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Subunit-Specific Rules Governing AMPA Receptor Trafficking to Synapses in Hippocampal Pyramidal Neurons

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Summary

AMPA-type glutamate receptors (AMPA-Rs) mediate a majority of excitatory synaptic transmission in the brain. In hippocampus, most AMPA-Rs are hetero-oligomers composed of GluR1/GluR2 or GluR2/GluR3 subunits. Here we show that these AMPA-R forms display different synaptic delivery mechanisms. GluR1/GluR2 receptors are added to synapses during plasticity; this requires interactions between GluR1 and group I PDZ domain proteins. In contrast, GluR2/GluR3 receptors replace existing synaptic receptors continuously; this occurs only at synapses that already have AMPA-Rs and requires interactions by GluR2 with NSF and group II PDZ domain proteins. The combination of regulated addition and continuous replacement of synaptic receptors can stabilize long-term changes in synaptic efficacy and may serve as a general model for how surface receptor number is established and maintained.

Introduction

A change in AMPA-R-mediated transmission underlies several developmental and adult forms of synaptic plasticity (Bear, 1999; Bliss and Collingridge, 1993; Cline et al., 1996; Linden and Connor, 1995; Nicoll and Malenka, 1995) that may play important roles in learning and memory (Martin et al., 2000). One proposed mechanism involves an activity-controlled trafficking of AMPA-Rs from nonsynaptic to synaptic sites (Lüscher et al., 2000; Lynch and Baudry, 1984; Malinow et al., 2000).

AMPA-Rs are hetero-oligomeric complexes composed of different combinations of four subunits, GluR1 to GluR4 (also referred as GluRA to GluRD) (Dingledine et al., 1999; Hollmann and Heinemann, 1994; Seeburg, 1993). Each subunit contains a large extracellular and four membrane-associated domains showing considerable homology among different subunits. In contrast, the cytoplasmic carboxyl termini of these subunits are either long (e.g., GluR1 and GluR4) or short (e.g., GluR2 and GluR3) (Köhler et al., 1994) (Figure 1A). In hippocampus, GluR4 is mainly expressed early in development while GluR1 to GluR3 expression increases with development (Zhu et al., 2000). In adult hippocampus, these three AMPA-R subunits combine to form two distinct populations, GluR1/GluR2 and GluR2/GluR3 (Wenthold et al., 1996). The functional distinction of these two AMPA-R populations or the role played by different carboxyl termini has not been clarified.

Our previous studies suggest that AMPA-Rs containing long carboxyl termini are delivered to synapses by activity-dependent mechanisms. GluR1-containing receptors are driven into synapses by long-term potentiation (LTP) or calcium/calmodulin-dependent protein kinase II (CaMKII) activity (Hayashi et al., 2000; Shi et al., 1999). AMPA-Rs containing GluR4 are driven into synapses by spontaneous activity during early development in a manner not requiring CaMKII activity (Zhu et al., 2000). Other studies suggest that AMPA-Rs undergo a rapid continual cycling from nonsynaptic to synaptic sites (Lüscher et al., 1999; Lüthi et al., 1999; Nishimune et al., 1998; Noel et al., 1999; Song et al., 1998). This cycling appears to require interactions between GluR2 and *N*-ethylmaleimide-sensitive factor (NSF), suggesting that GluR2-containing receptors participate in this trafficking process. The role that this cycling plays in plasticity is not clear since mice lacking GluR2 show both LTP and long-term depression (LTD) (Jia et al., 1996).

Are these two different synaptic regulation behaviors displayed by the same AMPA-Rs, or are there different AMPA-Rs with distinct properties? In this study we have examined the molecular determinants on AMPA-Rs that regulate their synaptic delivery. We find two distinct synaptic delivery processes that are controlled by the subunit composition of the receptor. One process requires subunits with long carboxyl termini and participates in plasticity by adding receptors to increase synaptic transmission. The other process requires subunits with only short carboxyl termini and replaces existing synaptic receptors in a manner not requiring neuronal activity. Such a process may preserve stable transmission for long periods of time even in the absence of neuronal activity.

Results

We initially examined the trafficking of recombinant homomeric receptors. We focused on GluR1 and GluR2 as representatives of long and short carboxy-terminal forms. This suggested subunit-specific rules controlling trafficking of AMPA-R. Subsequently, we examined trafficking of hetero-oligomeric recombinant receptors and endogenous receptors and found that they also conform to these rules.

Continuous Synaptic Delivery of Homomeric GluR2 Receptors

We have previously shown that GluR1-GFP, when expressed in organotypic hippocampal slices with the

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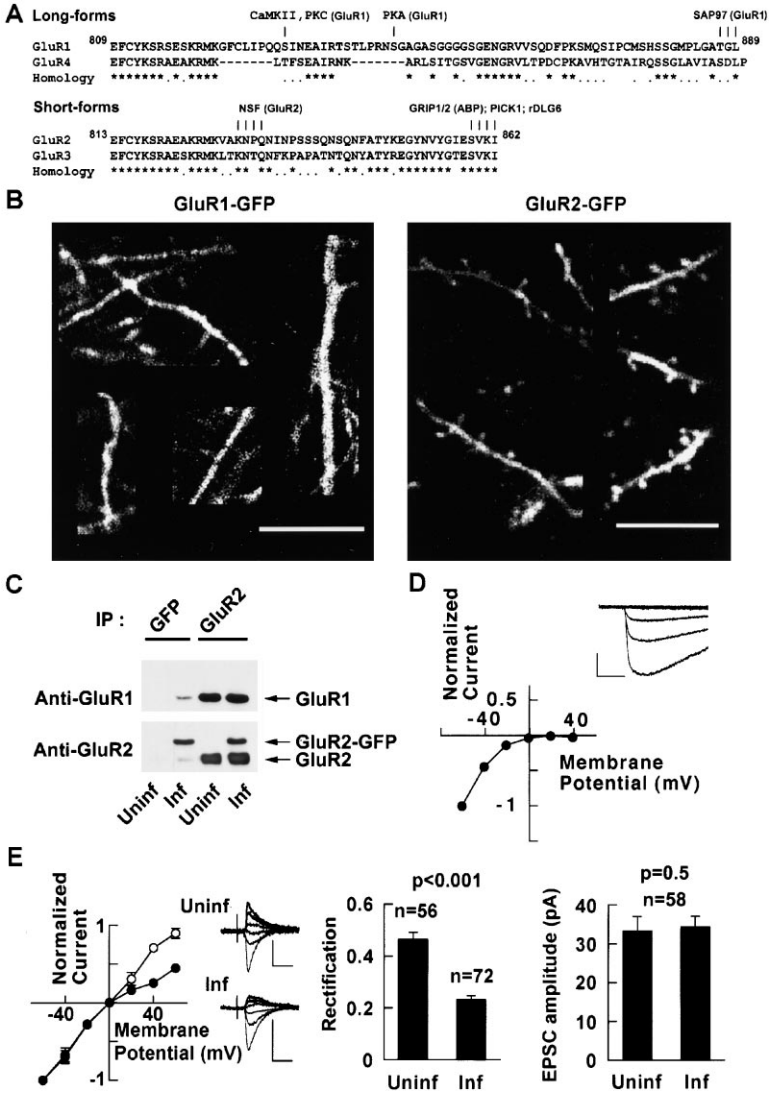

Figure 1. Homomeric GluR1 and GluR2 Receptors Show Different Synaptic Trafficking as Revealed by Optical and Electrophysiological Measurements

(A) Alignments of the cytoplasmic carboxyl termini of AMPA-R subunits. *-identical residues; •-homologous residues. Numbers indicate amino acids in GluR1 and GluR2 without signal peptides. Some known sites for protein interactions or phosphorylation are shown (see text).

(B) Two photon laser scanning microscope images of apical dendritic region of hippocampal CA1 pyramidal neurons expressing GluR1-GFP (left) or GluR2-GFP (right). Note spine delivery of GluR2-GFP but not GluR1-GFP. Scale bar: 10 μm.

(C) Formation of homomeric receptors by GluR2(R586Q)-GFP. GluR2(R586Q)-GFP expressed in CA1 region of hippocampal slice culture was immunoprecipitated with anti-GFP or anti-GluR2 antibodies and western blotted with antibodies against GluR1 and GluR2. GluR2-GFP coimmunoprecipitated with a marginal fraction of endogenous GluR1 or GluR2 while endogenous GluR1 and GluR2 coimmunoprecipitated very efficiently.

