

Figure 2 PKA is reduced in PSDs from D36 mice. (**A**) Subcellular fractions were prepared from forebrains of WT and D36 mice that were 8 weeks or older by differential centrifugation (P2), sucrose gradient centrifugation (enriched for synaptosomes, Syn), extraction with Triton X-100 and subsequent sucrose gradient centrifugation to remove presynaptic material (Tx-I), and a second extraction with Triton X-100 before ultracentrifugation to obtain purified PSDs (PSD). A 10 μg weight of total protein for each fraction (quantified by bicinchoninic acid protein assay) was separated by SDS–PAGE in parallel lanes for subsequent immunoblotting to monitor enrichment of PSD proteins with increasing purification of PSDs. Panels have been rearranged for clarity. (**B**) Immunoblot film signals were quantified by densitometry (see Materials and methods). For each individual protein, the signal was normalized against the WT signal from the same experiment. Dark (WT) and light (D36) bars reflect means ± s.e.m. from three independent experiments. Five different WT and five different D36 mice were used for each experiment. S.e.m. values for WT signals were obtained by comparison with the WT signal average from all three experiments for that protein. PSD-95, GluR1, and AKAP150 levels are similar in WT and D36 PSD fractions, but RIIα and RIIβ are strongly reduced in D36 (asterisk: $P < 0.01$; *t*-test).

(bicinchoninic acid), which ensured that identical amounts of total protein were present in all fractions from WT versus D36 mice. Both methods thus indicated that the amount of PSD material obtained from D36 versus WT mice was equal. The strong decrease in AKAP150 content by the first Triton X-100 extraction might have been partly due to removal of a substantial fraction of AKAP150 from PSDs. In support of this notion, considerable portions of a number of postsynaptic proteins are removed from PSDs by Triton X-100, including AMPARs and SAP97 (Valtschanoff *et al*, 2000; Sans *et al*, 2001; Vinade *et al*, 2003). Nevertheless, AKAP150 was consistently detectable in the Tx-1 fraction and the synaptic Triton-resistant AKAP150 pool enriched upon the second extraction, supporting its association with PSDs.

The two RII isoforms, RIIα and RIIβ, and the catalytic subunit, Cα, showed a distribution similar to that of AKAP150 in WT mice. They were prominent in Syn and RII subunits were present at a reduced level in Tx-1 and PSD (Figure 2A, middle). PKA subunits and especially C subunits might be more susceptible to Triton extraction than AKAP150, as Triton likely destabilizes binding of C to RII and of RII to AKAP150. Consequently, Cα was not observable in Tx-1 and PSD fractions. However, the nearly 60 and 90% decrease of RIIα and RIIβ, respectively, in the final PSD fractions from 8-week-old D36 versus WT mice indicates that AKAP150 is the main AKAP for targeting PKA to the PSD (Figure 2). Hence other AKAPs, including yotiao, which links PKA to the NMDA receptor (NMDAR) (Westphal *et al*, 1999), have a more modest role in recruiting PKA to the PSD.

Phosphorylation of GluR1 S845 and its postsynaptic targeting under basal conditions

AKAP150 connects PKA to GluR1 for S845 phosphorylation (Tavalin *et al*, 2002; Wong and Scott, 2004). A fraction of GluR1 is phosphorylated on S845 under basal conditions (Boehm *et al*, 2006; Oh *et al*, 2006). This fraction was unchanged in 1, 4, and 8-week-old D36 mice as was the total amount of GluR1 (Figure 3A and B). The enrichment of GluR1 in the PSD fraction from WT mice was modest as reported earlier (Sans *et al*, 2001; Vinade *et al*, 2003) and was unchanged in D36 mice (Figure 2). Collectively, these experiments demonstrate that GluR1, PSD-95, and C-terminally truncated AKAP150 are present in PSDs from D36 mice at WT levels, but PKA is reduced by more than 70%. GluR1 phosphorylation on S845 under basal conditions was unaltered in D36 mice, indicating that this phosphorylation does not depend on anchored PKA. Only 10–15% of GluR1 are actually phosphorylated on S845 under basal conditions (Boehm *et al*, 2006; Oh *et al*, 2006), and it is unclear whether this phosphorylation occurs at postsynaptic sites. It is conceivable, perhaps likely, that a pool of unbound, freely diffusible PKA has access to S845 mediating this basal phosphorylation throughout the cell. PKA holoenzymes containing regulatory RI subunits are in fact largely cytosolic and

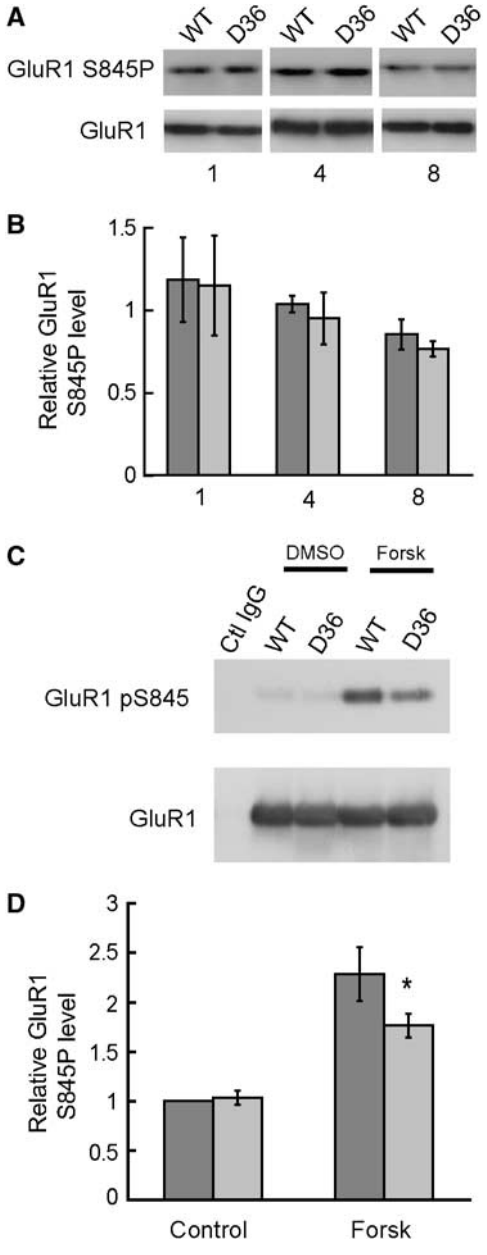

Figure 3 Activity-induced but not basal phosphorylation of GluR1 S845 is reduced in D36 mice. (**A**, **B**) Forebrains from 1, 4, and 8-week-old WT and D36 mice were solubilized with 1% deoxycholate in the presence of phosphatase inhibitors and cleared by ultracentrifugation before immunoprecipitation of GluR1, and immunoblotting with a S845 phosphospecific antibody, and after stripping with a general anti-GluR1 antibody (A, top and bottom panels, respectively). Immunosignals were quantified by densitometry (B). All signals were normalized within one experiment to the 4-week WT value. Bars reflect means±s.e.m. from three independent experiments with three different WT (dark bars) and three different D36 mice (light bars). S.e.m. for 4-week WT signals was obtained by comparison of the individual signals with the 4-week WT signal average from all experiments. Comparable amounts of S845-phosphorylated and total GluR1 were present in WT versus D36 mice at all age groups. (**C**, **D**) To chemically induce LTP, acute forebrain slices from WT and D36 mice were treated with forskolin followed by 5 min incubation with 30 mM K^+ and 0 Mg^{2+}. For control, slices were treated for 15 min with vehicle (0.01% DMSO) followed by 5 min incubation with standard ACSF. Immunoprecipitation, immunoblotting (C), and densitometry were as above (D). All signals were normalized within one experiment to the WT control value. Bars reflect means±s.e.m. from three independent experiments with three different WT (dark bars) and three different D36 mice (light bars). Induction of S845 phosphorylation by forskolin/high K^+ was significantly blunted in D36 mice ($^*P<0.05$ compared with WT treated with forskolin; *t*-test).

anchored to a much lesser degree than RII subunits (Carlisle Michel and Scott, 2002).

Phosphorylation of GluR1 S845 upon chemical induction of LTP

Acute forebrain slices were control treated (15 min vehicle followed by 5 min standard ACSF) or chemically potentiated (15 min forskolin followed by ACSF containing 30 mM K^+/0 Mg^{2+}) to induce LTP at most synapses (Makhinson *et al*, 1999; Kopec *et al*, 2006) (see also Otmakhov *et al*, 2004). The treatment increased S845 phosphorylation in WT and D36 slices (Figure 3C and D), but the increase was reduced by 44% in D36 slices. Hence, AKAP150 is important for optimal phosphorylation of GluR1 by PKA under conditions that induce LTP. Because LTP depends on phosphorylation of S845 (Esteban *et al*, 2003; Lee *et al*, 2003; Oh *et al*, 2006), we hypothesized that LTP is impaired in D36 mice. Such a finding would establish a critical functional role for AKAP150 in postsynaptic signaling and specifically LTP.

