Perisynaptic GluR2-lacking AMPA receptors control the reversibility of synaptic and spines modifications

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How persistent synaptic and spine modification is achieved is essential to our understanding of developmental refinement of neural circuitry and formation of memory. Within a short period after their induction, both types of modifications can either be stabilized or reversed, but how this reversibility is controlled is largely unknown. We have shown previously that AMPA receptors (AMPARs) are delivered to perisynaptic regions after the induction of long-term potentiation (LTP) but are absent from perisynaptic regions after the full expression of LTP. Here, we report that perisynaptic AMPARs are GluR2-lacking and they translocate to synapses in a protein kinase C (PKC)-dependent manner. Once entering synapses, these AMPARs quickly switch to GluR2-containing in an activity-dependent manner. Absence of postinduction activity or blocking interactions between GluR2 and NSF, or GluR2 and GRIP/PICK1 results in LTP mediated by GluR2-lacking AMPARs. However, these synaptic GluR2-lacking AMPARs are not sufficient to allow reversibility of LTP. On the other hand, postsynaptic inhibition of PKC activity holds AMPARs at perisynaptic regions. As long as perisynaptic AMPARs are present, both LTP and spine expansion remain labile: they can be reverted to the baseline state together with removal of perisynaptic AMPARs, or they can enter a stabilized state of persistent increase together with synaptic incorporation of perisynaptic AMPARs. Thus, perisynaptic GluR2-lacking AMPARs play a critical role in controlling the reversibility of both synaptic and spine modifications.

dendritic spine | long-term potentiation | two-photon imaging

Persistent functional and structural changes in synaptic connections are generally believed to underlie long-lasting modifications in neuronal networks, such as developmental refinement of neural circuitry and memory formation in the adult (1–3). One widely studied form of such long-lasting changes is long-term potentiation (LTP), which is accompanied by long-lasting expansion of dendritic spines (2–4). Within a short time window after LTP induction, both types of modifications can be reversed (5, 6). What controls the labile period of these modifications is of great importance to our understanding of the consolidation of synaptic and spine modifications.

Modification of existing synaptic AMPA receptors (AMPARs) and/or addition of new AMPARs to synapses appear to underlie the expression of LTP. Evidence supports a model that newly added AMPARs first appear at extrasynaptic/perisynaptic regions and are subsequently incorporated into synapses (7–11). These perisynaptic AMPARs can be removed by moderate synaptic activity (10) and this removal prevents the full expression of LTP and reverses spine expansion (6, 10). It has been suggested that perisynaptic AMPARs could play a critical role in regulating the persistence of LTP and spine expansion (10, 12). This notion is consistent with the observation that stabilization of spine expansion requires synaptic incorporation of new AMPARs (6, 13). A few studies showed that induction of LTP leads to synaptic appearance of GluR2-lacking AMPARs (14–16) whose removal may allow reversal of potentiation (17). These GluR2-lacking AMPARs are quickly replaced by GluR2-containing AMPARs and this switch in subunit composition makes synaptic AMPARs impermeable to Ca^{2+} and hence not vulnerable to glutamate excitotoxicity (18). Synaptic addition of GluR2 subunits may also stabilize structural remodeling associated with LTP because overexpression of GluR2 leads to increases in spine density and spine head width (19). Thus, GluR2-lacking AMPARs might keep both LTP and spine expansion in a labile state whereas switching to GluR2-containing AMPARs stabilizes both forms of modifications. However, whether GluR2-lacking AMPARs underlie LTP expression remains highly controversial (20–22).

In this study, we found that perisynaptic AMPARs are GluR2-lacking and switch to GluR2-containing after translocating to synapses. No reversal of LTP could be induced when GluR2-lacking AMPARs are present at synapses indicating that they are not sufficient to support reversal. On the other hand, both LTP and spine expansion remain labile when perisynaptic AMPARs are held in place by inhibiting PKC activity. Together, these results demonstrate that GluR2-lacking AMPARs can control the reversibility of LTP and spine expansion only when they are at perisynaptic region.