(D) Current-voltage relationship of kainate-evoked whole-cell responses obtained from HEK 293 cells expressing GluR2(R586Q)-GFP. Inset: representative responses at membrane potentials from −60 to +40 mV (20 mV step). Scale bars: 25 pA, 250 ms.

(E) Homomeric GluR2 receptor was inserted into synapses as detected by GluR2(R586Q)-GFP mutant. (Left) Current-voltage relationship of synaptic AMPA-R-mediated response from −60 to +60 mV (20 mV step) of infected (inf, closed circles) and uninfected (uninf, open circles) neurons. Sample traces are shown as inset. Scale bar: 25 pA, 25 m. (Middle) Average of the rectification ($I_{+40\ mV}/I_{-60\ mV}$) of AMPA-R-mediated responses of uninfected (uninf) and infected (inf) neurons. (Right) Amplitude of synaptic AMPA-R responses (measured at −60 mV) from nearby uninfected neurons and neurons expressing GluR2(R586Q)-GFP elicited with the same stimulation condition. Same symbols and graph axes are used in subsequent figures.

Sindbis virus expression system, primarily forms homomeric AMPA-Rs (Malinow et al., 1999; Shi et al., 1999) that mainly distribute to intracellular compartments within dendrites of CA1 pyramidal neurons (Shi et al., 1999). Expression of GluR2-GFP using the same system also led to the formation of homomeric recombinant receptors (Figure 1C). However, in contrast to the distribution of GluR1-GFP (Figure 1B, left), GluR2-GFP was clearly visible in dendritic spines (Figure 1B, right).

To determine if the homomeric GluR2-GFP receptors seen in spines participate in synaptic transmission, we engineered them to have a distinct electrophysiological signature that can be measured during synaptic transmission (electrophysiological tagging; Hayashi et al., 2000). In this case, we mutated Arg(R)586 to Gln(Q) in the GluR2 channel pore. As expected (Dingledine et al., 1992; Sudo et al., 1999), GluR2 with this mutation, GluR2(R586Q)-GFP, formed functional receptors in transfected human embryonic kidney (HEK) 293 cells and showed complete inward rectification ($n = 4$, Figure 1D). Hippocampal pyramidal neurons expressing this construct showed marked inward rectification in evoked AMPA-R-mediated synaptic transmission (Figure 1E, left and middle), indicating the delivery of these receptors into synapses (Sudo et al., 1999). Similar results were obtained with GluR2(R586Q) separately coexpressed with GFP using an internal ribosomal entry site (IRES) sequence (rectification, uninfected: 0.46 ± 0.02, $n = 18$; infected: 0.28 ± 0.02, $n = 15$; $p < 0.001$; amplitude, uninfected: 34.5 ± 4.5 pA; infected: 31.9 ± 3.7 pA; $n = 17$; $p = 0.6$).

Homomeric GluR2 Receptors Replace Existing Synaptic AMPA-Rs

Interestingly, the amplitude of synaptic transmission at −60 mV onto cells expressing GluR2(R586Q)-GFP was

not different from that of nearby control uninfected cells receiving the same stimulation (Figure 1E, right). To test the possibility that the synaptic delivery of these recombinant GluR2 receptors is accompanied by synaptic removal of endogenous receptors, we expressed GluR2(R586E)-GFP in pyramidal neurons. This point mutation at the channel pore prevents conductance (pore-dead mutant, Dingledine et al., 1992). If these receptors are added to synapses without removal of existing receptors, there should be no change in the amplitude of AMPA-R-mediated transmission. However, if these receptors replace existing synaptic receptors, AMPA-R-mediated transmission should decrease. Consistent with the second prediction, AMPA-R-mediated transmission onto cells expressing GluR2(R586E)-GFP was significantly smaller than that onto nearby control uninfected cells (Figure 2A). In a control experiment, expression of a similar mutant in the GluR1 channel pore region, GluR1(Q582E)-GFP, had no effect on AMPA-R-mediated synaptic transmission (Figure 2B). This confirms our previous results (Hayashi et al., 2000; Shi et al., 1999) showing that mere expression of homomeric GluR1 receptors is not sufficient for their incorporation into synapses. On the other hand, expression of homomeric GluR2 receptors is sufficient for their incorporation into synapses. Apparently, synaptic delivery of this recombinant receptor is accompanied by removal of some fraction of previously existing synaptic receptors. These two processes are likely not causally linked, as we show below that the delivery of GluR2-containing receptors can be blocked while the removal process continues.

Homomeric GluR2 Receptor Is Not Delivered to Silent Synapses

A significant proportion of excitatory synapses, particularly early in development, appear to contain only *N*-methyl-D-aspartate type glutamate receptors (NMDA-Rs) and have been termed "silent synapses" (Durand et al., 1996; Isaac et al., 1995, 1997; Liao et al., 1995, 1999; Liao and Malinow, 1996; Petralia et al., 1999; Racca et al., 2000; Rumpel et al., 1998; Takumi et al., 1999; Wu et al., 1996). We have recently found that spontaneous activity in immature hippocampal slices can drive GluR4-containing AMPA-Rs into synapses including silent synapses (Zhu et al., 2000). If, as indicated above, homomeric GluR2 receptors merely replace AMPA-Rs that are already at synapses, then one would expect that homomeric GluR2 receptors would not be delivered to silent synapses. To test this prediction, we measured the fraction of silent synapses onto cells expressing GluR2(R586Q)-GFP (the conducting form of GluR2) and compared this to uninfected cells from the same slices. Cells expressing GluR2(R586Q)-GFP had a similar proportion of silent synapses as uninfected cells (Figure 2C), indicating that homomeric GluR2 receptors are not delivered to silent synapses (in contrast to GluR4-containing receptors; Zhu et al., 2000). Rather, these results indicate that homomeric GluR2 receptors are selectively delivered to synapses with existing AMPA-Rs. Previously, we have shown that homomeric GluR1 receptors are delivered to synapses by LTP or CaMKII activity (Hayashi et al., 2000). To examine if they are delivered to silent synapses, we coexpressed GluR1-GFP and a

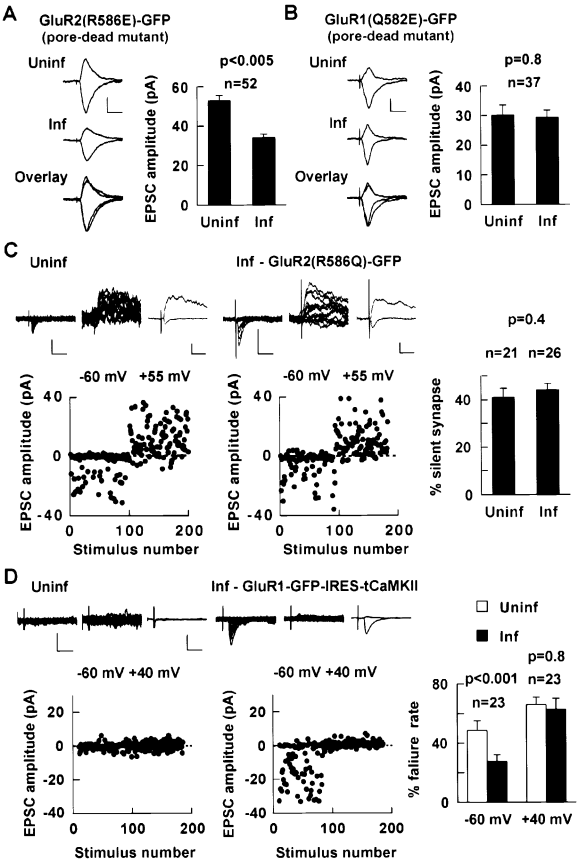

Figure 2. Homomeric GluR2 Receptor Is Inserted into Synapses by Replacing Existing AMPA-Rs and Is Not Delivered to Silent Synapses, while Homomeric GluR1 Receptor Is Driven to Silent Synapses by tCaMKII

(A) Synaptic AMPA-R-mediated response was decreased in neurons expressing the nonconducting GluR2 mutant, GluR2(R586E)-GFP. (Left) Sample traces from nearby uninfected and infected neurons and the scaled responses (overlay) by the value at −60 mV. Scale bars: 25 pA, 25 ms.

(B) Synaptic AMPA-R-mediated response was not decreased in neurons expressing the nonconducting GluR1 mutant, GluR1(Q582E)-GFP. Same panels as in (A). Scale bars: 25 pA, 25 ms.