Age-dependent effect of the D36 mutation on LTP

LTP induced by a 1 s/100 Hz tetanus was intact in 4–5 but absent in 7- to 12-week-old D36 mice (Figure 4A and B). Paired-pulse facilitation was virtually identical over a wide range of interpulse intervals for D36 and WT mice within both age groups (Figure 5A). These data suggest that the observed LTP deficit in the older mice is due to a postsynaptic rather than presynaptic mechanism (Zalutsky and Nicoll, 1990; Foster and McNaughton, 1991; Schulz *et al*, 1994; Han *et al*, 2006; Pelkey *et al*, 2006). Stimulus–response curves were obtained by plotting the initial slope of the field excitatory post-synaptic potential (fEPSP) as a measure of the strength of the postsynaptic response against the fiber volley amplitude, which reflects the level of presynaptic activation. These curves were not different for WT versus D36 mice (Figure 5B). Amplitudes of AMPARs mEPSC were obtained by whole-cell patch recording from acute hippocampal slices, again with no change in D36 mice (Figure 5C and D).

Amplitudes of NMDAR mEPSCs are below the noise level in acute hippocampal slices. To test whether NMDAR activity is affected in D36 mice, we recorded fEPSPs with CNQX present to eliminate AMPAR currents. Mg^{2+} was omitted to remove its NMDAR block. This approach allows measurement of NMDAR-mediated fEPSPs without activation of AMPARs (Huang *et al*, 2006; see also Figure 6G–I). We monitored presynaptic fiber volleys and subsequent NMDAR fEPSPs using increasing stimulus strengths in hippocampal slices from 8-week-old WT and D36 mice. The fEPSPs were mediated by NMDAR as addition of the NMDAR antagonist APV (20 μM) eliminated any fEPSP responses in the presence of CNQX at the strongest stimulus intensities applied (data not shown). Plotting initial slopes of NMDAR fEPSPs against fiber volley amplitudes did not reveal any significant difference in NMDAR responses between the two genotypes (Figure 5E1).

We also performed whole-cell patch recordings of evoked EPSCs (eEPSCs) before and after perfusion with APV in hippocampal slices from 8-week-old WT and D36 mice. The holding potential was +40 mV to remove the Mg^{2+} block of NMDARs. In the absence of APV, eEPSCs are a combination of AMPAR and NMDAR currents. Evoked EPSCs recorded 15 min after perfusion with APV decayed much faster than eEPSCs before APV, indicating that the slow NMDAR component was blocked by APV. Recordings with APV thus yielded AMPAR currents and the difference between total eEPSCs and those with APV NMDAR currents. The ratio of their amplitudes and thus overall channel activity of postsynaptic NMDAR was unaltered in D36 versus WT mice (Figure 5E2). Accordingly, the lack of LTP in 7- to 12-week-old D36 mice is not due to alterations in total NMDAR current.

PKA is required for LTP in 8 but not 4-week-old mice

The abrogation of LTP in 7–12 but not 4- to 5-week-old D36 mice suggested an age-dependent requirement of PKA in LTP. To systematically test this hypothesis, hippocampal slices from 4 and 8-week-old WT mice were tetanized (1 × 1 s/100 Hz tetanus) in the absence and presence of H89 and KT5720, two structurally different inhibitors of PKA. Both drugs inhibited LTP in 8 but not 4-week-old mice (Figure 6A–D). Neither inhibitor affected baseline fEPSPs even during prolonged application (Figure 6E and F). PKA can positively

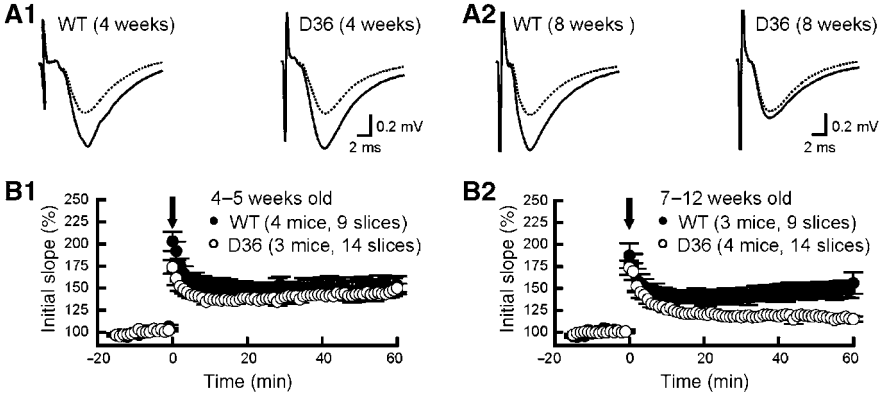

Figure 4 LTP is affected in 7–12 but not 4- to 5-week-old D36 mice. LTP was induced by a single tetanus (1 s/100 Hz; arrows in B) in CA1 of acute hippocampal slices from D36 and littermate-matched WT control mice. (**A**) Example fEPSP recordings before (dashed lines) and 60 min after LTP induction (solid lines) from mice 4–5 (A1) and 7–12 weeks old (A2). (**B**) Averages of the complete time courses are shown from mice 4–5 (B1) and 7–12 weeks old (B2). At 4–5 weeks LTP is comparable in hippocampal slices from D36 and control mice (145 ± 4% s.e.m. versus 152 ± 11% at 55–60 min, open and closed circles, respectively). However, at 7–12 weeks LTP is nearly absent in D36 mice (116 ± 3%, open circles; control mice, 149 ± 9%, closed circles; this difference is highly significant: $P < 0.001$, *t*-test).