Results

Perisynaptic AMPARs Are GluR2-Lacking. We have shown previously that pairing presynaptic theta burst stimulation with postsynaptic depolarization, termed theta burst pairing (TBP), induces a slowly developing LTP (6, 10). This slowly developing LTP is not accompanied by and hence not mediated by changes in the intrinsic excitability of postsynaptic neurons (Fig. S1) (23, 24). With TBP, AMPARs appear at perisynaptic region and can be activated by spillover of synaptically released glutamate using glutamate uptake blocker TFB-TBOA (TBOA) (100 nM) (6). In control neurons, TBOA caused a significant increase in EPSPs, indicating the presence of perisynaptic AMPARs (Fig. 1*A*). In interleaved experiments performed in the presence of polyamine toxin philanthotoxin 433 (PhTx433) [10 μM, a selective antagonist to GluR2-lacking AMPARs (25)], TBOA did not have much effect on EPSPs (Fig. 1*A*), indicating that perisynaptic AMPARs do not contain GluR2 (*SI Text, Note 1*). Another selective antagonist to GluR2-lacking AMPARs, IEM-1460 (IEM) (50 μM), was applied in the same manner as PhTx433, and again TBOA lost its effect on EPSPs (Fig. 1*B*). When PhTx433 was added 30 min after TBP, at a time when LTP had reached a plateau, no changes in EPSPs were observed (Fig. 1*C*). These results indicate that perisynaptic AMPARs do not contain GluR2, but the newly added synaptic AMPARs do and hence a switch in receptor composition occurs. PhTx433 did not have an effect on basal synaptic transmission (Fig. S2) (25, 26).

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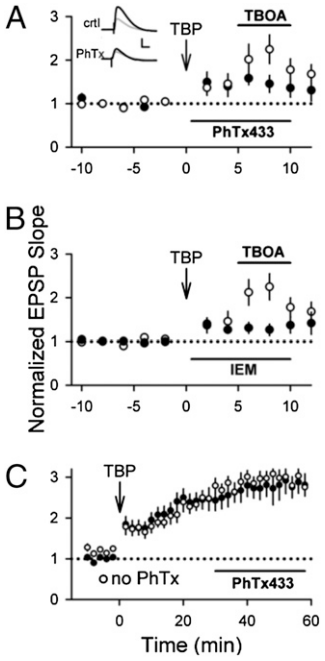

Fig. 1. Transient presence of GluR2-lacking AMPARs after LTP induction. (*A*) TBOA significantly enhanced EPSPs after TBP in control neurons (○; 225 ± 33%; n = 6). PhTx433 blocked TBOA-induced enhancement of EPSPs (●: 145 ± 19%, n = 8, $P < 0.05$ comparing to that without PhTx433). The arrow indicates TBP given at time 0. (*Inset*) Sample synaptic responses during baseline and in the presence of TBOA, in gray and black, respectively. (Scale bars: 5 mV and 20 ms.) (*B*) Similar to PhTx433, IEM also blocked the enhancement of TBOA on EPSPs [●: 131 ± 15%, n = 8, $P < 0.05$ comparing to that without IEM (○: 227 ± 30%, n = 5)]. (*C*) PhTx433 had no effect on LTP expression when added 30 min after TBP [●: 283 ± 30%, n = 8, P = 0.58 comparing with control LTP (○: 292 ± 15%, n = 7) 45–50 min after TBP].

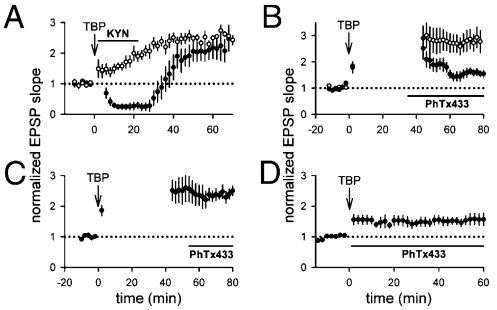

Fig. 2. Postinduction synaptic activity is required for switch in AMPAR subunit composition but not synaptic appearance of new AMPARs. (*A*) Kyneuric acid (KyN) was bath-applied 2 min after TBP. Synaptic responses were almost completely blocked during the presence of KyN (●). After washout of KyN, EPSPs developed gradually and reached a level comparable to that in the control neurons (control: ○, 244 ± 10%, n = 7; Kyn: ●, 222 ± 40%, n = 6, P = 0.35). (*B*) Pausing synaptic stimulation for 45 min, 2 min after TBP, did not affect LTP expression (○: 280 ± 38%, n = 9). However, bath application of PhTx433 10 min before resuming stimulation significantly reduced LTP (●: 159 ± 13%, n = 8, $P < 0.001$ comparing with control). (*C*) Bath application of PhTx433 10 min after resuming synaptic stimulation had no significant effect on LTP (240 ± 18%, n = 6). (*D*) PhTx433 applied immediately after TBP blocked the full expression of LTP (154 ± 17%, n = 8; $P < 0.001$ comparing to control LTP in *A*).

Postinduction Activity Is Required for Switching to GluR2-Containing AMPARs but Not Synaptic Incorporation of AMPARs. We next examined the effect of blocking of all synaptic activity on LTP expression using bath application of kyneuric acid (KyN) (3 mM) starting 2 min after TBP. After washout of KyN, EPSPs developed gradually and reached a level comparable to that in control neurons (Fig. 2*A*; *SI Text*, *Note 2*). Thus, synaptic activity is not required for LTP expression. Consistent with this conclusion, LTP expression was not affected by pausing synaptic stimulation for 45 min, 2 min after TBP (Fig. 2*B*).