(C) No change in the fraction of silent synapses in neurons expressing GluR2(R586Q)-GFP, compared to uninfected neurons. (Top) 15 consecutive responses recorded at −60 mV (left), +55 mV (middle), and average (right) from uninfected neuron (uninf) and neuron expressing GluR2(R586Q)-GFP (inf). Scale bars (from left to right): 30 pA, 25 ms; 10 pA, 25 ms; 50 pA, 25 ms; 10 pA, 25 ms. (Bottom) Plots of excitatory postsynaptic current (EPSC) amplitude versus trial number at indicated membrane potentials for uninfected neurons (left) and neurons expressing GluR2(R586Q)-GFP (middle). Fraction of silent synapses (see Experimental Procedures) for uninfected neurons and neurons expressing GluR2(R586Q)-GFP (right).

(D) Homomeric GluR1-GFP receptor is driven to silent synapses by tCaMKII. (Top) 15 consecutive synaptic responses recorded in the presence of APV at −60 mV (left), +40 mV (middle), and average (right) simultaneously from nearby uninfected neuron (uninf) and neuron expressing GluR1-GFP-IRES-tCaMKII (inf). Scale bars (from left to right): 25 pA, 25 ms; 10 pA, 25 ms. (Bottom) Plots of EPSC amplitude versus trial number at indicated membrane potentials for nearby uninfected neuron (left) and neuron expressing GluR1-GFP-IRES-tCaMKII (middle). Note responses only in infected neuron and only at −60 mV. Failure rate recorded simultaneously in nearby pairs of uninfected neurons and neurons expressing GluR1-GFP-IRES-tCaMKII at −60 mV and +40 mV (right).

constitutively active form of CaMKII (GluR1-GFP-IRES-tCaMKII; Hayashi et al., 2000). In addition to the enhanced synaptic transmission (uninfected: 4.1 ± 0.7 pA; infected: 7.4 ± 1.0 pA; n = 20; p < 0.005; Hayashi et al., 2000), we also saw a reduction of synaptic failures at hyperpolarized potentials in neurons expressing GluR1-GFP-IRES-tCaMKII, compared to nearby uninfected control neurons (Figure 2D). To test specifically if this reduction was due to delivery of homomeric GluR1 receptors to synapses containing no AMPA receptors, we measured failure rates of AMPA-R-mediated responses at depolarized potentials also since homomeric GluR1 recombinant receptors do not conduct at depolarized potentials. Failure rates at depolarized potentials were unchanged (Figure 2D) in neurons expressing GluR1-GFP-IRES-tCaMKII compared to nearby uninfected neurons, indicating the delivery of recombinant homomeric GluR1 receptors to silent synapses by active CaMKII.

Synaptic Delivery of Homomeric GluR2 Receptor Does Not Require Neuronal Activity

We next examined if neuronal activity is required for replacement of synaptic receptors with homomeric GluR2 receptors. After infecting slices with the virus expressing GluR2(R586Q)-GFP, we incubated slices with either tetrodotoxin (to block voltage-gated sodium channels), high Mg^{2+} (to depress synaptic activity; Zhu et al., 2000), DL-APV (to block NMDA-Rs), or NBQX (to block AMPA-Rs). In all cases, slices were maintained with drugs from the time of infection until recordings were obtained. None of these pharmacological treatments blocked synaptic delivery of this receptor, as infected cells still showed marked rectification in AMPA-R-mediated response compared to uninfected controls (Figure 3A). These results indicate that neither presynaptic nor postsynaptic action potentials, nor spontaneous synaptic activity acting on AMPA-Rs or NMDA-Rs, are required for the continuous synaptic AMPA-R replacement mediated by GluR2. We note that similar treatments do block synaptic delivery of homomeric GluR4 receptors (Zhu et al., 2000).

A Group II PDZ Domain Interaction Is Necessary for Delivery of Homomeric GluR2 Receptor to Synapses

We next investigated the molecular interactions with GluR2 that may be necessary for the continuous synaptic delivery of receptors. Several proteins have been identified which interact with the carboxyl terminus of GluR2, including GRIP1/2(ABP), PICK1, and rDLG6 (Dong et al., 1997; Inagaki et al., 1999; Srivastava et al., 1998; Xia et al., 1999). These proteins are all PDZ (*P*SD-95, *D*LG, *Z*O-1) domain-containing proteins, and the carboxyl terminus of GluR2 corresponds to a group II PDZ domain binding ligand. To examine if such interactions are necessary for the synaptic delivery of GluR2, we generated a mutant GluR2 with a tyrosine (Y) added at the end of the carboxyl terminus (+863Y), a mutation that prevents interaction between GluR2 and PDZ domain-containing proteins (Xia et al., 1999). Whole-cell recordings from transfected HEK 293 cells indicated that the recombinant receptor made of this mutant was functional and showed inward rectification similar to

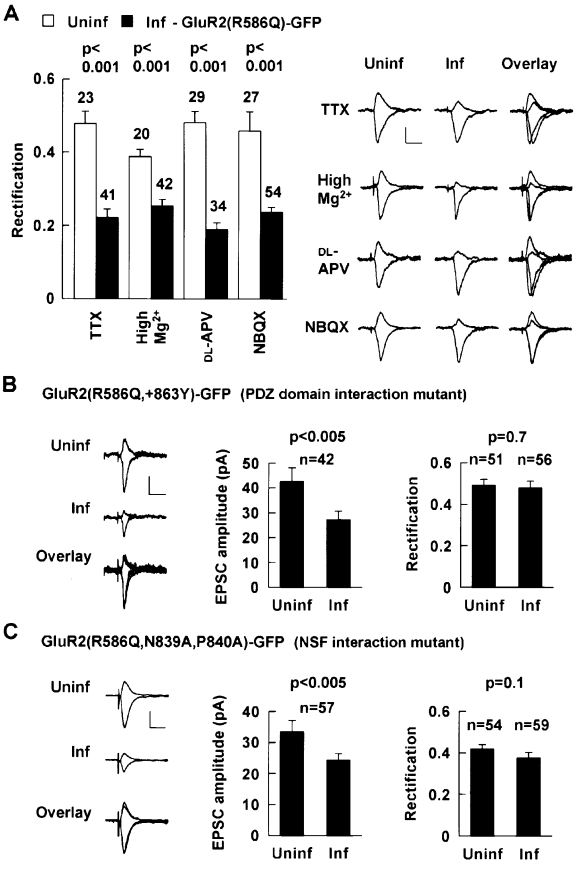

Figure 3. Synaptic Delivery of Homomeric GluR2 Receptors Requires Interactions with Group II PDZ Domain Proteins and with NSF, But No Neuronal Activity

(A) Neuronal activity is not required for synaptic delivery of homomeric GluR2 receptor. (left) Rectification of AMPA-R-mediated response of uninfected neurons (open bars) and neurons expressing GluR2(R586Q)-GFP (closed bars) in slices maintained in 1 μM tetrodotoxin (TTX), 10 mM Mg^{2+} (High Mg^{2+}), 100 μM DL-APV, or 3.3 μM NBQX after infection. (right) Sample traces from uninfected (uninf), infected (inf) neurons, and the scaled responses (overlay). Scale bar: 10 pA, 25 ms.

(B) A mutation at the PDZ domain protein interaction site, GluR2 (R586Q,+863Y)-GFP, blocked synaptic delivery of GluR2 as indicated by no change in rectification (right) of AMPA-R-mediated responses. However, it depressed the amplitude of the response (middle). (Left) Sample traces of uninfected (uninf), infected neurons expressing GluR2(R586Q,+863Y)-GFP (inf), and the scaled responses (overlay). Scale bar: 10 pA, 25 ms.

(C) Mutations at the NSF interacting site, GluR2(R586Q, N839A,P840A)-GFP, also blocked synaptic delivery of GluR2 indicated by no change in the rectification (right), and a decrease in the amplitude (middle), of AMPA-R-mediated responses. (Left) Sample traces of uninfected (uninf), infected neurons expressing GluR2(R586Q,N839A,P840A)-GFP (inf), and the scaled responses (overlay). Scale bar: 25 pA, 25 ms.