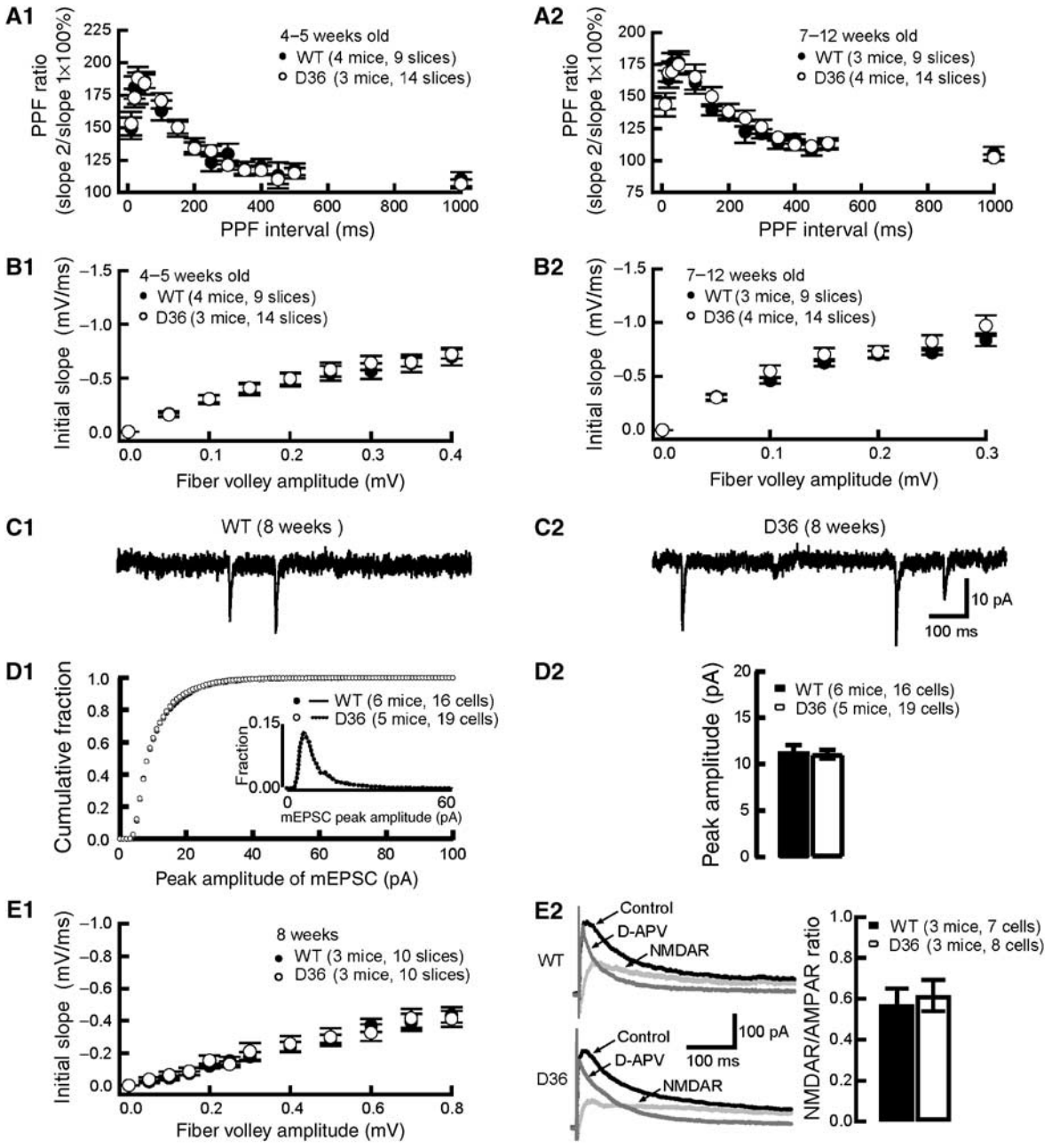

Figure 5 Basal synaptic transmission is normal in 4–5 and 7- to 12-week-old D36 mice. (**A**) Paired-pulse facilitation is virtually identical for D36 and control mice within both age groups over the whole range of interstimulus intervals. (**B**) Input–output curves with the postsynaptic response (initial slope of fEPSP) plotted as a function of the presynaptic fiber volley amplitude are indistinguishable between D36 and control mice within both age groups. (**C**) Examples of AMPAR mEPSC recordings from WT (C1) and D36 mice (C2). (**D**) Cumulative fraction (D1), histogram distribution (D1, insert), and overall average (D2) of mEPSC peak amplitudes (and frequencies; not illustrated) are virtually identical in 8-week-old WT versus D36 mice. (**E1**) Field EPSPs were recorded in the presence of CNQX (10 μM) to block AMPAR and in the absence of Mg^{2+} to eliminate NMDAR block by Mg^{2+}. Input–output curves with the postsynaptic NMDAR response (initial slope of fEPSP) plotted as a function of the presynaptic fiber volley amplitude are indistinguishable between D36 and control mice at the age of 8 weeks. (**E2**) Ratio of NMDAR- to AMPAR-mediated EPSCs in D36 mice (0.61 ± 0.08) is not distinguishable from WT (0.57 ± 0.06) at the age of 8 weeks (right panel). Left panel shows recorded traces at $+40$ mV; black traces are the evoked EPSCs recorded under control condition; dark gray traces are the AMPAR-evoked EPSCs recorded in the presence of 100 μM D-APV; light gray traces are the NMDAR-evoked EPSCs computed by subtraction of AMPAR evoked EPSCs (dark gray trace) from the control-evoked EPSCs (black trace).

modulate NMDAR activity (Skeberdis *et al*, 2006). To test whether H89 and KT5720 affect NMDAR activity under our conditions, we monitored fEPSPs in the presence of CNQX and in the absence of Mg^{2+} (see Figure 5E1). Presynaptic fiber volleys and the resulting NMDAR fEPSPs were recorded with increasing stimulus strengths before and 20 min after the onset of perfusion with H89 and KT5720. The initial slopes of the resulting NMDAR fEPSP plotted against the fiber volley amplitude did not show any significant difference in postsynaptic NMDAR response over the whole range of stimuli before

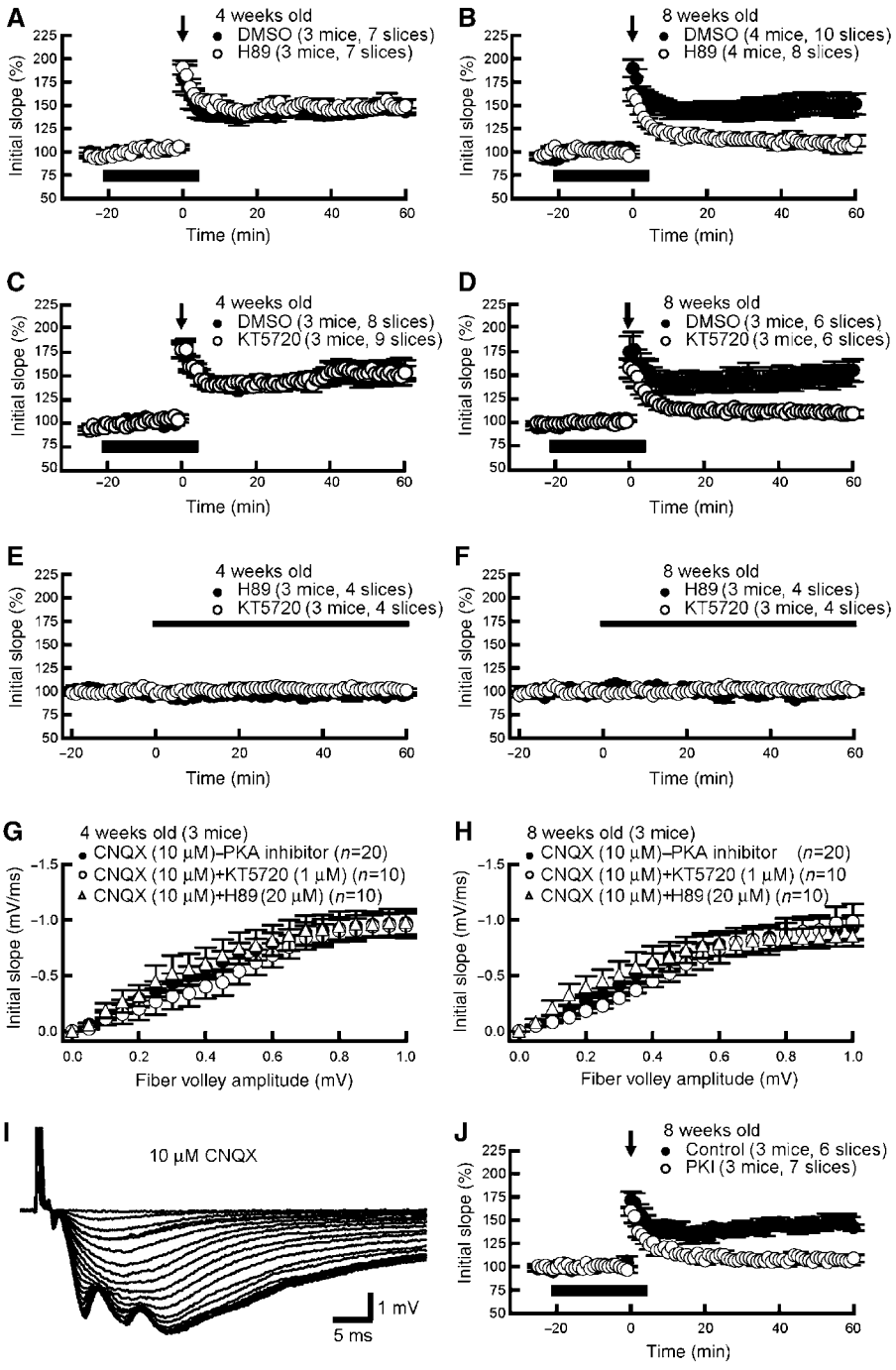

Figure 6 PKA is necessary for LTP in 8 but not 4-week-old WT C57BL/6 mice. (**A**) Inhibition of PKA with H-89 (20 μM in DMSO) does not affect LTP in hippocampal slices from 4-week-old mice (150±7% s.e.m., at 55–60 min) compared with vehicle control (0.025% DMSO; 148%±4%). (**B**) In-8-week-old mice, LTP is blocked by H-89 (107±7% compared with 150±11% for vehicle control at 55–60 min: $P<0.01$; *t*-test). (**C**, **D**) Inhibition of PKA with KT5720 (1 μM in DMSO) does not affect LTP in 4-week-old mice (C; 151±9% at 55–60 min) compared with vehicle control (0.1% DMSO; 155±10%), but inhibits LTP at 8 weeks (D; 110±4 versus 155±10% for vehicle control; $P<0.004$; *t*-test). (**E**, **F**) Bath application of H89 or KT5720 does not affect baseline fEPSP responses at 4 or 8 weeks over extended periods. (**G–I**) Field EPSPs were recorded in the presence of CNQX (10 μM) to block AMPARs and in the absence of Mg^{2+} for full NMDARs currents. Increasing stimulus strength increased fEPSPs to the point were population spikes started to appear (upward deflections in I at highest stimulus intensities; shown are fEPSP responses of increasing amplitude with increasing stimulus strength). Input–output curves were not different for any stimulus strength before versus after application of H89 or KT5720 at 4 (G) and 8 weeks (H). (**J**) At 8 weeks LTP is also blocked by the highly specific membrane-permeable PKA-inhibitory peptide 11R-PKI (Matsushita *et al*, 2001) (10 μM; 107±3% compared with 148±5% for the protease inhibitor only control: $P<0.0001$). Arrows indicate tetanus (1 s/100 Hz); black bars show times of drug applications.

versus after application of PKA inhibitors (Figure 6G–I). These results indicate that block of PKA does not affect overall NMDAR current in this system. We confirmed that LTP requires PKA at 8 weeks of age, using a membrane-permeable version of the PKI inhibitory peptide. This peptide is derived from the endogenous protein kinase A inhibitor (PKI) and is highly specific for PKA. This peptide nearly abolished LTP in slices from 8-week-old mice (Figure 6J).