To our surprise, when PhTx433 was added 10 min before resuming synaptic stimulation, EPSPs were quickly reduced to a level similar to that before pausing stimulation (Fig. 2*B*). This suggests that LTP is mainly mediated by GluR2-lacking AMPARs. In other words, in the absence of postinduction activity, GluR2-lacking AMPARs can enter synapses, but they remain GluR2-lacking. Furthermore, LTP was not affected when PhTx433 was applied 10 min after resuming synaptic stimulation (Fig. 2*C*). Application of PhTx433 shortly after TBP blocked the full expression of LTP, suggesting that subunit switch requires activation of GluR2-lacking AMPARs (Fig. 2*D*; *SI Text*, *Note 3*). Put together, the above results are consistent with a model in which after TBP, GluR2-lacking AMPARs first appear at the perisynaptic region, they are then incorporated into synapses and switch to GluR2-containing shortly after their activation at synapses. Alternatively, synaptic activity may be required to maintain GluR2 subunits, and absence of activity results in switching to GluR2-lacking AMPARs (*SI Text*, *Note 4*).

Requirement of NSF and PICK1/GRIP in the Switch of Subunit Composition. Switching from GluR2-lacking to GluR2-containing requires interactions between GluR2 and NSF, GluR2, and PICK1/GRIP in the cerebellar stellate cells (27, 28). PICK1 also plays a critical role in subunit switch in the hippocampal CA1 neurons (26). We loaded postsynaptic neurons with short, synthetic peptides to disrupt these interactions. In neurons loaded with pep-2m that disrupts GluR2–NSF interaction (29), a reduction in baseline EPSPs was observed (Fig. 3*A*). After TBP, normal LTP was seen (Fig. 3*A* and *SI Text*, *Note 5*) but was largely reversed by PhTx433 (Fig. 3*A*). Hence, in the absence of GluR2–NSF interaction, LTP is mediated by GluR2-lacking AMPARs, similar to LTP expressed in the absence of synaptic stimulation (Fig. 2*B*). Because PhTx433 did not affect EPSPs after pep-2m-induced rundown had reached a plateau in control cells (no LTP induced) (Fig. S3)

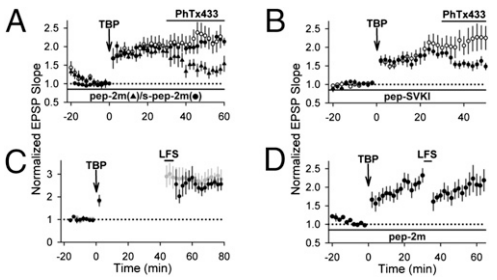

Fig. 3. Inhibiting subunit switch leads to LTP mediated by GluR2-lacking AMPARs. (*A*) To disrupt GluR2-NSF interaction, neurons were loaded with pep-2m through the recording patch pipette. Although baseline EPSPs were reduced by this procedure, normal LTP was seen after TBP (○: 220 ± 27%, n = 11, P = 0.68 comparing with control LTP), this LTP was largely abolished by bath application of PhTx433 (▲; 139 ± 14%; n = 5; $P < 0.01$ comparing with control LTP). Loading with scrambled pep-2m had no effect on either baseline responses or LTP (●: 217 ± 12%, n = 11, P = 0.55 comparing with ○). (*B*) Internal loading of pep-SVKI had no effects on basal synaptic transmission and LTP (○: 199 ± 18%, n = 10, P = 0.75 comparing with control LTP); however, this LTP was significantly reduced by PhTx433 (●: 153 ± 8%, n = 11, $P < 0.05$ comparing with control LTP). (*C*) LFS given immediately before resuming test stimulation did not affect LTP (black: 254 ± 23%, n = 7, P = 0.93), comparing to those without receiving LFS (gray: data from Fig. 2*B*). (*D*) In neurons loaded with pep-2m, LFS given 30 min after TBP did not affect LTP (216 ± 30%, n = 6, P = 0.85 comparing with control LTP).

(23), the PhTx433 sensitivity after LTP induction is most likely mediated by newly added AMPARs. Thus, GluR2–NSF interaction is required for subunit switch but not synaptic incorporation of AMPARs. Loading of pep-SVKI, which disrupts interactions between GluR2 and GRIP/PICK1, did not alter baseline synaptic transmission or LTP (Fig. 3*B*), but LTP was significantly reduced by PhTx433 (Fig. 3*B*). Therefore, GluR2–PICK1/GRIP interaction is required for subunit switch but not synaptic incorporation of AMPARs.