GluR2(R586Q)-GFP (data not shown). However, when expressed in neurons, this mutation blocked synaptic delivery of this receptor as cells expressing GluR2(R586Q, +863Y)-GFP showed no change in rectification of AMPA-R-mediated responses (Figure 3B, right). This indicates that the interactions between GluR2 and group II PDZ domain proteins are necessary for its continuous synaptic delivery. In addition, the amplitude

of transmission onto these cells was depressed (Figure 3B, middle). The suppression of AMPA-R-mediated transmission may be explained as a dominant negative effect caused by GluR2(R586Q,+863Y)-GFP. This protein may still bind to other proteins (e.g., NSF, see below) required for receptor synaptic delivery and thus compete with endogenous receptors for interactions with the delivery machinery and thereby block the delivery arm of the cycling of endogenous synaptic AMPA-Rs. The removal process may continue, leading to a synaptic depression.

Interaction between GluR2 and NSF Is Necessary for Delivery of Homomeric GluR2 Receptors to Synapses

GluR2 also interacts with NSF (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998), a homo-hexameric ATPase required in some membrane fusion processes (Rothman, 1994). To examine if such an interaction is required to deliver GluR2 to synapses, we introduced a double mutation on the GluR2 carboxyl terminus (N839A, P840A) that largely abolishes its interaction with NSF in an in vitro assay (Osten et al., 1998). This recombinant protein, GluR2(R586Q, N839A, P840A)-GFP, formed functional receptors that were inwardly rectified when expressed in HEK 293 cells (data not shown). Again, receptors with such mutations failed to show synaptic delivery, as there was no change in rectification of AMPA-R-mediated responses in neurons expressing this mutant form of GluR2 (Figure 3C, right). This indicates that an interaction between GluR2 and NSF is also likely critical for its synaptic delivery, although interactions between GluR2 and other proteins at this site could also produce such effects. This NSF binding mutant also acted in a dominant negative fashion as the amplitude of AMPA-R-mediated transmission onto these cells was significantly depressed (Figure 3C, middle).

Carboxyl Terminus Controls the Mode of Synaptic Delivery of Homomeric Receptors

We next wished to determine if the cytoplasmic carboxyl termini of GluR1 and GluR2, which bind to different intracellular proteins (Figure 7A), direct the different synaptic trafficking of receptors described above. To address this, we swapped the GluR1 and GluR2 cytoplasmic carboxyl termini and generated two chimeric receptor proteins: GluR1(1–822)-GluR2(827–862)-GFP and GluR2(1–826)-GluR1(823–889)-GFP. Whereas full-length GluR1-GFP was retained in dendrites (Figure 1B, left), GluR1 with GluR2-carboxyl terminus, GluR1(1–822)-GluR2(827–862)-GFP, was clearly detected in dendritic spines (Figure 4A). Furthermore, electrophysiological recordings from these cells showed marked inward rectification (Figure 4B, right) in AMPA-R-mediated transmission compared to nearby uninfected neurons, while the amplitude of transmission at −60 mV was not affected (Figure 4B, left). Thus, AMPA-Rs composed of GluR1(1–822)-GluR2(827–862)-GFP appear to show synaptic trafficking similar to those composed of GluR2-GFP, that is, they continuously replace existing synaptic AMPA-Rs. We also tested GluR1(1–822)-GFP, which lack most of the carboxyl terminus yet formed functional receptors (data not shown) when expressed in HEK 293 cells. Ex-

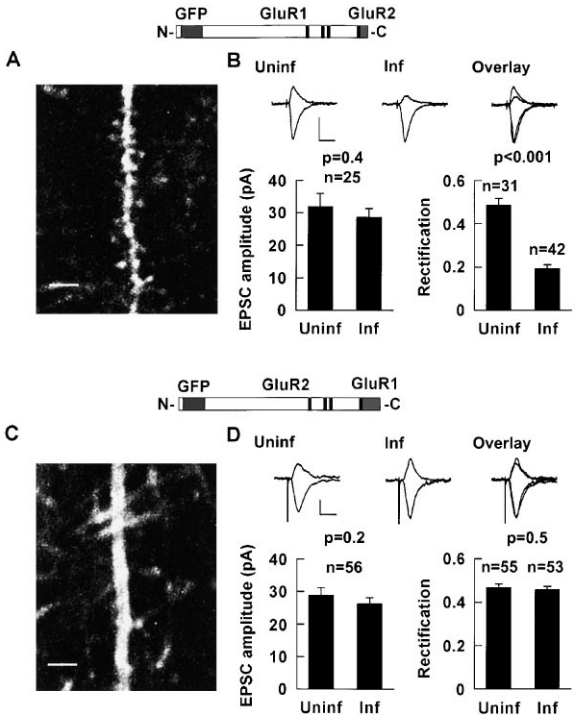

Figure 4. Cytoplasmic Carboxyl Termini of GluR2 and GluR1 Mediate Continuous and Regulated Synaptic Delivery of AMPA-Rs, Respectively

(A and B) Analysis of chimeric protein made of GluR1 with GluR2 carboxyl terminus, GluR1(1–822)-GluR2(827–862)-GFP. (A) Two photon laser scanning microscope image of the apical dendrite of a neuron expressing this construct shows spines. Scale bar: 2 μm. (B) Expression of this chimera increased the rectification (right) with no change in the amplitude (left) of AMPA-R-mediated responses, indicating continuous delivery to synapses in a similar manner to GluR2. (Top) Sample traces from uninfected (uninf), infected neurons (inf), and the scaled responses (overlay). Scale bars: 20 pA, 25ms. (C and D) Analysis of chimeric protein made of GluR2 with GluR1 carboxyl terminus, GluR2(1–826)-GluR1(823–889)-GFP. (C) Two photon laser scanning microscope image of the apical dendrite of neurons expressing this construct show no spines. Scale bar: 2 μm. (D) Expression of this chimeric receptor did not change the rectification (right) or the amplitude (left) of AMPA-R-mediated responses, indicating that it was not delivered to the synapse. Same panels as in (B). Scale bars: 10 pA, 25 ms.

pression of these receptors in neurons did not change either rectification (uninfected: 0.48 ± 0.02, n = 16; infected: 0.49 ± 0.02, n = 21; p = 0.5) or amplitude (uninfected: 33.2 ± 2.9 pA; infected: 35.3 ± 2.7 pA; n = 51; p = 0.3) of AMPA-R-mediated transmission, indicating that the GluR2 carboxyl terminus was providing critical interactions required for synaptic delivery of the chimeric GluR1(1–822)-GluR2(827–862)-GFP receptors.

As a complement to these studies, we examined GluR2 with GluR1-carboxyl terminus, GluR2(1–826)-GluR1(823–889)-GFP. Such receptors were excluded from dendritic spines (Figure 4C), similar to GluR1-GFP. Furthermore, electrophysiological recordings from neurons expressing GluR2(1–826, R586Q)-GluR1(823–889)-GFP showed no evidence of a change in rectification or amplitude of AMPA-R-mediated responses (Figure 4D). Thus, this chimeric receptor was not incorporated into

synapses continuously, similar to GluR1-GFP. We conclude that the distinct synaptic trafficking of GluR1 and GluR2 is controlled by their respective carboxyl termini.

GluR2 Carboxyl Terminus Is Required for Continuous Delivery of Endogenous AMPA-Rs to Synapses

The studies described above with recombinant homomeric receptors support the existence of two distinct synaptic delivery pathways for AMPA-Rs involving GluR1 and GluR2. This view is consistent with a previous subcellular fractionation study of endogenous AMPA-Rs (Hayashi et al., 1997), indicating that GluR2/GluR3 is relatively enriched in the postsynaptic density (PSD) fraction, compared to GluR1. To test directly if endogenous AMPA-Rs use similar two pathway trafficking mechanisms, we expressed in pyramidal neurons the cytoplasmic carboxy-terminal region of GluR1 or GluR2 tagged with GFP at the amino terminus, GluR1(809–889)-GFP and GluR2(813–862)-GFP. Two photon laser scanning images of neurons expressing these constructs showed similar homogenous distribution throughout the dendritic tree (cell body: GluR1(809–889)-GFP, 201 $\pm$ 17 arbitrary fluorescence units (AU), n = 15; GluR2(813–862)-GFP, 196 $\pm$ 15 AU, n = 15; p = 0.8; spines: GluR1(809–889)-GFP, 263 $\pm$ 9 AU, n = 44; GluR2(813–862)-GFP, 270 $\pm$ 12 AU, n = 42; p = 0.8; n.b. spine values and cell body values cannot be directly compared). Biochemical assays in vitro have shown that the carboxyl termini of AMPA-Rs expressed as fusion proteins can interact with the proteins that interact with full-length receptors (Leonard et al., 1998; Osten et al., 1998). We predict that such fusion constructs, when expressed in neurons, will act in a dominant negative manner and prevent synaptic delivery of endogenous receptors. If endogenous receptors use similar two pathway synaptic trafficking mechanisms as described for recombinant receptors above, one should expect to see different effects when the cytoplasmic carboxyl terminus of GluR1 or GluR2 is expressed. Expression of GluR2(813–862)-GFP caused a marked depression of transmission mediated by AMPA-Rs, with no significant effect on NMDA-R-mediated transmission (Figure 5A). In contrast, expression of GluR1(809–889)-GFP had no discernable effects on basal synaptic transmission mediated by either AMPA-R or NMDA-R (Figure 5B). This supports the view that endogenous synaptic AMPA-Rs recycle continuously in a manner requiring interactions mediated by GluR2 carboxyl terminus. In contrast, this recycling does not depend on the GluR1 carboxyl terminus.