GluR2-lacking AMPARs are required for LTP in 8 but not 4-week-old mice

Recent work indicates that LTP in 2- to 3-week-old mice and rats depends on the temporary insertion of GluR2-lacking AMPARs (Plant *et al*, 2006) (but see Adesnik and Nicoll, 2007). Furthermore, PKA is necessary for postsynaptic localization of AMPARs formed by ectopically expressed GluR1 during LTP in organotypic hippocampal slices (Esteban *et al*, 2003). These receptors are rectifying and lack GluR2. We tested whether expression of LTP at 8 weeks depends on GluR2-lacking AMPARs. Philanthotoxin (PhTx) inhibits both GluR2-lacking AMPARs and NMDARs (e.g., Adesnik and Nicoll, 2007). Hence we applied PhTx after LTP induction. This strategy prevents maintenance of LTP in 2- to 3-week-old mice and rats as observed by Plant *et al* (2006) (but see Adesnik and Nicoll, 2007). PhTx reversed the initial potentiation and completely inhibited LTP as quantified after 55–60 min at 8 but not 4 weeks (Figure 7A and B). To further scrutinize the requirement for GluR2-lacking AMPARs, slices were incubated with the membrane-permeable spermine derivative 1-naphthylacetyl spermine (NASPM), which selectively inhibits GluR2-lacking AMPARs. Application of NASPM before and during LTP induction or immediately after LTP induction also inhibited LTP (Figure 7C and D).

GluR2-lacking AMPARs are required for LTP in 2 but not 3-week-old mice

Contrasting Plant *et al* (2006), Adesnik and Nicoll (2007) did not observe that GluR2-lacking AMPARs are required during the early phase of LTP. Both groups state that they used 2- to 3 –week-old mice and rats. Because we found that GluR2-lacking AMPARs are required for LTP in 8 but not 4-week-old mice (Figure 7) and that PKA inhibitors block LTP in 8 but not in 4-week-old (Figure 6) and also not in 3-week-old mice (data not shown), we hypothesized that a similar age dependence applies to 2 versus 3-week-old mice. We used precisely 12–14 and 20- to 22-day-old C57BL/6 mice. LTP was induced with 2 trains of 1 s/100 Hz stimuli, 20 s apart. PhTx and NASPM significantly inhibited LTP by 60–80% in the 2 but not 3-week-old mice (Figure 8).

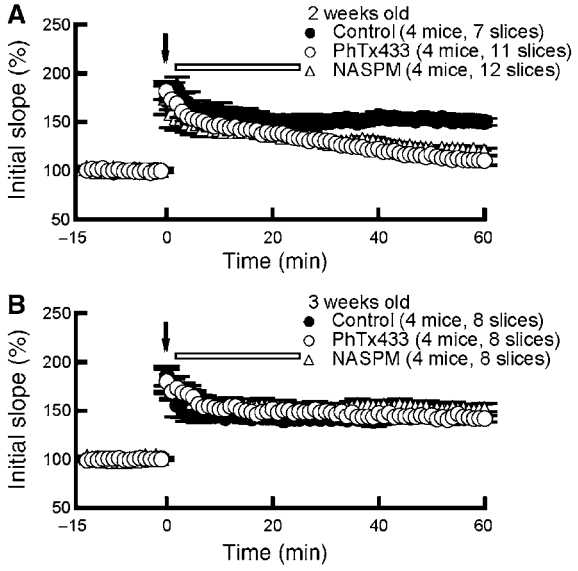

Figure 8 GluR2-lacking AMPAR are necessary for LTP in 2 but not 3-week-old WT C57BL/6 mice. LTP was induced by two tetani (1 s/100 Hz) 20 s apart in the CA1 area of acute hippocampal slices. (**A**) In slices from 12- to 14-day-old mice, PhTx-433 (2.5 μM) and NASPM (20 μM) inhibit LTP (111±4% s.e.m. and 119±3%, respectively, compared with 151±4% for control at 55–60 min; in both cases, $P<0.00005$; *t*-test). (**B**) PhTx-433 and NASPM do not affect LTP in hippocampal slices from 20- to 22-day-old mice (143±3 and 151±6%, respectively) compared with control (144±6%).

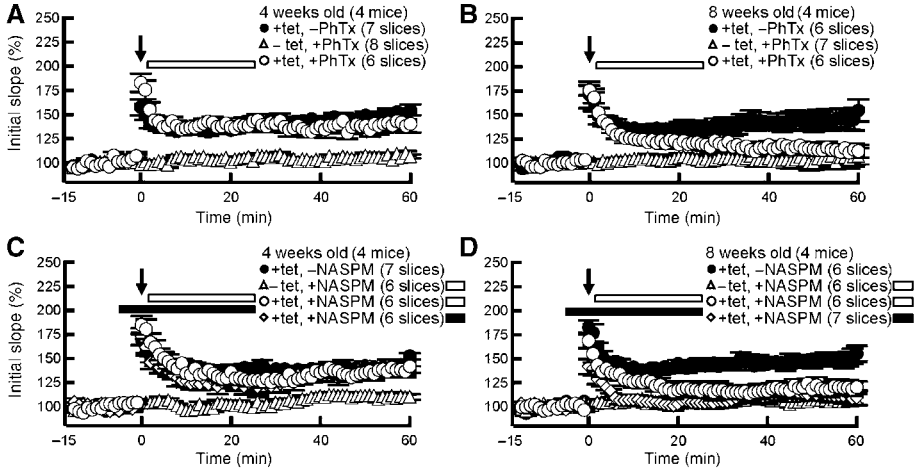

Figure 7 GluR2-lacking AMPARs are necessary for LTP in 8 but not 4-week-old WT C57BL/6 mice. (**A**) Inhibition of GluR2-lacking AMPARs with PhTx-433 (2.5 μM) does not affect LTP in hippocampal slices from 4-week-old mice (140±7% s.e.m., at 55–60 min) compared with control (150±5%). PhTx-433 has also no effect on baseline responses (107±2%). (**B**) In slices from 8-week-old mice, PhTx-433 inhibits LTP nearly completely (113±5% compared with 146±10% for control at 55–60 min; $P<0.02$; *t*-test) but has no effect on baseline (105±9%). (**C**) Inhibition of GluR2-lacking AMPARs with NASPM (20 μM) does not affect LTP in hippocampal slices from 4-week-old mice (139±5% s.e.m., at 55–60 min, when NASPM is applied during stimulation; 138±6%, when NASPM is applied after stimulation) compared with control (142±4%). NASPM has also no effect on baseline (110±2%). (**D**) In slices from 8-week-old mice, LTP is inhibited by NASPM (111±7% when NASPM is applied during stimulation; 120±7% when NASPM is applied after stimulation; *t*-test: $P<0.003$ and $P<0.02$, respectively, compared with 150±7% for control at 55–60 min). NASPM has no effect on baseline (106±4%). Arrows indicate tetanus (1 s/100 Hz); bars show times of drug applications starting before (black) or immediately after (open) tetanus.

Discussion

We show that AKAP150-anchored PKA is critical for LTP in 7–12 but not 4-week-old mice under our conditions. LTP also depends fully on PKA in 8–9 days and still partially in 2-week-old rats (Wikstrom *et al*, 2003; Yasuda *et al*, 2003). Together these findings indicate two developmental switches for PKA dependency. The first transformation occurs when PKA dependency changes from an absolute at 8–9 days to a partial requirement at 2 weeks of age and no requirement at 3 weeks (Y Lu and JW Hell, data not shown) and 4 weeks of age (Figure 6A and C). The second transformation takes place between 4 and 7 weeks, as PKA is again required at the latter age for LTP induced by a single tetanus.

In agreement with our observations that PKA is critical for LTP in 8-week-old mice, pharmacological or genetic interference with signaling by PKA blocks early LTP in CA1 induced by several stimulus trains of 1 s/100 Hz or theta burst stimulation in rats and mice older than 5–6 weeks (Frey *et al*, 1993; Blitzer *et al*, 1995; Winder *et al*, 1998; Woo *et al*, 2000; Duffy and Nguyen, 2003). This inhibition of LTP by PKA blockers is more modest in 5- to 6-week-old rats (about 70% inhibition; Huang and Kandel, 1994) and further reduced if not absent in 3- to 5-week-old animals (Nayak *et al*, 1998; Lee *et al*, 2000). Other factors such as the number of tetani also influence PKA dependency (Huang and Kandel, 1994; Winder *et al*, 1998; Wong *et al*, 1999; Bortolotto and Collingridge, 2000; Duffy and Nguyen, 2003). The variations in the precise age, recording conditions, and LTP induction protocols between these studies preclude a stringent direct comparison among them. Our systematic analysis shows that in C57BL/6 mice, and under our conditions, single tetanus LTP depends on PKA at 8 but not 4 weeks of age. Although PKA dependency might be more or less pronounced under other conditions or in other mouse strains, our findings lead to the important conclusion that mechanisms for LTP induction differ with age.