GluR2-lacking AMPARs have been suggested to endow neurons with the capacity to reverse synaptic potentiation (17). We previously found that brief, low-frequency stimulation (LFS) given shortly after TBP removed perisynaptic AMPARs (10) and prevented the full expression of LTP (6). To test whether LFS can disrupt LTP-mediated by GluR2-lacking synaptic AMPARs, we gave LFS before resuming test stimulation when new synaptic AMPARs do not contain GluR2. Despite a transient depression, LTP was not affected by LFS (Fig. 3*C*). Because subunit switch is activity-dependent, it is possible that during LFS subunit switch may occur faster than the removal of AMPARs. Neurons were loaded with pep-2m to block subunit switch, and LFS was still ineffective in depressing EPSPs (Fig. 3*D*). Thus, once AMPARs are incorporated into synapses they cannot be removed by moderate synaptic activity and hence it is unlikely that GluR2-lacking synaptic AMPARs play a major role in the reversal of LTP.

PKC Activity Is Required for the Full Expression of LTP but Not Spine Expansion. Our previous studies showed that removing perisynaptic AMPARs leads to the reversal of spine expansion and prevents the full expression of LTP (6, 10). Thus, perisynaptic AMPARs may control the reversibility of both LTP and spine expansion. If this is the case, incorporation of perisynaptic AMPARs to synapse should terminate the reversal period. A direct prediction of this hypothesis is that inhibiting synaptic incorporation of perisynaptic AMPARs should extend the reversal window.

Protein kinase C (PKC) may play an important role in AMPAR translocation because PKC activity is required for the persistent expression of LTP (30–33) and synaptic incorporation of AMPARs (ref. 34, but see ref. 35), and enhanced AMPAR trafficking is seen with elevated PKC activity (36). Thus, we tested the effect of PKC inhibition on LTP and spine expansion. When TBP was delivered in the presence of a reversible PKC inhibitor chelerythrine (Chel) (5 μM), the full expression of LTP was inhibited (Fig. 4*A*). Loading pyramidal neurons with a peptide inhibitor of PKC, PKCI(19–36) (50 μM), through the recording patch pipette also inhibited the full expression of LTP (Fig. 4*A*). Chel or PKCI (19–36) did not have any significant effects on basal synaptic transmission (Fig. S4 *A* and *C*) or the size of naïve spines (Fig. S4 *B* and *D*). Spine expansion occurred normally and persisted for at least 60 min after TBP when PKC activity was inhibited by either Chel or PKCI(19–36) (Fig. 4*B*). Bath application of Chel 30 min after TBP had no significant effect on EPSPs (Fig. S5), suggesting that sustained PKC activity is not required to maintain new synaptic AMPARs or LTP expression. Loading pyramidal neurons with PKC activator phorbol 12,13-dibutyrate (PDBU) (5 μM) did not affect LTP expression, spine expansion, basal synaptic transmission, or basal spine size (Fig. S6), suggesting that elevated postsynaptic PKC activity does not lead to accelerated synaptic incorporation of AMPARs.

PKC Activity Is Not Required for the Delivery or Maintenance of Perisynaptic AMPARs. PKC is unlikely to affect the induction of LTP (34). Our previous results showed that inhibiting the delivery or removing perisynaptic AMPARs abolished the gradual development of LTP (6, 10). Thus, it is conceivable that PKC activity may affect the delivery, maintenance, or translocation of perisynaptic AMPARs. In the presence of Chel or PKCI(19–36), EPSPs were enhanced by TBOA to an extent indistinguishable