Previous studies have suggested that GluR2-containing AMPA-Rs participate in continuous synaptic delivery largely based on the actions of a short peptide (G10/pep2m) that mimics the predicted interaction site on GluR2 with NSF (Lüscher et al., 1999; Nishimune et al., 1998; Song et al., 1998). We confirmed these studies by showing that intracellular infusion of G10/pep2m into neurons from mouse hippocampal organotypic slice cultures caused a depression in AMPA-R-mediated transmission, while a scrambled control peptide, S10, did not produce such depression (Figure 5C). To test for the specificity of G10/pep2m, we examined its effects on transmission in neurons from mice lacking GluR2 (Jia et al., 1996). Infusion of G10/pep2m, or S10, in such neurons produced no decrement of AMPA-R-mediated transmission (Figure 5D). This supports the view that G10/pep2m is exerting its effects specifically on interactions mediated by GluR2 with NSF.

GluR1 Carboxyl Terminus Mediates Regulated Delivery of Endogenous AMPA-Rs to Synapses

To examine regulated synaptic delivery of endogenous AMPA-Rs, we tested the effects of the carboxyl termini of GluR1 and GluR2 on LTP. Slices were infected with one of these three constructs: GFP, GluR1(809–889)-GFP, or GluR2(813–862)-GFP. LTP was tested about 36 hr after infection in a blind manner. Following a baseline period of transmission, LTP was induced with a pairing protocol (Hayashi et al., 2000). While cells expressing GFP showed $\sim$100% stable enhancement, cells expressing GluR1(809–889)-GFP showed only a brief potentiation that decayed to the baseline level by $\sim$20 min (Figure 5E). This supports the view that GluR1-mediated delivery of receptors to synapses is critical for stable LTP. Of interest, cells expressing GluR2(813–862)-GFP showed very large LTP ($\sim$400% increase). This can be explained by a depressed basal transmission and a normal amount of LTP. Such a view can also account for the large amount of LTP exhibited in mice lacking GluR2 (Jia et al., 1996). Our results also suggest that GluR2-mediated interactions are not critical for the first 40–60 min of LTP (as long as LTP was examined).

Hetero-oligomeric AMPA-Rs Show Two Distinct Modes of Synaptic Delivery

AMPA-Rs in adult hippocampus are mainly GluR1/GluR2 or GluR2/GluR3 hetero-oligomers (Wenthold et al., 1996). The studies above examining recombinant homomeric receptors or endogenous receptors identified two different synaptic trafficking pathways. To investigate directly the synaptic trafficking mechanisms for hetero-oligomeric AMPA-Rs, we coexpressed GluR2 (R586Q)-GFP and GluR1 in neurons and then examined the rectification properties of AMPA-R-mediated responses onto these neurons (we used biolistic gene transfer methods for the simultaneous expression of several proteins; see Experimental Procedures; Lo et al., 1994). As indicated from previous experiments, coexpression of GluR1 and GluR2 in HEK 293 cells generates predominantly hetero-oligomeric receptors, instead of two populations of homomeric channels (Shi et al., 1999). In neurons coexpressing GluR2(R586Q)-GFP and GluR1, there was no significant difference in rectification of AMPA-R-mediated responses between transfected and untransfected neurons (Figure 6A). This confirms the hetero-oligomer formation between GluR2 (R586Q)-GFP and GluR1 (if GluR2(R586Q)-GFP remains in homomeric form, this would modify rectification, as it is continuously delivered to synapses) and indicates that GluR1/GluR2(R586Q)-GFP hetero-oligomers are restricted from synapses in the absence of activity just as GluR1 homomeric receptors. To test if such receptors could be driven into synapses, we coexpressed GluR2 (R586Q)-GFP, GluR1, and tCaMKII-GFP. In recordings from neurons expressing this combination of recombi-

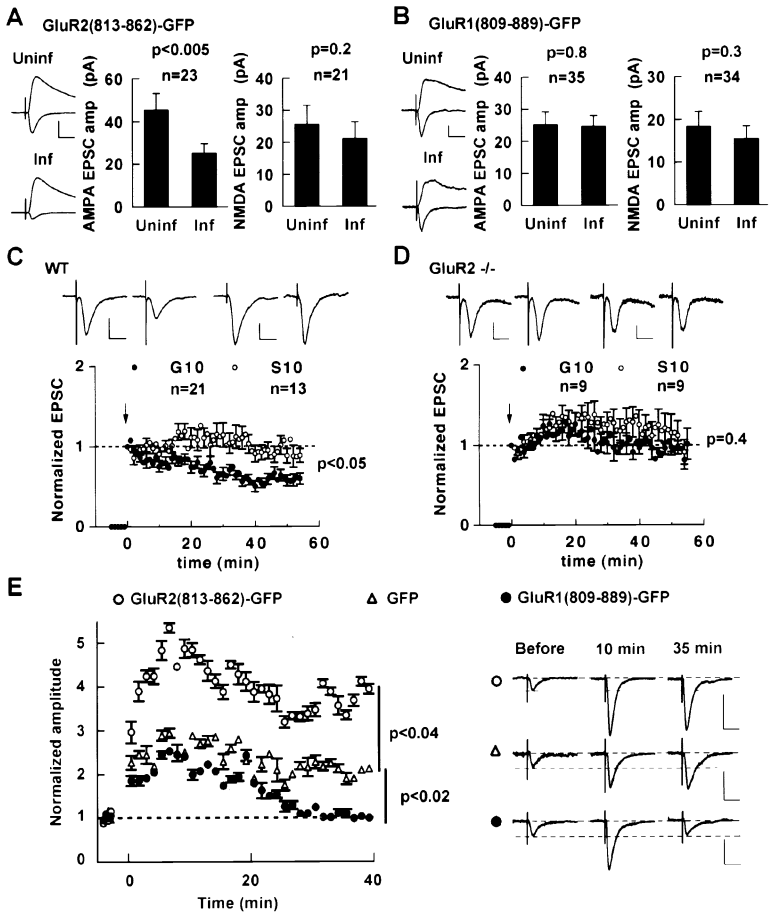

Figure 5. Differential Effects of the Carboxyl Termini of GluR1 or GluR2 on Basal Transmission and Synaptic Plasticity

Continuous synaptic delivery of endogenous AMPA-Rs is selectively blocked by GluR2 carboxyl terminus, but not GluR1 carboxyl terminus; regulated synaptic delivery of endogenous AMPA-Rs is selectively blocked by GluR1 carboxyl terminus, but not GluR2 carboxyl terminus.

(A) (Left) Evoked AMPA-R- and NMDA-R-mediated whole-cell synaptic responses recorded at +40 mV and −60 mV from pairs of nearby neurons, one expressing GluR2(813–862)-GFP (inf) and the other uninfected (uninf). AMPA-R-mediated (middle) and NMDA-R-mediated (right) synaptic responses from uninfected and infected cells are plotted. (Left) Sample trace from nearby pairs of uninfected (uninf) and infected neurons expressing GluR2(813–862)-GFP. Scale bar: 50 pA, 25 ms.

(B) Expression of GluR1 carboxyl terminus, GluR1(809–889)-GFP, has no effect on basal synaptic transmission mediated by AMPA-R or NMDA-R. Panels were the same as in (A). Scale bar: 10 pA, 25 ms.

(C) Amplitude of AMPA-R-mediated synaptic response in hippocampal slice culture neurons from wild-type mice plotted versus time after gaining whole-cell access. Intracellular solution contained either the peptide that blocks interactions between GluR2 and NSF (G10/pep2m) or control scrambled peptide (S10). (Top) Sample traces; G10 (left) and S10 (right) at 2 min and 40 min; Scale bar: 30 pA, 25 ms.