The age dependency of LTP on PKA and its anchoring by AKAP150 at 4 versus 8 weeks shows similarities with the age dependency on GluR1 (Zamanillo *et al*, 1999; Jensen *et al*, 2003) and phosphorylation of GluR1 on S845 (Lee *et al*, 2003); LTP is impaired in adult GluR1 S831/845A double-knock-in mice and absent in GluR1 knock-out mice that are older than 5 weeks. These defects are less pronounced in the corresponding younger animals.

PKA and phosphorylation of S845 and potentially other sites are important for accumulation of GluR1-containing AMPARs at the cell surface and specifically at the synapse during LTP (Esteban *et al*, 2003; Swayze *et al*, 2004; Sun *et al*, 2005; Oh *et al*, 2006; Man *et al*, 2007). PKA increases surface expression of GluR1 by inhibiting internalization and promoting recycling back to the plasma membrane after ligand-induced endocytosis (Ehlers, 2000; Sun *et al*, 2005; Man *et al*, 2007). AMPARs reach by lateral diffusion postsynaptic sites, where mechanisms that require Ca^{2+} influx retain receptors (Borgdorff and Choquet, 2002; Tardin *et al*, 2003). An increase in the number of functional AMPARs at postsynaptic sites is a critical mechanism in LTP (Bredt and Nicoll, 2003; Collingridge *et al*, 2004; Malenka and Bear, 2004; Lisman and Raghavachari, 2006). Thus PKA-mediated augmentation in GluR1 at the surface and ultimately postsynaptic site likely contributes to LTP. AKAP150-anchored PKA could therefore play an important role in LTP in 8-week-old mice by fostering GluR1 phosphorylation on S845 and thereby surface expression of GluR1-containing AMPARs.

The temporary functional expression of GluR2-lacking AMPARs at postsynaptic sites is emerging as a common mechanism in different forms of synaptic plasticity. In 2- to 3-week-old mice and rats, inward rectification and sensitivity to PhTx increase during the very early phase of LTP (Plant *et al*, 2006). GluR2-lacking AMPARs are replaced by GluR2-containing AMPARs over the subsequent 10–20 min (Plant *et al*, 2006). However, Adesnik and Nicoll (2007) did not find that GluR2-lacking AMPARs are detectable or required during the early phase of LTP in 2- to 3-week-old mice . Using mice at more precisely defined ages, we now demonstrate that GluR2-lacking AMPARs are necessary for LTP in 10–12 but not 20- to 22-day-old mice (Figure 8). These results explain the above discrepancy if mice of Plant and co-workers were functionally or *de facto* closer to our 2-week-old mice and mice of Adesnik and Nicoll closer to our 3-week-old mice.

Postsynaptic expression of GluR2-lacking GluR1 homomeric AMPARs requires phosphorylation of S845 by PKA in organotypic hippocampal slice cultures (Esteban *et al*, 2003). These results were obtained upon overexpression of GluR1, which leads to the formation of mainly homomeric GluR1 receptors, and cannot be directly applied to the *in vivo* situation where only a minority of GluR1 forms homomeric receptors. However, they provide evidence for a critical role of PKA and S845 in promoting postsynaptic accumulation of GluR1-containing AMPARs during potentiation. GluR1 in general and specifically its phosphorylation on S845 is important for LTP in adult mice (Zamanillo *et al*, 1999; Jensen *et al*, 2003; Lee *et al*, 2003). Furthermore, GluR1 homomeric receptors are the main if not the only GluR2-lacking AMPAR in adult rodents (Wenthold *et al*, 1996). Our results that PhTx and NASPM block LTP in 8-week-old mice thus indicate that at this age LTP depends on the PKA-dependent postsynaptic expression of GluR1 homomeric AMPARs during its initial phase.

Our findings show that LTP dependence on GluR2-lacking AMPARs switches twice during development in parallel to the PKA dependency. The first transformation happens when LTP changes from a mechanism that depends on GluR2-lacking AMPARs at 2 weeks to one that does not depend on GluR2-lacking AMPARs at 3–4 weeks (Figures 7 and 8). The second transformation occurs between 4 and 8 weeks of age as GluR2-lacking AMPARs again become necessary at the latter age. Together with the parallel change in PKA dependency of LTP, our findings strongly argue that the LTP mechanisms vary for different developmental stages. Accordingly, PKA and GluR2-lacking AMPARs are important for LTP in 2 and again 8-week but not 3- to 4-week-old mice under our conditions. However, this covariance in dependence on PKA and GluR2-lacking AMPARs does not necessarily mean that molecular mechanisms are identical at 2 and 8 weeks. Similar to GluR1, postsynaptic clustering of GluR4 requires activity of PKA, which phosphorylates S842 at the C-terminus of GluR4 (Esteban *et al*, 2003). This phosphorylation plays a role analogous to S845 phosphorylation in driving GluR4 to postsynaptic sites (Zhu *et al*, 2000; Esteban *et al*, 2003). GluR4 is expressed in the hippocampus of young mice and rats until 6 weeks of age and declines thereafter to lower levels (Wenthold *et al*, 1996; Jensen *et al*, 2003). In adult mice, homomeric GluR1 receptors are therefore likely the main AMPARs that mediate the early phase of LTP that depends on PKA and

GluR2-lacking AMPARs. It is however possible that GluR4-containg AMPARs contribute to LTP at P12–P14.

Association of PKA via AKAP150 and SAP97 with GluR1 is crucial for phosphorylation of GluR1 at S845 by PKA (Leonard *et al*, 1998; Tavalin *et al*, 2002; Wong and Scott, 2004). Because AKAP150-anchored PKA is required for LTP at 8 weeks (Figure 4), we propose that PKA acts, at least in part, by phosphorylating GluR1, thereby driving it to the postsynaptic sites of synapses that are undergoing LTP. This model is not inconsistent with the observations that S845 phosphorylation as well as the amount of GluR1 in PSD fractions is unaltered under basal conditions in 8-week-old D36 versus WT mice. Basal GluR1 surface expression is unaltered in S831/845A knock-in mice, suggesting that without stimulation surface expression is not controlled by S845 phosphorylation (Lee *et al*, 2003). Furthermore, only 10–15% of GluR1 are phosphorylated on S845 under basal conditions (Boehm *et al*, 2006; Oh *et al*, 2006). A small pool of unbound, freely diffusible active PKA C subunit might have access to S845 for basal phosphorylation, which might occur in intracellular compartments or on GluR1/R2 heteromeric AMPARs, whose subcellular distribution might depend less on GluR1 than GluR2 phosphorylation (Chung *et al*, 2000; Daw *et al*, 2000; Seidenman *et al*, 2003; Gardner *et al*, 2005; Lu and Ziff, 2005). Such scenarios would explain the lack of effect on GluR1 distribution in GluR1 and AKAP150 mutant mice.

In support of a critical role of AKAP150 in PKA-mediated GluR1 S845 phosphorylation, the substantial increase in this phosphorylation upon chemical induction of LTP was blunted by 44% in D36 mice (Figure 3C and D). We used forskolin followed by high K^+/0 Mg^{2+} to induce chemical LTP. This protocol is perhaps the best suited protocol for chemical induction of LTP without a requirement for any electrical stimulation including test stimuli (Makhinson *et al*, 1999; Otmakhov *et al*, 2004; Kopec *et al*, 2006). However, because it directly activates adenylyl cyclase, we cannot be certain that molecular changes, including S845 phosphorylation, are strictly related to LTP. Changes could reflect LTP-specific mechanisms as well as general responses to PKA activation. Nevertheless, the results clearly show that activity-induced S845 phosphorylation is blunted in D36 mice. Thus, PKA anchoring by AKAP150 is important for S845 phosphorylation.

PKA controls other signaling cascades that are involved in synaptic plasticity, further complicating the picture. For instance, PKA phosphorylates inhibitor-1, which in turn inhibits the protein phosphatase PP1, preventing it from dephosphorylating a number of substrates of various serine/threonine kinases (Blitzer *et al*, 1998). Although we still do not know all the mechanistic details that underlie LTP, the absence of LTP in 8-week-old D36 mice indicates that PKA anchoring by AKAP150 is a critical element in multiple signaling cascades that govern long-term synaptic plasticity.