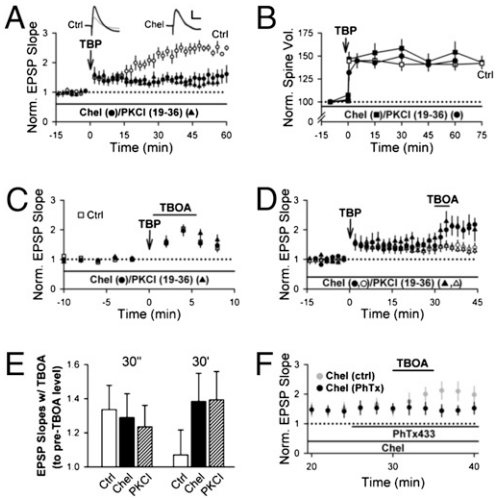

Fig. 4. PKC activity is required for the full expression of LTP but not delivery or maintenance of perisynaptic AMPARs. (*A*) Blocking PKC activity, by either bath application of chelerythrine (Chel, ●: 158 ± 27% at 45–50 min after TBP; $n = 7$; $P < 0.05$, comparing with control LTP) or internal loading of PKCI (19–36) (▲: 126 ± 8%, $n = 8$, $P < 0.01$), prevented the full expression of LTP, comparing with that in control neurons (open circles: 252 ± 13%, $n = 7$). (*Insets*) Sample voltage traces [control (*Left*) and Chel-treated (*Right*)]. EPSPs during baseline and after TBP are shown in gray and black, respectively. (Scale bars: 3 mV and 50 ms.) (*B*) Population data showing that neither the initial nor persistent spine expansion is affected by inhibiting PKC activity. There is no difference between Chel (■: 154 ± 11%, $n = 59$ spines/6 cells, $P = 0.35$), PKCI(19–36) (●: 145 ± 10%, $n = 51$ spines/5 cells, $P = 0.73$), or control (□; 142 ± 6%, $n = 76$ spines/8 cells). (*C*) Enhancement in EPSPs were seen when TBOA was bath-applied 30 s after TBP in the presence of Chel (●: 197 ± 14%, $n = 9$) or PKCI(19–36) (▲: 197 ± 18%, $n = 6$), not different comparing to the enhancement in control neurons (open squares: 205 ± 13%, $n = 5$). (*D*) Similar TBOA-induced enhancement was observed in either Chel-bathed (●: 212 ± 30%, $n = 8$) or PKCI-loaded (▲: 228 ± 34%, $n = 8$) cells where TBOA was added 30 min after TBP. Responses in the absence of TBOA were shown for comparison (open symbols, $n = 8$). (*E*) Bar graphs comparing the effects of TBOA on EPSPs when added 30 s or 30 min after TBP. Comparable increases were seen at 30 s in both control and PKC-inhibited neurons, but increases at 30 min were observed only in PKC-inhibited neurons (black and hatched bars). The values were computed by dividing EPSP slopes in the presence of TBOA over the values from baseline period immediately before TBOA application. (*F*) When TBOA was added in the presence of PhTx433, in Chel-bathed neurons (●), it did not enhance EPSPs (153 ± 21%, $n = 9$, $P < 0.05$ comparing with *D*). These experiments were done in the same manner as those in *D*, and the period between 20 and 40 min after TBP was presented to show details. The data from *D* are shown by the gray circles for comparison.

from those in control neurons (Fig. 4 *C* and *E*), indicating that delivery of perisynaptic AMPARs to spine surface does not require PKC activity. We added TBOA 30 min after TBP in the presence of PKC blockers and found that EPSPs were enhanced similarly as when TBOA was added 30 s after TBP (Fig. 4 *D* and *E*). This result suggests that perisynaptic AMPARs are maintained in the absence of PKC activity, and importantly they are present for longer period (at least 30 min) than they are under control conditions (10–15 min). In addition, TBOA did not have much of an effect on EPSPs in the presence of PhTx433 (Fig. 4*F*). Put together, in the absence of PKC activity, new AMPARs are held at perisynaptic region and do not change their subunit composition.

Synaptic Translocation of Perisynaptic AMPARs Stabilizes Both Synaptic and Spine Modifications. The above results suggest that in the absence of PKC activity, AMPARs are delivered to and maintained at perisynaptic regions after TBP, but they are not incorporated

into synapse. Hence, it is likely that translocation of perisynaptic AMPARs to synapses requires PKC activity. If this is the case, perisynaptic AMPARs may move to synapse once PKC inhibition is removed. Shortly after washout of Chel, EPSPs gradually increased and reached a plateau similar to that in control neurons (Fig. 5*A*), consistent with the long-lasting elevation in PKC activity after LTP induction (37). In control neurons, after the full expression of LTP perisynaptic AMPARs are absent at spine surface, and LFS does not affect LTP (10). To test whether this is also the case after the removal of Chel, TBOA was added at the plateau of increase in EPSPs and did not alter EPSPs (Fig. 5*B*). When LFS was given at the plateau of increase in EPSPs, only a transient depression was seen (Fig. 5*C*), likely presynaptic in nature. These results suggest that after restoring PKC activity, LTP develops in a manner indistinguishable from the LTP induced without prior PKC inhibition. No further spine expansion was observed following Chel removal (Fig. 5*D*), suggesting that spine expansion may have been saturated. Spine size was not altered after LFS given at the plateau of increase in EPSPs (Fig. 5*E*). These results support the notion that once perisynaptic AMPARs have entered the synapse, both LTP and spine modifications enter a stabilized, potentiated state that is resistant to reversal by moderate synaptic activity.