(D) Same experiments as in (C) performed in neurons from mice lacking GluR2. Scale bar (from left to right): 10 pA, 25 ms; 5 pA, 25 ms.

(E) (Left) Evoked AMPA-R-mediated synaptic responses from cells expressing GluR1(809–889)-GFP (closed circles), GluR2(813–862)-GFP (open circles), or GFP (open triangles) are plotted versus time. At time 0, an LTP-inducing pairing protocol was delivered (see Experimental Procedures). At 20–25 min after pairing, LTP is blocked in neurons expressing GluR1(808–889)-GFP ($n = 12$; $p < 0.02$, compared to GFP-expressing neurons, t test) but not with other constructs (GFP-expressing, $n = 8$; GluR2(813–862)-GFP, $n = 7$, $p < 0.04$, compared to GFP-expressing neurons, t test). (Right) Sample responses from neurons infected with different constructs obtained at indicated times relative to pairing protocol. Scale bars (from top to bottom): 50 pA, 25 ms; 20 pA, 25 ms; 50 pA, 25 ms.

nant proteins, AMPA-R-mediated transmission showed both significant inward rectification and potentiation (Figure 6B). This supports the view that GluR1/GluR2 hetero-oligomers require activity for their synaptic delivery and, once delivered, they enhance transmission. This delivery did not require GluR2-PDZ domain interactions as neurons coexpressing GluR2(R586Q, +863Y)-GFP, GluR1, and tCaMKII-GFP also showed both significant inward rectification (untransfected: 0.46 ± 0.03, $n = 16$; transfected: 0.24 ± 0.04, $n = 15$; $p < 0.001$) and potentiation (untransfected: 14.8 ± 2.6 pA; transfected: 28.5 ± 3.2 pA; $n = 18$; $p < 0.0005$).

We also coexpressed GluR2(R586Q)-GFP and GluR3 in neurons. AMPA-R-mediated responses were clearly inwardly rectified in these neurons (Figure 6C). These results suggest that GluR2/GluR3 hetero-oligomers are delivered in a continuous manner to the synapse. To test if the change in rectification in neurons cotransfected with GluR2(R586Q)-GFP and GluR3 was due to existence of homomeric GluR3 receptors, we investigated the synaptic trafficking mechanisms of GluR3. When expressed in HEK 293 cells, the homomeric recombinant GluR3-GFP receptor was functional and showed the expected inward rectification ($n = 5$; Figure 6D). When expressed in neurons, GluR3-GFP could be seen in dendritic spines (Figure 6E). However, when examined by whole-cell recording, we found that there was no significant difference in rectification in neurons expressing GluR3-GFP, compared to nearby uninfected control neurons (Figure 6F, right). Even more surprisingly, AMPA-R-mediated transmission onto those neurons was depressed (Figure 6F, middle). This suggests that GluR3 homomeric receptors are able to traffic to spines, but cannot be inserted into synapses. Indeed, they appear to block the continuous synaptic delivery of endogenous, presumably GluR2/GluR3 receptors. Taken together, these results indicate that GluR2 (R586Q)-GFP/GluR3 hetero-oligomers were formed and delivered to synapses continuously in neurons coexpressing GluR2(R586Q)-GFP and GluR3.

Discussion

The molecular and cellular mechanisms that control the synaptic delivery of glutamate receptors are likely to be a major site of regulation during plasticity as well as

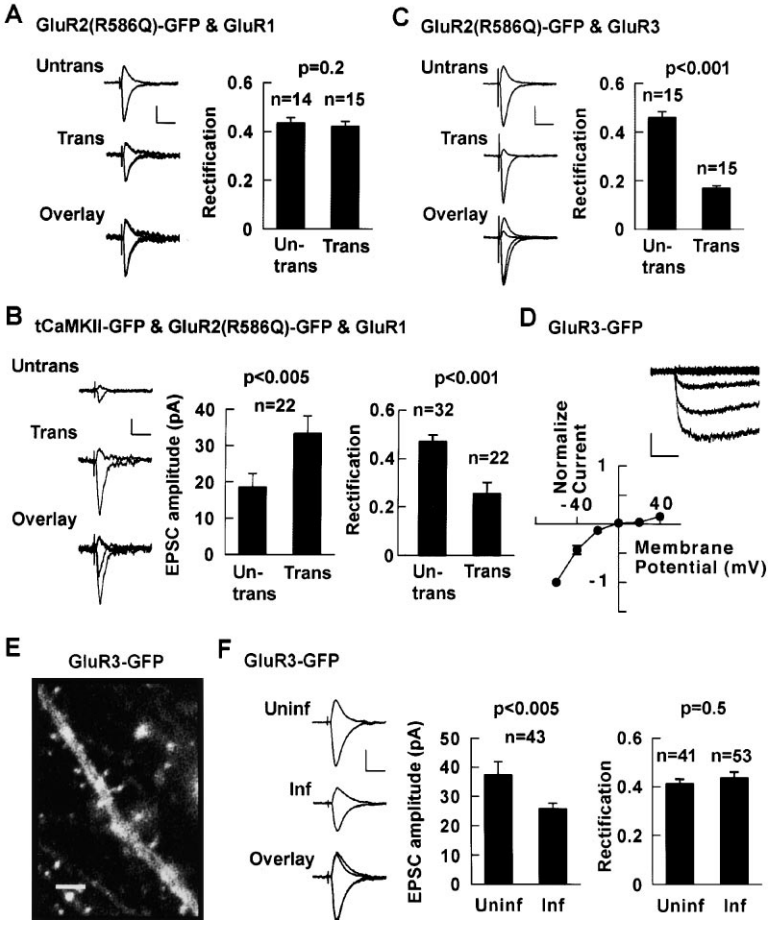

Figure 6. Hetero-Oligomeric Receptors Show Regulated Synaptic Delivery (GluR1/GluR2), and Continuous Synaptic Delivery (GluR2/GluR3)

(A) GluR1 is dominant over GluR2, with respect to synaptic trafficking. Recordings from neurons expressing both GluR2(R586Q)-GFP and GluR1 showed no change in the rectification (right) of AMPA-R-mediated responses, indicating that GluR1/GluR2 hetero-oligomers were not delivered to synapse. (Left) Sample traces from untransfected (untrans), transfected (trans) neurons, and the scaled responses (overlay). Scale bar: 20 pA, 25 ms.

(B) Recordings from neurons coexpressing three constructs, tCaMKII-GFP, GluR2(R586Q)-GFP, and GluR1. AMPA-R-mediated synaptic response was both potentiated (middle) and inwardly rectified (right) compared to untransfected control cells. (Left) Sample responses from nearby untransfected (untrans), transfected (trans) neurons, and the scaled responses by the value at +40 mV (overlay).

(C) Recording from neurons expressing both GluR2(R586Q)-GFP and GluR3 showed inward rectification in AMPA-R-mediated response, indicating that GluR2/GluR3 hetero-oligomers are continuously delivered to the synapse. Same panels as in (A). Scale bar: 20 pA, 25 ms.

(D) Current-voltage relationship of kainate-evoked whole-cell responses obtained from HEK 293 cells expressing GluR3-GFP. Inset: representative responses at different membrane potentials from −60 to +40 mV (20 mV step). Scale bars: 10 pA, 250 ms.

(E) Two photon laser scanning microscope image of the apical dendrite of a neuron expressing GluR3-GFP revealed spine delivery of this protein. Scale bar: 2 μm.

(F) Expression of GluR3-GFP in neurons did not change the rectification (right), but resulted in the depression of AMPA-R-mediated response (middle). (Left) Sample responses from nearby uninfected (uninf), infected neurons expressing GluR3-GFP (inf), and the scaled responses (overlay). Scale bar: 20 pA, 25 ms.

important in the maintenance of stable transmission (Braithwaite et al., 2000; Garner et al., 2000; Lüscher et al., 2000; Lynch and Baudry, 1984; Malinow et al., 2000; Sheng and Pak, 2000; Turrigiano, 2000; Ziff, 1999). Here we find evidence for two distinct, subunit-specific synaptic delivery mechanisms for AMPA-type glutamate receptors in hippocampal pyramidal neurons (Figure 7B). The AMPA-R subunits controlling these two processes are ligands to group I and group II PDZ domains (Figure 7A; Songyang et al., 1997). Such duality may also occur in the trafficking of *Caenorhabditis elegans* glutamate receptors (Rongo et al., 1998), suggesting evolutionary conservation among species.