In conclusion, LTP at 2 and 8, but not 3–4 weeks, depends on AKAP-150-anchored PKA and GluR2-lacking AMPARs. LTP at 4 weeks likely invokes an alternate mechanism involving a more direct effect on postsynaptic delivery or retention of GluR2-containing AMPARs than at the other ages. The two switches before and after 4 weeks of age in LTP requirements might reflect a general developmental change of the principal CA1 neurons. During immature stages when neurons are not ready to firmly establish the majority of their synaptic connections, neurons might restrain LTP by augmenting requirements (here PKA and GluR2-lacking AMPARs). After a period of heightened susceptibility to LTP, neurons might again restrict LTP as the brain matures and stabilizes. In support of this hypothesis, susceptibility to synaptic potentiation varies at different developmental periods as for LTP and synaptic scaling in the cortex (Crair and Malenka, 1995; Kirkwood *et al*, 1995; Desai *et al*, 2002).

Materials and methods

Generation of AKAP150 mutant mice

All animal procedures had been approved by the University of Iowa and followed NIH guidelines. To truncate the last 36 residues of AKAP150, a stop codon was engineered into the mouse AKAP150 (AKAP5) gene, replacing the triplet encoding leucine at position 710 (see Supplementary data for more details). The neomycin phosphotransferase gene used for positive selection was flanked by loxP sites and removed by crossing the first generation of mutants to mice expressing Cre recombinase. Resulting mice were backcrossed eight times with WT C57BL/6 (Taconic Farms) to breed a nominally >99% genetic C57BL/6 background. Heterozygous breeder pairs provided homozygous control and D36 mice.

Genotyping

DNA strands were isolated from supernatants of RNase A- and Proteinase K-digested tail clips and used to amplify the 3′ coding region of AKAP150. The stop codon engineered into the mutant gene to truncate AKAP150 residues also created an *Xba*I restriction site. The primers 5′-CCCACAGATACAGAGAAACCGAG-3′ and 5′-GGAAACGAAGTCACTGGAACAGCG-3′, respectively, correspond to sequences upstream and downstream of the newly created stop codon. The 327 bp product was purified with PCR purification columns (Qiagen) and digested with *Xba*I. Only alleles with the engineered stop codon gave a cleaved product of 208 and 119 bp (Figure 1).

Isolation of PSDs, immunoprecipitation, and immunoblotting

PSDs were isolated on ice (Carlin *et al*, 1980) using five mice per preparation. Briefly, forebrains were homogenized in 15 ml solution A (0.32 M sucrose, 1 mM $MgCl_2$, 1 mM HEPES, pH 7.0, plus protease inhibitors; Leonard *et al*, 1998) and centrifuged (2500 r.p.m., 10 min), and then pellets were re-extracted. Supernatants were combined and centrifuged at low (2000 r.p.m., 10 min) and high speed (20 000 r.p.m., 20 min) to obtain a synaptic membrane-enriched pellet (P2). P2 was resuspended in solution A lacking $MgCl_2$, loaded onto a 0.85/1/1.25 M sucrose step gradient, and spun (24 000 r.p.m., 2 h). The synaptosome fraction (Syn) at the 1/1.25 M interface was treated for 15 min in 0.5% Triton X-100 and centrifuged (24 000 r.p.m., 30 min). The pellet (Tx-1) was resuspended in solution A lacking $MgCl_2$, loaded onto a 1/1.5/2.0 M sucrose gradient, and spun (35 000 r.p.m., 2 h). The PSD-enriched fraction at the 1.5/2.0 interface was treated once more with 0.5% Triton X-100 and centrifuged (35 000 r.p.m., 1 h) to obtain purified PSDs. Protein concentrations were determined with bicinchoninic acid and 10 μg of total protein of each fraction analyzed by immunoblotting (Leonard *et al*, 1998). Solubilization of GluR1 with 1% deoxycholate and the subsequent immunoprecipitation and immunoblotting were described earlier (Leonard *et al*, 1998). Chemiluminescent immunosignals were quantified (Adobe Photoshop) after digitalization by scanning films as described (Merrill *et al*, 2007). Comparing signals from increasingly longer exposures ensured that signals were in the linear range as described (Davare and Hell, 2003; Hall *et al*, 2006). Signals for phosphorylated S845 were corrected for variations in total GuR1, which were never larger than 10%. The following antibodies were used: anti-AKAP150 (Ndubuka *et al*, 1993), anti-PKA Cα (Ndubuka *et al*, 1993), anti-PKA RIIα (Burton *et al*, 1999), anti-PKA RIIβ (Burton *et al*, 1999), anti-PSD-95 (Valtschanoff *et al*, 2000), anti-synaptophysin (Davare *et al*, 2001), and anti-GluR1 (Leonard *et al*, 1998). The phosphospecific antibody against GluR1 phosphorylated on S845 was produced and purified as described (Mammen *et al*, 1997).

Preparation of acute hippocampal slices

All mice were male. WT mice for testing inhibitors of PKA and GluR2-lacking AMPAR were C57BL/6 (Taconic Farms). After

decapitation, brains were rapidly removed, glued onto the plateau of the slicing chamber, and immersed into ice-cold slicing buffer (in mM: 127 NaCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.9 KCl, 1 $CaCl_2$, 2 $MgSO_4$, 10 dextrose) saturated with 95% O_2 and 5% CO_2. Transverse slices (350 μM thick) were prepared with a vibrating microtome (VT1000S, Leica Microsystems, Nussloch, Germany), kept in oxygenated slicing buffer for 30 min at 34°C and for another 30 min at 22°C, and transferred to a submersion recording chamber continually perfused with 32°C oxygenated artificial cerebrospinal fluid (ACSF; in mM: 127 NaCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.9 KCl, 2.2 $CaCl_2$, 1 $MgSO_4$, 10 dextrose; rate: 2 ml/min). Slices were equilibrated for at least 15 min before recording.

S845 phosphorylation in forebrain slices

Forebrain slices from >8-week-old WT and D36 mice were prepared in parallel (see above), allowed to recover for 1.5 h at 22°C, transferred to submersion chambers with ACSF, equilibrated for 30 min at 30°C, treated with either DMSO (vehicle control; final concentration 0.01%) or forskolin (50 μM) for 15 min, and transferred to a second chamber. For non-potentiated control samples, the second chamber contained normal ACSF and for forskolin-treated slices 30 mM K^+ and 0 Mg^{2+}, to allow chemical induction of LTP by sequential forskolin/high K^+/0 Mg^{2+} treatment (Makhinson *et al*, 1999). After 5 min in the second chamber, slices were extracted with ice-cold 1% deoxycholate buffer for subsequent immunoprecipitation and immunoblotting (see above).

Electrophysiology

ACSF-filled glass electrodes (resistance <1 MΩ) were positioned in the stratum radiatum of area CA1 for extracellular recording. Synaptic responses were evoked by stimulating Schaffer collaterals with 0.1 ms pulses with a bipolar tungsten electrode (WPI Inc., Sarasota, FL) once every 15 s (Lim *et al*, 2003). The stimulation intensity was systematically increased to determine the maximal fEPSPslope and then adjusted to yield 40–60% of the maximal (fEPSP) slope. Experiments with maximal fEPSPs of less than 0.5 mV or with substantial changes in the fiber volley were rejected. After recording of a stable baseline for 15–25 min, LTP was induced by one 1 s/100 Hz stimulus train unless stated otherwise. Drugs were applied in perfusion buffer. *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89, Sigma) and KT5720 (AG Scientific) were dissolved in DMSO (80 and 1 mM, respectively) and diluted to a final concentration of 20 and 1 μM, respectively, immediately before perfusion. PhTx and NASPM (both Sigma) were dissolved in water (1 and 10 mM, respectively). The PKI peptide (RRRRRRRRRRGFIASGRTGRRNAI) carried 11 arginines at the N-terminus to make it membrane permeable (Matsushita *et al*, 2001; Lim *et al*, 2003) (custom-synthesized by the WM Keck Biotechnology Resource Center, Yale University, New Haven, CT, and purified by HPLC). 11R-PKI was dissolved in water (1 mM) and diluted before perfusion (final concentration: 10 μM); protease inhibitors (PMSF, 35 μg/ml; leupeptin, 10 μg/ml; aprotinin, 10 μg/ml) were added to perfusion solutions for 11R-PKI and corresponding control experiments to prevent degradation of the peptide.

Field EPSPs were recorded (AxoClamp 2B amplifier, Axon Instruments, Foster City, CA), filtered at 1 kHz, digitized at 10 kHz (Axon Digidata 1200), and stored for off-line analysis (Clampfit 9). Initial slopes of fEPSPs were expressed as percentages of baseline averages. In summary graphs, each point represents the average of four consecutive responses. The time-matched, normalized data were averaged across experiments and expressed as means±s.e.m. For statistical analysis, the last 5 min in each measurement were average and normalized to the average of the 5 min preceding LTP induction before comparison with the corresponding control values.