Reversal Time Window Is Extended in the Absence of PKC Activity. If reversal is controlled by the presence of perisynaptic AMPARs, removal of perisynaptic AMPARs should disable the reversal. To test this prediction, we gave LFS 30 min after TBP in the presence of Chel, then washed out Chel and found that the full expression of LTP was blocked (Fig. 6*A*). When LFS was given at the same time point in control neurons, only a transient depression was seen (Fig. S7), and no changes in EPSPs were seen after LFS with sustained inhibition of PKC (Fig. S8). When added immediately after LFS, TBOA did not affect EPSPs (Fig. 6*B*), indicating the absence and hence removal of perisynaptic AMPARs. With PKC inhibited, expanded spines collapsed back to baseline level after LFS (Fig. 6 *C* and *D*), in clear contrast to the persistent expansion in control neurons (Fig. 6*D*). This result suggests that removal of perisynaptic AMPARs is also associated with the reversal of spine expansion. Importantly, without PKC activity, the reversal period for spine expansion is extended to at least 30 min after TBP. LFS given 15 min after TBP prevented the full development of LTP in the PDBU-loaded neurons (Fig. S9*A*) (6), indicating that reversal time window is not shortened by enhanced PKC activity. Thus, postsynaptic PKC activity appears to play a permissive role in the translocation of perisynaptic AMPARs. The above results support the notion that perisynaptic AMPARs sustain the reversal time window of both LTP and spine expansion.

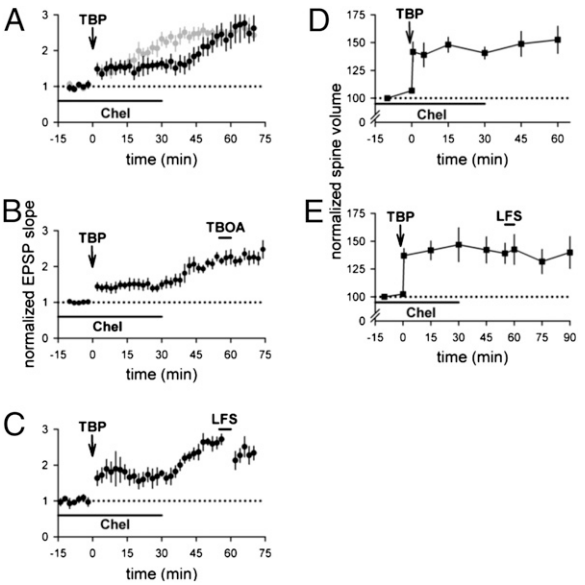

Fig. 5. Synaptic translocation of perisynaptic AMPARs stabilizes both synaptic and spine modifications. (*A*) Inhibition of the full development of LTP by PKC inhibitors is reversible. After removal of Chel, EPSPs increased gradually and persistent LTP was seen (245 ± 31% at 55–60 min after TBP, n = 8). For comparison, data from control neurons (from Fig. 4*A*) are depicted by the gray circles. (*B*) Full development of LTP is accompanied by the absence of TBOA-induced enhancement in EPSPs (227 ± 12%, n = 9) after washout of Chel. (*C*) LFS does not affect EPSPs after the full development of LTP with Chel removed 30 min after TBP (230 ± 23%, n = 5). (*D*) No change in spine expansion occurs after restoring PKC activity (152 ± 12%, n = 67 spines/8 cells). (*E*) LFS does not affect spine expansion when giving at the plateau of increase in EPSPs (140 ± 14%, n = 68 spines/6 cells).

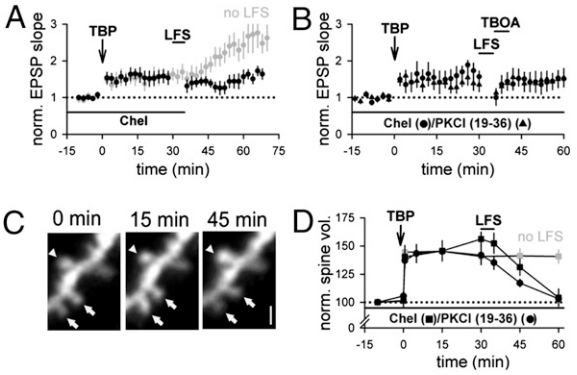

Fig. 6. Removal of perisynaptic AMPARs leads to reversal of synaptic and spine modifications. (*A*) LFS suppresses the full expression of LTP when given in the presence of PKC inhibitors. The gradual increase in EPSPs seen in control neurons (gray: from Fig. 5*A*) was absent in neurons receiving LFS (black: 155 ± 18%, n = 8). Chel was removed after LFS to restore PKC activity. (*B*) LFS, given 30 min after TBP, abolished the effect of TBOA that was added immediately after LFS in the presence of Chel (●: 144 ± 24%, n = 5) or PKCI (19–36) (▲: 142 ± 26%, n = 10). (*C*) Sample images showing the reversal of spine expansion after LFS in a neuron bathed in Chel. Arrows and arrowheads marked the expanded and stable spines, respectively. TBP was given at 0 min. (Scale bar: 1 μm.) (*D*) Population data showing that LFS reverses TBP-induced spine expansion in the presence of Chel (■: 104 ± 8%, n = 55 spines/7 cells) or PKCI(19–36) (●: 103 ± 2%, n = 40 spines/5 cells), comparing with the persistent expansion in control neurons (gray squares: from Ctrl in Fig. 4*B*).