We find that one delivery process does not require activity and serves to recycle continuously synaptic receptors. This process is mediated by GluR2/GluR3 hetero-oligomers. The other process is activity dependent and delivers additional receptors during plasticity. This process is mediated by GluR1/GluR2 hetero-oligomers. While differences in transmitter-activated kinetics and conductance have been described for different subunits (Dingledine et al., 1999), our results support the view that a major difference between AMPA-R subunits is their contribution to synaptic receptor trafficking dynamics.

Continuous Exchange of Receptors Mediated by GluR2/GluR3 Hetero-oligomers

Our studies demonstrate that homomeric GluR2 receptors and hetero-oligomeric GluR2/GluR3 receptors can be readily incorporated into synapses in the absence of activity (although a potential role for neuronal activity in the modulation of this process is not ruled out by our data). This incorporation is prevented if GluR2 is mutated at the site that interacts with NSF. We confirm that a peptide (G10/pep2m), with homology to a region of GluR2 that interacts with NSF, does depress AMPA-R-mediated transmission (Lüscher et al., 1999; Nishimune et al., 1998; Song et al., 1998). We show this occurs only in tissue from animals that have GluR2, indicating a specific effect of G10/pep2m on GluR2-NSF interactions. Interestingly, GluR3, which shares considerable homology with GluR2 at the cytoplasmic carboxyl terminus (Figure 1A) but does differ at this NSF interaction site (and does not bind NSF), reaches spines but is not delivered to synapses as a homomeric receptor. The GluR2 carboxyl terminus is sufficient to drive GluR1-GluR2 chimeric receptors to synapses, and overexpression of the GluR2 carboxyl terminus can prevent endogenous receptors from trafficking to synapses. Together, these studies provide strong evidence that endogenous

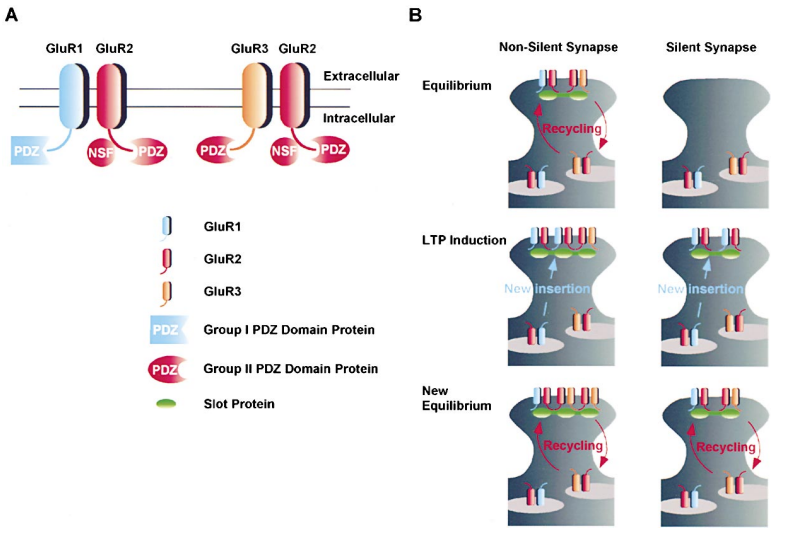

Figure 7. A Model of Subunit-Specific AMPA-R Dynamics at the Synapse

(A) GluR1/GluR2 and GluR2/GluR3 hetero-oligomers are the two major native types of AMPA-Rs expressed in hippocampal CA1 pyramidal cells (Wenthold et al., 1996). Binding proteins are schematically illustrated. Note that GluR1/GluR2 hetero-oligomers contain both long and short forms of carboxyl termini and thus can interact with the binding proteins of both forms. In contrast, GluR2/GluR3 hetero-oligomers can interact with the binding proteins of short forms only.

(B) Dynamics at nonsilent (left) and silent (right) synapses are shown. There are two populations of AMPA receptors: Hetero-oligomers of GluR1/GluR2 require activity for delivery into the synapse and this delivery is dependent on the GluR1 carboxyl terminus; hetero-oligomers of GluR2/GluR3 continuously replace existing synaptic receptors in a manner not requiring activity and mediated by the GluR2 carboxyl terminus. In the basal state, there is equilibrium between synaptic and nonsynaptic receptors in nonsilent synapses that is maintained by a balanced cycling of receptors in and out of synapses. The number of surface receptors is determined by the number of available putative "slot proteins" which serve as binding sites for receptors. A silent synapse stays silent as GluR2/GluR3 hetero-oligomers can be inserted only by replacing existing surface AMPA receptors (top). LTP induction inserts GluR1/GluR2 hetero-oligomers into both types of synapses. This also inserts more "slots" into synapses, which then set a new equilibration level of surface receptor number (middle). The new equilibrium is again maintained by a receptor exchanging and recycling mechanism in which GluR1/GluR2 hetero-oligomers can be replaced by GluR2/GluR3 hetero-oligomers. The subsequent recycling process mediated by GluR2/GluR3 hetero-oligomers requires the GluR2 carboxyl terminus (bottom).

hetero-oligomeric receptors composed of GluR2 and GluR3 are continuously cycling between nonsynaptic and synaptic pools. The delivery arm of the cycling requires interactions between GluR2 and NSF, probably in one of the last steps before insertion into the synapse. Interactions between GluR2 and group II PDZ domain protein(s) also appear to be required for proper synaptic delivery, as assessed with our electrophysiological assay. In dissociated cultured pyramidal neurons, GluR2 may accumulate at synapses to a limited degree in a manner not requiring PDZ domain interactions (Osten et al., 2000). These authors suggest a role for interactions between AMPA-Rs and PDZ domain proteins in the synaptic anchoring aspects of delivery. Alternatively, receptor synaptic trafficking in dissociated neurons may differ from that in neurons maintained within a slice environment. The rules controlling synaptic AMPA-R delivery may also differ among cell types (Li et al., 1999; Liu and Cull-Candy, 2000).

Activity-Dependent Delivery of GluR1/GluR2 Hetero-oligomers

Our previous results show that homomeric GluR1 receptors require activity, either LTP or increased CaMKII activity, to be driven into synapses (Hayashi et al., 2000; Shi et al., 1999). This process requires interactions between GluR1 and PDZ domain proteins. Here we show that hetero-oligomeric receptors composed of GluR1 and GluR2, which represent the majority of endogenous GluR1 in hippocampus (Wenthold et al., 1996), also require activity for their delivery. These results are consistent with a model in which GluR1 interacts with proteins that restrict hetero-oligomeric GluR1/GluR2 receptors from synaptic delivery. CaMKII activity may relieve this restriction. Mere relief of such restriction appears not to be sufficient for synaptic delivery, however, since GluR1 receptors lacking their carboxyl terminus (this study) or GluR1 receptors with a point mutation at the PDZ interaction site (Hayashi et al., 2000) are not delivered to synapses. Thus, there appear to be additional protein interactions that effect synaptic delivery.

We have shown that expression of the GluR1 carboxyl terminus can prevent LTP. This supports the view that GluR1 is critical for the expression of LTP (Shi et al., 1999; Zamanillo et al., 1999). This result also strengthens the view that delivery of GluR1-containing receptors to synapses is a dominant modification underlying LTP (Hayashi et al., 2000). We do, however, see a transient synaptic potentiation in cells expressing the GluR1 carboxyl terminus lasting ~20 min following pairing. This likely reflects mechanisms other than delivery, possibly phosphorylation of synaptic receptors by CaMKII (Barria et al., 1997; Lee et al., 2000) producing an increase in their conductance (Benke et al., 1998; Derkach et al., 1999). Of interest, the GluR2 carboxyl terminus does not prevent LTP, consistent with LTP in mice lacking GluR2 (Jia et al., 1996). Furthermore, GluR1/GluR2 oligomers are delivered by CaMKII even if GluR2 contains a mutation at its PDZ interaction site. These results indicate that delivery of endogenous GluR1/GluR2 hetero-oligomers relies primarily on interactions between GluR1 and its specific delivery machinery.