Whole-cell recordings from pyramidal neurons in CA1 with a holding potential at −70 mV provided mEPSCs. Patch pipettes (3–6 MΩ) were pulled from KG-33 glass capillaries (1.1 mm I.D., 1.7 mm O.D., Garner Glass Company, Claremont, CA) on a Flaming–Brown electrode puller (P-97, Sutter Instruments Co., Novato, CA) and filled with internal solution (in mM: 125 K-gluconate, 20 KCl, 10 NaCl, 2 Mg-ATP, 0.3 Na-GTP, 2.5 QX314, 10 PIPES, 0.2 EGTA, pH 7.3 adjusted with KOH). Slices were perfused with ACSF. $GABA_A$ receptor currents were blocked with 50 μM picrotoxin (Sigma), NMDAR currents with 10 μM APV (Sigma), and Na^+ channel currents with 1 μM tetrodotoxin (TTX, Sigma). Recordings were made using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, digitized at 10 kHz via an Axon Digidata 1322A, and stored for off-line analysis. The threshold for mEPSCs was set as 5 pA.

Evoked EPSCs (eEPSCs) were recorded at holding potential of +40 mV to determine NMDAR to AMPAR current ratio. K^+ was replaced by Cs^+ in the internal solution. Picrotoxin (50 μM) was added to ACSF for perfusion. Ten traces were recorded in the absence of APV to determine the combined AMPAR and NMDAR eEPSCs. Subsequently, 10 traces were recorded in the presence of 100 μM APV to determine AMPAR eEPSCs. The average NMDAR eEPSC amplitude was calculated by subtracting the averaged AMPAR eEPSC from the averaged combined AMPAR/NMDAR eEPSC.

Note added in proof

O'Dell and colleagues recently found that LTP induced by two 1s/100 Hz tetani 10 s apart in 8-week-old wild-type C57Black/6 mice is not sensitive to inhibitors of GluR2-lacking AMPAR (Gray EE, Fink AE, Sarinana J, Vissel B, O'Dell TJ (2007) LTP in the hippocampal CA1 region does not require insertion and activation of GluR2-lacking AMPA receptors. *J Neurophysiol*, in press). We observed under our conditions using a single tetanus that the resulting LTP does require GluR2-lacking AMPAR (Figure 7). We tested whether the use of one versus two tetani constitutes a critical difference. In fact, in our hands, LTP induced by the two tetani protocol yields LTP that is not sensitive to inhibition of GluR2-lacking receptors and also not to inhibition of PKA (Supplementary Figure 2). We suggest that under our conditions using mild, perhaps more physiological induction protocols, LTP depends on both GluR2-lacking AMPAR and PKA, whereas stronger stimuli recruit other signaling mechanisms that render LTP induction independent of GluR2-lacking AMPAR and PKA.

Two other recent publications provide further support for a critical role of GluR1 S845 phosphorylation in two different forms of LTP induced by modest stimuli: (A) Hu H, Real E, Takamiya K, Kang M-G, Ledoux J, Huganir RL, Malinow R (2007) Emotion enhance learning via norepinephrine regulation of AMPA-receptor trafficking. *Cell* **131**, 160–173; (B) Seol GH, Ziburkus J, Huang SY, Song L, Kim IT, Takamiya K, Huganir RL, Lee H-K, Kirkwood A (2007) Neuromodulators control the polarity of spike-timing-dependent synaptic plasticity. *Neuron* **55**, 919–929. Hu *et al* further show that fear conditioning depends also on S845 phosphorylation when mice were exposed for relatively short time periods to the conditioning environment (2 min). It does not require S845 phosphorylation when mice were exposed for 8 min. It thus appears that S845 phosphorylation is important for both LTP and fear conditioning under conditions of limited stimulation but stronger stimuli or extended conditioning can circumvent this requirement.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of Interest

The authors indicate no conflict of interest.

References

Adesnik H, Nicoll RA (2007) Conservation of glutamate receptor 2-containing AMPA receptors during long-term potentiation. *J Neurosci* **27:** 4598–4602

Bellone C, Luscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed *in vivo* by mGluR-dependent long-term depression. *Nat Neurosci* **9:** 636–641

Blitzer RD, Connor JH, Brown GP, Wong T, Shenolikar S, Iyengar R, Landau EM (1998) Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. *Science* **280:** 1940–1942

Blitzer RD, Wong T, Nouranifar R, Iyengar R, Landau EM (1995) Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. *Neuron* **15:** 1403–1414

Boehm J, Kang MG, Johnson RC, Esteban J, Huganir RL, Malinow R (2006) Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. *Neuron* **51:** 213–225

Borgdorff AJ, Choquet D (2002) Regulation of AMPA receptor lateral movements. *Nature* **417:** 649–653

Bortolotto ZA, Collingridge GL (2000) A role for protein kinase C in a form of metaplasticity that regulates the induction of long-term potentiation at CA1 synapses of the adult rat hippocampus. *Eur J Neurosci* **12:** 4055–4062

Bowie D, Mayer ML (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* **15:** 453–462

Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* **40:** 361–379

Burton KA, Treash-Osio B, Muller CH, Dunphy EL, McKnight GS (1999) Deletion of type IIalpha regulatory subunit delocalizes protein kinase A in mouse sperm without affecting motility or fertilization. *J Biol Chem* **274:** 24131–24136

Carlin RK, Grab DJ, Cohen RS, Siekevitz P (1980) Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. *J Cell Biol* **86:** 831–845

Carlisle Michel JJ, Scott JD (2002) AKAP mediated signal transduction. *Annu Rev Pharmacol Toxicol* **42:** 235–257

Chung HJ, Xia J, Scannevin RH, Zhang X, Huganir RL (2000) Phosphporylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *J Neurosci* **20:** 7258–7267

Collingridge GL, Isaac JT, Wang YT (2004) Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci* **5:** 952–962

Crair MC, Malenka RC (1995) A critical period for long-term potentiation at thalamocortical synapses. *Nature* **375:** 325–328

Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW (2001) A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. *Science* **293:** 98–101

Davare MA, Hell JW (2003) Increased phosphorylation of the neuronal L-type Ca2+ channel Cav1.2 during aging. *Proc Natl Acad Sci USA* **100:** 16018–16023

Daw MI, Chittajallu R, Bortolotto ZA, Dev KK, Duprat F, Henley JM, Collingridge GL, Isaac JT (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. *Neuron* **28:** 873–886

Desai NS, Cudmore RH, Nelson SB, Turrigiano GG (2002) Critical periods for experience-dependent synaptic scaling in visual cortex. *Nat Neurosci* **5:** 783–789

Duffy SN, Nguyen PV (2003) Postsynaptic application of a peptide inhibitor of cAMP-dependent protein kinase blocks expression of long-lasting synaptic potentiation in hippocampal neurons. *J Neurosci* **23:** 1142–1150

Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* **28:** 511–525

Esteban JA, Shi SH, Wilson C, Nuriya M, Huganir RL, Malinow R (2003) PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat Neurosci* **6:** 136–143

Foster TC, McNaughton BL (1991) Long-term enhancement of CA1 synaptic transmission is due to increased quantal size, not quantal content. *Hippocampus* **1:** 79–91

Frey U, Huang YY, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* **260:** 1661–1664

Gardner SM, Takamiya K, Xia J, Suh JG, Johnson R, Yu S, Huganir RL (2005) Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF. *Neuron* **45:** 903–915

Gomez LL, Alam S, Smith KE, Horne E, Dell'Acqua ML (2002) Regulation of A-kinase anchoring protein 79/150-cAMP-dependent protein kinase postsynaptic targeting by NMDA receptor activation of calcineurin and remodeling of dendritic actin. *J Neurosci* **22:** 7027–7044

Gorski JA, Gomez LL, Scott JD, Dell'Acqua ML (2005) Association of an A-kinase-anchoring protein signaling scaffold with cadherin adhesion molecules in neurons and epithelial cells. *Mol Biol Cell* **16:** 3574–3590

Hall DD, Feekes JA, Arachchige Don AS, Shi M, Hamid J, Chen L, Strack S, Zamponi GW, Horne MC, Hell JW (2006) Binding of protein phosphatase 2A to the L-type calcium channel Cav1.2 next to Ser1928, its main PKA site, is critical for Ser1928 dephosphorylation. *Biochemistry* **45:** 3448–3459

Han J, Mark MD, Li X, Xie M, Waka S, Rettig J, Herlitze S (2006) RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating G(i/o)-mediated inhibition of presynaptic Ca2+ channels. *Neuron* **51:** 575–586

Huang YY, Kandel ER (1994) Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. *Learn Mem* **1:** 74–82

Huang Z, Shimazu K, Woo NH, Zang K, Muller U, Lu B, Reichardt LF (2006) Distinct roles of the beta 1-class integrins at the developing and the mature hippocampal excitatory synapse. *J Neurosci* **26:** 11208–11219