Discussion

Our results provide insights into how persistent expression of LTP and spine expansion is regulated. We found that after inducing LTP with TBP, GluR2-lacking AMPARs first appear at perisynaptic region and then at synapse where they quickly switch to GluR2-containing upon activation. This synaptic incorporation requires postsynaptic PKC activity but not post-LTP synaptic activity. Absence of activity or disrupting interactions between GluR2 and NSF, or GluR2 and GRIP/PICK1 prevents switching to GluR2-containing AMPARs and results in LTP mediated by GluR2-lacking AMPARs. Thus, postinduction synaptic activity does not affect the expression of LTP but determines the subunit composition of AMPARs that underlies LTP. The reversal time window of both LTP and spine expansion can be extended by holding perisynaptic AMPARs in place via inhibition of postsynaptic PKC activity. We propose a model in which perisynaptic AMPARs play a critical role in the long-term expression of both functional and structural modifications (Fig. S9*B*).

The simplest and most likely explanation of our results is that translocation of perisynaptic AMPARs to synapse underlies synaptic incorporation of new AMPARs and expression of LTP. This model is supported by the findings that new synaptic AMPARs are devoid of GluR2, synaptic incorporation of new AMPARs is abolished by removal of perisynaptic AMPARs, and AMPARs can exchange between synaptic and extrasynaptic/perisynaptic regions (10, 38). This model is further supported by recent findings of Makino and Malinow (11) that extrasynaptic GluR1-containing AMPARs traffic to synapse with LTP induction. Makino and Malinow (11) also showed that LTP induction is accompanied by a delayed exocytosis of new AMPARs to dendrites, and they suggested that these AMPARs contribute to the expression of future LTP. This delayed dendritic exocytosis could escape our detection with TBOA because they are likely further away from the synapse than perisynaptic AMPARs and hence beyond the reach by spilled synaptic glutamate. On the other hand, we and others have suggested that LTP induction triggers exocytosis of AMPARs, which are subsequently incorporated into synapse and underlie LTP expression (6, 39–41). Future experiments will be required to resolve this discrepancy. On the other hand, although we think it unlikely, we cannot rule out the possibility that perisynaptic AMPARs are internalized after a short presence and that this internalization is coupled to direct insertion of GluR2-containing AMPARs to synapses from an intracellular pool (*SI Text*, *Note 6*).

Our results may provide potential resolution to the controversy of whether GluR2-lacking AMPARs mediate LTP expression (14–16, 20–22, 42). There are two potential resolutions: (*i*) there is one form of LTP but it is expressed with different kinetics, or (*ii*) two different forms of LTP exist. For the first possibility, LTP induction triggers delivery of GluR2-lacking AMPARs to perisynaptic regions followed by their synaptic incorporation and switching to Glu2-containing AMPARs. Differences in induction protocols may alter the kinetics of these events and contribute to the reported discrepancy. It is possible that translocation of perisynaptic AMPARs and subunit switch occur very rapidly with pairing protocol and that these processes were already completed by the time PhTx433 was added (20). This scenario is consistent with more GluR2-containing AMPARs being present after LTP induction (42). An alternative scenario is that different induction protocols leads to two different forms of LTP, one that requires GluR2-lacking AMPARs and another that does not. Supporting this notion, theta burst stimulation led to the transient synaptic appearance of GluR2-lacking AMPARs (16) and required postsynaptic SNARE-dependent exocytosis (6, 10); but high-frequency, stimulation-induced LTP is mediated by synaptic addition of GluR2/3-containing AMPARs (43) and not GluR2-lacking AMPARs (ref. 16, but see ref. 15), and does not require exocytosis (43). Further testing is required to determine whether different forms of LTP coexist and, if so, under what conditions one form or the other is selectively induced, and whether the expression is synapse-specific given the known heterogeneity of synaptic compositions (44).

Our results indicate that reversibility of synaptic and spine modifications is controlled by both the localization (perisynaptic) and subunit composition (GluR2-lacking) of the newly added AMPARs. The proposal that perisynaptic AMPARs mediates reversal of LTP (depotentiation) is different from the conventional view that removing AMPARs underlies depotentiation (*SI Text*, *Note 7*). How can perisynaptic AMPARs keep LTP and spine expansion in a labile state? We suggest that interaction between AMPARs and spines/cytoskeleton may play a key role. Spine expansion is sustained in the presence of perisynaptic AMPARs, suggesting an interaction between perisynaptic AMPARs and the newly added cytoskeletal elements (such as F-actin) (45–47). This scenario can also explain why these perisynaptic AMPARs can stay in place for a sustained period amid the high mobility demonstrated for nonsynaptic AMPARs (38). This interaction, however, is weak and can be disrupted by moderate activity (such as LFS) that does not affect naïve synapses or spines (6). In addition, a recent study suggests that phosphorylation of S845 of GluR1 is critical to maintain perisynaptic AMPARs (48). With elevated postsynaptic PKC activity, perisynaptic AMPARs can traffic into synapses (*SI Text*, *Notes 8*). Once there, these AMPARs become associated with PSD proteins and trapped there, and this attachment to strong scaffolds offers them resistance to removal by LFS. This stronger anchoring may also enable spine expansion to be insensitive to LFS via a mechanism yet to de elucidated. It is also possible that anchoring of these new AMPARs at synapses requires the delivery of new scaffolding/anchoring proteins (49, 50). Thus, our findings further highlight a possibility that both functional and structural plasticity are eventually consolidated in a coordinated manner despite their independent initial expression.