A Two Pathway Model for the Synaptic Delivery of AMPA-Rs

These studies provide direct evidence for two distinct mechanisms by which AMPA-Rs can be delivered to synapses (Figure 7B). These two mechanisms can contribute to important aspects of synaptic function. GluR1/GluR2 delivery provides additional receptors following

plasticity-inducing stimuli thereby effecting synaptic enhancement. These receptors can be delivered to silent synapses, converting them to functional ones. GluR2/GluR3 receptors can continuously replace synaptic receptors. Thus, this second process can act to preserve plastic changes in the face of protein turnover (Zhu et al., 2000). How can the number of synaptic receptors be maintained during this continuous replacement? One possibility is that several proteins, in addition to GluR1/GluR2 hetero-oligomers, are delivered in tandem to synapses during plasticity. These proteins could serve as placeholders (i.e., "slots") that could be filled with nonsynaptic GluR2/GluR3 hetero-oligomers if synaptic GluR1/GluR2 or GluR2/GluR3 hetero-oligomers leave the synapse. GluR1/GluR2 hetero-oligomers may leave "slots" more slowly (in days) compared to GluR2/GluR3 hetero-oligomers (in minutes), thus explaining why expression of GluR2 carboxyl terminus or infusion of G10/pep2m depresses transmission only partially and the GluR2 carboxyl terminus does not block LTP at 1 hr. Some of these delivered proteins could also serve as "slots" for the eventual addition of NMDA-Rs (Watt et al., 2000). Delivery of proteins will likely increase the physical size of synaptic contact (Toni et al., 1999) and could possibly communicate to the presynaptic side (Irie et al., 1997) eventually leading to the matching of pre- and postsynaptic size and function (Lisman and Harris, 1993; Scheiffele et al., 2000).

This two pathway delivery process may also serve as a general model addressing an important signal transduction problem in cell biology: how is the appropriate number of cell surface receptors established and maintained? One may speculate that receptors in other signal transduction pathways also have two mechanisms for their delivery: one mechanism responds to external cues and controls the number of receptors, while another mechanism simply replaces surface receptors in a manner that maintains receptor number. It may be interesting to look for such duality in systems where stimuli lead to long-lasting changes in number of surface effector molecules.

In the future, it will be important to determine the molecules that mediate and modulate these two different modes of AMPA-R synaptic delivery. One important problem is to identify the mechanism by which receptors with long carboxyl tails are retained away from spines and synapses, and how activity drives them to synapses. Another key problem is to identify potential "slot" proteins, how they can control replacement of receptors in a manner that maintains receptor number, and how the slots themselves may be replenished with fidelity. It is possible that slots are not always fully filled with receptors; variable filling of slots may be one way in which activity could modulate the continuous cycling pathway. It is also of interest to compare these two delivery mechanisms with AMPA-R removal mechanisms; receptor removal may also have continuous and regulated processes (Beattie et al., 2000; Carroll et al., 1999a, 1999b; Ehlers, 2000; Hirai, 2001; Lin et al., 2000; Lüthi et al., 1999; Man et al., 2000; Wang and Linden, 2000; Xia et al., 2000; Zhou et al., 2001). Lastly, this study attempts to define the molecular signatures of plasticity; in this manner, it may be possible to examine when and where synaptic plasticity occurs in the brain during behavioral modification.

Experimental Procedures

Molecular Biology

Constructs of AMPA-R subunits tagged with GFP were made as previously described (Shi et al., 1999). Briefly, GFP coding sequence (enhanced GFP, Clontech) was inserted three amino acids downstream of the predicted signal peptide cleavage site of the corresponding AMPA-R subunit. Fusion proteins of carboxyl termini of GluR1 and GluR2 with GFP were constructed by PCR amplification of the GluR1 (809–889) or GluR2 (813–862) and in-frame ligation into pEGFP-C1 (Clontech), placing the GluR fragment at the carboxyl end of GFP. The chimeric GluR1 with GluR2 carboxyl terminus (GluR1(1–822)-GluR2(827–862)-GFP) was made by fusing the carboxyl terminus (827–862) of GluR2 after amino acid 822 of GluR1 with a PCR method. The chimeric GluR2 with GluR1 carboxyl terminus (GluR2(1–826)-GluR1(823–889)-GFP) was made similarly. Point mutations were carried out using Quick Change mutagenesis kit (Stratagene). Truncation of GluR1 was carried out by digesting GluR1 with the unique XmnI site and ligating to the StuI site of pSinRep5. Thus, the resultant mutant protein has three extra amino acids (Ala-Leu-Gln) after GluR1(1–822). Amino acid numbering here does not include the signal peptide (18 for GluR1, 21 for GluR2) for consistency. The fusion protein of truncated CaMKII (1–290) with GFP has been described previously (Shi et al., 1999). These constructs were transferred to pSinRep5 (for Sindbis virus production) or a plasmid mammalian expression vector with a CMV promoter (for biolistics method). Individual recombinant proteins were expressed in CA1 neurons in rat hippocampal culture slices, using Sindbis virus expression system (Malinow et al., 1999). In some experiments, simultaneous expression of multiple constructs (Figure 6) was achieved using biolistics transfection (BioRad; Lo et al., 1994). Typically, slices were prepared from postnatal 5–7 day (P5–7) old animals, infected with virus or transfected using gene gun after 5–14 days in vitro.

Imaging and Electrophysiological Recordings

Two photon laser scanning imaging (Mainen et al., 1999) and electrophysiological recording experiments were performed 1–3 days after infection or transfection. In general, no differences were found between these different time points and data were pooled. The recording chamber was perfused with physiological solution (22°C–25°C), and unless otherwise stated, contained (in mM): NaCl 119, KCl 2.5, $CaCl_2$ 4, $MgCl_2$ 4, $NaHCO_3$ 26, NaH_2PO_4 1, glucose 11, picrotoxin 0.1, bicuculline 0.01, DL-APV 0.1, and 2-chloroadenosine 0.002, at pH 7.4 and gassed with 5% CO_2/95% O_2. 2-Chloroadenosine was included to prevent bursting. DL-APV was not included in the bath solution during experiments in which NMDA currents were recorded. Patch recording pipettes (3–6 MΩ) were filled with internal solutions containing (in mM): cesium methanesulfonate 115, CsCl 20, HEPES 10, $MgCl_2$ 2.5, Na_2ATP 4, Na_3GTP 0.4, sodium phosphocreatine 10, EGTA 0.6, and spermine 0.1, at pH 7.25. Whole-cell recordings were made with Axopatch-1D amplifier (Axon Instruments). Synaptic responses were evoked by one or two bipolar electrodes with single voltage pulse (200 μs, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers ~300–500 μm from the CA1 cells. Stimulus level was set to produce a synaptic response of ~30 pA. Synaptic AMPA-R-mediated responses at −60 mV and +40 mV were averaged over 50–100 trials and their ratio was used as an index of rectification. In order to compare the size of amplitude of synaptic response, whole-cell recordings were first made from an infected or uninfected cell and the stimulus level was set to produce a synaptic response of ~30 pA. Upon termination of that recording, a whole-cell recording was immediately obtained from the nearby control uninfected or infected cell with the same location and intensity of stimulus. Infected and uninfected cells were identified under visual guidance using fluorescence and transmitted light illumination (Shi et al., 1999). LTP was induced in pairing postsynaptic depolarization at −10 mV with presynaptic stimulation at 2 Hz for 1.5 min. Failure rate of synaptic transmission was calculated from ~100 trials at each potential, similar to the method previously described (Liao et al., 1995). The fraction

of silent synapses was calculated by the equation *1−(lnFd)/(lnFh),* where *Fd* and *Fh* are failure rates at +55 mV and −60 mV, respectively (Liao et al., 1995). The peptides G10/pep2m (KRMKVAKNAQ, custom synthesis by Research Genetics) or S10 (VRKKNMAKQA) were dissolved (2 mM) in Cs-based internal solution (Lüscher et al., 1999; Nishimune et al., 1998). All results are reported as mean ± SEM and statistical significance was set at $p < 0.05$. Statistical differences of the means were determined using Wilcoxon and paired t test for paired measurements and Mann-Whitney and nonpaired t test for nonpaired measurements unless otherwise stated in the text. In all experiments where multiple tests were used, significance matched all tests.

Expression and Recording from HEK 293 Cells

HEK 293 cells were transfected by different AMPA-R constructs with Lipofectin reagent (Gibco BRL, Life technologies). After ~36 hr, the transfected cells were recorded with patch pipettes containing Cesium-based internal solution. The AMPA-R-mediated responses were evoked by a brief pulse (10–30 ms) of agonist (kainate, 1 mM) in the presence of cyclothiazide (0.1 mM) and averaged for 5–10 trials at different holding potentials (from −60 to +40 mV at 20 mV step).

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