Jensen V, Kaiser KM, Borchardt T, Adelmann G, Rozov A, Burnashev N, Brix C, Frotscher M, Andersen P, Hvalby O, Sakmann B, Seeburg PH, Sprengel R (2003) A juvenile form of postsynaptic hippocampal long-term potentiation in mice deficient for the AMPA receptor subunit GluR-A. *J Physiol* **553:** 843–856

Kamboj SK, Swanson GT, Cull-Candy SG (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainate receptors. *J Physiol* **486:** 297–303

Kirkwood A, Lee HK, Bear MF (1995) Co-regulation of long-term potentiation and experience-dependent synaptic plasticity in visual cortex by age and experience. *Nature* **375:** 328–331

Koh DS, Burnashev N, Jonas P (1995) Block of native Ca2+-permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. *J Physiol* **486:** 305–312

Kopec CD, Li B, Wei W, Boehm J, Malinow R (2006) Glutamate receptor exocytosis and spine enlargement during chemically induced long-term potentiation. *J Neurosci* **26:** 2000–2009

Lee HK, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405:** 955–959

Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112:** 631–643

Leonard AS, Davare MA, Horne MC, Garner CC, Hell JW (1998) SAP97 is associated with the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit. *J Biol Chem* **273:** 19518–19524

Lim IA, Merrill MA, Chen Y, Hell JW (2003) Disruption of the NMDA receptor–PSD-95 interaction in hippocampal neurons with no obvious physiological short-term effect. *Neuropharmacology* **45:** 738–754

Lisman J, Raghavachari S (2006) A unified model of the presynaptic and postsynaptic changes during LTP at CA1 synapses. *Sci STKE* **2006:** re11

Lu W, Ziff EB (2005) PICK1 interacts with ABP/GRIP to regulate AMPA receptor trafficking. *Neuron* **47:** 407–421

Makhinson M, Chotiner JK, Watson JB, O'Dell TJ (1999) Adenylyl cyclase activation modulates activity-dependent changes in synaptic strength and Ca2+/calmodulin-dependent kinase II autophosphorylation. *J Neurosci* **19:** 2500–2510

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. *Neuron* **44:** 5–21

Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. *J Biol Chem* **272:** 32528–32533

Man H-Y, Sekine-Aizawa Y, Huganir R (2007) Regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking through PKA phosphorylation of the Glu receptor 1 subunit. *Proc Natl Acad Sci USA* **104:** 3579–3584

Martin SJ, Grimwood PD, Morris RG (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci* **23:** 649–711

Matsushita M, Tomizawa K, Moriwaki A, Li ST, Terada H, Matsui H (2001) A high-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation. *J Neurosci* **21:** 6000–6007

McCormack SG, Stornetta RL, Zhu JJ (2006) Synaptic AMPA receptor exchange maintains bidirectional plasticity. *Neuron* **50:** 75–88

Merrill MA, Malik Z, Akyol Z, Bartos JA, Leonard AS, Hudmon A, Shea MA, Hell JW (2007) Displacement of alpha-actinin from the NMDA receptor NR1 C0 domain By Ca2 + /calmodulin promotes CaMKII binding. *Biochemistry* **46:** 8485–8497

Nayak A, Zastrow DJ, Lickteig R, Zahniser NR, Browning MD (1998) Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. *Nature* **394:** 680–683

Ndubuka C, Li Y, Rubin CS (1993) Expression of a kinase anchor protein 75 depletes type II cAMP-dependent protein kinases from the cytoplasm and sequesters the kinases in a particulate pool. *J Biol Chem* **268:** 7621–7624

Oh MC, Derkach VA, Guire ES, Soderling TR (2006) Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. *J Biol Chem* **281:** 752–758

Otmakhov N, Khibnik L, Otmakhova N, Carpenter S, Riahi S, Asrican B, Lisman J (2004) Forskolin-induced LTP in the CA1 hippocampal region is NMDA receptor dependent. *J Neurophysiol* **91:** 1955–1962

Pelkey KA, Topolnik L, Lacaille JC, McBain CJ (2006) Compartmentalized Ca2 + channel regulation at divergent mossy-fiber release sites underlies target cell-dependent plasticity. *Neuron* **52:** 497–510

Plant K, Pelkey KA, Bortolotto ZA, Morita D, Terashima A, McBain CJ, Collingridge GL, Isaac JT (2006) Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. *Nat Neurosci* **9:** 602–604

Rosenmund C, Carr DW, Bergeson SE, Nilaver G, Scott JD, Westbrook GL (1994) Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. *Nature* **368:** 853–856

Sans N, Racca C, Petralia RS, Wang YX, McCallum J, Wenthold RJ (2001) Synapse-associated protein 97 selectively associates with a subset of AMPA receptors early in their biosynthetic pathway. *J Neurosci* **21:** 7506–7516

Schulz PE, Cook EP, Johnston D (1994) Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. *J Neurosci* **14:** 5325–5337

Seidenman KJ, Steinberg JP, Huganir R, Malinow R (2003) Glutamate receptor subunit 2 serine 880 phosphorylation modulates synaptic transmission and mediates plasticity in CA1 pyramidal cells. *J Neurosci* **23:** 9220–9228

Skeberdis VA, Chevaleyre V, Lau CG, Goldberg JH, Pettit DL, Suadicani SO, Lin Y, Bennett MV, Yuste R, Castillo PE, Zukin RS (2006) Protein kinase A regulates calcium permeability of NMDA receptors. *Nat Neurosci* **9:** 501–510

Sun X, Zhao Y, Wolf ME (2005) Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. *J Neurosci* **25:** 7342–7351

Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* **125:** 785–799

Swayze RD, Lise MF, Levinson JN, Phillips A, El-Husseini A (2004) Modulation of dopamine mediated phosphorylation of AMPA receptors by PSD-95 and AKAP79/150. *Neuropharmacology* **47:** 764–778

Tardin C, Cognet L, Bats C, Lounis B, Choquet D (2003) Direct imaging of lateral movements of AMPA receptors inside synapses. *EMBO J* **22:** 4656–4665

Tavalin SJ, Colledge M, Hell JW, Langeberg LK, Huganir RL, Scott JD (2002) Regulation of GluR1 by the A-kinase anchoring protein 79 (AKAP79) signaling complex shares properties with long-term depression. *J Neurosci* **22:** 3044–3051

Thiagarajan TC, Lindskog M, Tsien RW (2005) Adaptation to synaptic inactivity in hippocampal neurons. *Neuron* **47:** 725–737

Valtschanoff JG, Burette A, Davare MA, Leonard AS, Hell JW, Weinberg RJ (2000) SAP97 concentrates at the postsynaptic density in cerebral cortex. *Eur J Neurosci* **12:** 3605–3614

Vinade L, Chang M, Schlief ML, Petersen JD, Reese TS, Tao-Cheng JH, Dosemeci A (2003) Affinity purification of PSD-95-containing postsynaptic complexes. *J Neurochem* **87:** 1255–1261

Wenthold RJ, Petralia RS, Blahos II J, Niedzielski AS (1996) Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J Neurosci* **16:** 1982–1989

Westphal RS, Tavalin SJ, Lin JW, Alto NM, Fraser IDC, Langeberg LK, Sheng M, Scott JD (1999) Regulation of NMDA receptors by an associated phosphatase–kinase signaling complex. *Science* **285:** 93–96

Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. *Science* **313:** 1093–1097

Wikstrom MA, Matthews P, Roberts D, Collingridge GL, Bortolotto ZA (2003) Parallel kinase cascades are involved in the induction of LTP at hippocampal CA1 synapses. *Neuropharmacology* **45:** 828–836

Winder DG, Mansuy IM, Osman M, Moallem TM, Kandel ER (1998) Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. *Cell* **92:** 25–37

Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR (1999) Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. *Neuron* **23:** 787–798

Wong W, Scott JD (2004) AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol* **5:** 959–970

Woo NH, Duffy SN, Abel T, Nguyen PV (2000) Genetic and pharmacological demonstration of differential recruitment of cAMP-dependent protein kinases by synaptic activity. *J Neurophysiol* **84:** 2739–2745

Yasuda H, Barth AL, Stellwagen D, Malenka RC (2003) A developmental switch in the signaling cascades for LTP induction. *Nat Neurosci* **6:** 15–16

Zalutsky RA, Nicoll RA (1990) Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* **248:** 1619–1624

Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, Kaiser KM, Koster HJ, Borchardt T, Worley P, Lubke J, Frotscher M, Kelly PH, Sommer B, Andersen P, Seeburg PH, Sakmann B (1999) Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science* **284:** 1805–1811

Zhu JJ, Esteban JA, Hayashi Y, Malinow R (2000) Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat Neurosci* **3:** 1098–1106