Materials and Methods

Slice Preparation and Recordings. These procedures have been published (6, 51). Briefly, coronal hippocampal sections (350 μm) were taken from postnatal day 13 (P13)–P18 rat pups (Sprague–Dawley) using a Leica VT1000 tissue slicer in 4 °C artificial cerebral spinal fluid (ACSF) containing 110 mM choline chloride, 25 mM $NaHCO_3$, 25 mM D-glucose, 7 mM $MgSO_4$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 11.6 mM Na ascorbate, 3.1 mM Na pyruvate, and 0.5 mM $CaCl_2$. The recording ACSF contained 127 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM D-glucose, 2 mM $CaCl_2$, and 1 $MgCl_2$. Slices were allowed to recover for 30 min at 32 °C. They were then transferred to a holding chamber at room temperature in ACSF containing 127 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM D-glucose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. Recording and imaging started at least 1 h after recovery. Slices were placed in a custom-made recording chamber on the stage of an Olympus BX61 W microscope and perfused at a rate of 1–2 mL/min with ACSF. All recording and imaging experiments were performed at 30–32 °C. Whole-cell patch-clamp recordings were made from pyramidal neurons in CA1 of hippocampus under visual guidance. CA1 pyramidal cells in the hippocampus were held in current clamp mode throughout the experiments. EPSPs were recorded with an Axopatch 700B amplifier and analyzed with pClamp 9.0 software (Molecular Devices). The initial slope of EPSPs was used to measure synaptic responses. The recording pipette solution contained 128 mM K-gluconate, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM Hepes, 0.5 mM EGTA, 4 mM Na_2ATP, 0.4 mM NaGTP, 15 mM phosphocreatine, 1 mM calcein, and 0.1 mM spermine (pH 7.3). Calcein is a biologically inert fluorescent dye that we used for labeling of the dendritic spines. All experiments were carried out in the presence of a GABAA antagonist, picrotoxin (50 μM). For the electrophysiology experiments without imaging, glass pipettes (tip diameter, 4–6 μm) filled with ACSF were placed in the stratum radiatum to stimulate the presynaptic inputs. For combined recording and imaging experiments, synaptic inputs were stimulated using a glass pipette with a 3-μm opening positioned ~20–30 μm away from the imaged spines. Stimulation at 0.05 Hz was used to establish baseline synaptic responses. LTP was induced using theta-burst pairing protocol. Briefly, the stimulation strength was set to evoke EPSPs between 5 and 8 mV. LTP was induced using TBS protocol. A train of TBS consisted of five bursts of stimuli at 5 Hz, and each burst contained five pulses at 100 Hz. Each train was repeated twice with a 20-s interval. During TBS, the postsynaptic cells were depolarized through current injection to ensure that at least three spikes were generated during each burst. LFS consisted of 1-Hz stimulation for 5 min.

Image Acquisition and Analysis. Image acquisition and analysis images were taken every 15 min at a resolution of 512 × 512 pixels per frame, and an average of two were used in some experiments. For each time point, a stack of images covering the entire 3D range of the spines were taken with a *z*-step size ~0.5 μm. Two-dimensional projections of 3D image stacks containing dendritic spines of interest were used for display. Image analysis was performed blind with the person analyzing the images having no knowledge of the identity of the samples during the analysis. Spines were distinguished from filopodia based on our previous criteria (52). Analysis was performed on all spines in the image field that were well resolved—i.e., protruding tangentially from the dendrite and clearly separated from other spines. The volumes of spine heads were used as measurements of the size. Images were first thresholded to eliminate background fluores-

cence. The integrated fluorescence intensity inside a spine head was measured for individual spines at different time points and normalized to the fluorescence intensity of the dendrites from the same image stack to correct for potential changes in excitation (52). This fluorescence intensity is expected to be proportional to the accessible spine volume (52). Fold change (volume) was determined by averaged values after TBP over the averaged values before TBP.

Statistical Analysis. All data were expressed as mean ± SEM. Paired Student's *t* tests, Wilcoxon's signed ranks tests, and Wilcoxon's rank sum tests were used as appropriate. $P < 0.05$ was used to determined significance.

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