with wire mesh (8 mm between wires), and 42 cm high. The water level was 1.6 cm above the level of the platform, which was placed in the middle of one quadrant (SE) of the pool, midway between wall and pool center. The water (21 ± 2°C) was made opaque by the addition of 7.2 kg of powdered low-fat milk. Touching the edge of the pool was as described [51]. A month before the actual experiment, the mice swam daily for 13 days (platform NW) in a smaller pool (60 cm), which accustomed them to mounting the platform. Spatial navigation trials: Mice (19 wild type, 21 GluR-A+/−) were gently placed into the water at the edge of the pool at one of four start positions arbitrarily named N, S, E, and W. In a block of four trials all start positions were used in a semirandom order that differed every day. Finding the platform was defined as climbing onto it and staying for at least 5 s. Once on the platform, the mouse was allowed to stay for 30 s. For any mouse that failed to find the platform within 90 s, a latency of 90 s was recorded, and the mouse was placed on the platform. To ensure that the mouse did not use cues inside the pool, we rotated the pool daily and removed floating debris and feces before every trial. Transfer trials: On transfer trials the mice were placed onto the edge of the pool and allowed to swim with no platform present. The video system recorded the percentage of time spent in the various quadrants. The experimenter recorded from a video monitor the number of times the mouse crossed the previous position of the platform; crosses were defined by the head fully entering the square representing the platform position. Transfer trial in the absence of visual cues: To hide distal visual cues, we hung white curtains from the ceiling in a circle (2 m diameter around the pool).

Excitatory synaptic transmission in the vertebral central nervous system is mediated by activation of AMPA- and NMDA-type glutamate receptors. Repetitive synaptic activity transiently activates NMDA receptors and triggers long-lasting plasticity (1), expressed, at least in part, as an increase in AMPA receptor function (2, 3). The molecular basis for activity-induced changes in AMPA receptor function is not known and may include changes in channel conductance (4), possibly after receptor phosphorylation (5), or delivery of AMPA receptors to synapses, as has been documented during development (6). We investigated if an increase in AMPA receptor number at synapses may occur rapidly during NMDA receptor–dependent synaptic plasticity.

AMPA receptors are oligomers formed by a combination of four different subunits, GluR1 to 4 (GluRA to D) (7). A substantial proportion of endogenous AMPA receptors in hippocampal neurons have the GluR1 subunit (8). We constructed a recombinant GluR1 tagged with green fluorescent protein (GFP) at the putative extracellular NH2-terminus (GluR1-GFP; Fig. 1A) (9). This protein was expressed in human embryonic kidney (HEK) 293 cells; extracts showed a single band by protein immunoblotting of the expected molecular mass (Fig. 1B). Whole-cell recordings from GluR1-GFP–transfected HEK 293 cells showed inwardly rectifying responses to puffed agonist (Fig. 1C) (7). Cotransfection of GluR1-GFP with wild-type GluR2 yielded responses with no rectification (Fig. 1C), indicating effective hetero-oligomerization between GluR1-GFP and GluR2, as homomeric GluR2 can produce little current (7).

GluR1-GFP was introduced into neurons with Sindbis virus expression system (10, 11). In hippocampal dissociated cultured neurons (Fig. 2) (12), GluR1-GFP showed distribution throughout the dendritic tree with expression levels in dendrites approximately three times that of endogenous GluR1 (13). Immunostaining for surface (Fig. 2D) (14)
Two kinds of changes in GluR1-GFP distribution were detected after tetanic stimulation: delivery to spines and clustering in the dendritic shaft. Delivery of GluR1-GFP was measured in 38 spines from five experiments (Fig. 4). In about half of these spines (17 of 38), the amount of fluorescence at the corresponding location in images obtained before a tetanus was near background (23) (termed “empty” spines, Fig. 4A, arrow a), whereas in...
the remaining 21 of 38 spines (termed “active” spines, Fig. 4A, arrow b), there was a detectable amount of GluR1-GFP before tetanus. The intensity distribution of these analyzed spines is shown in Fig. 4, B and C. The GluR1-GFP signal at “empty” spines increased from 200 ± 43 AU to 1737 ± 235 AU [measured in arbitrary fluorescence units (AU); mean ± SD, N = 17] after a tetanic stimulus. At “active” spines, the increase was from 1023 ± 101 AU to 2210 ± 235 AU (mean ± SD, N = 21). Such increases in GluR1-GFP signals at spines were never observed in the absence of tetanic stimulation (24).

In addition to spine delivery, tetanic stimulation produced clustering of GluR1-GFP in the dendritic shaft. The clustered receptor
could be seen at the base of a spine (Fig. 5A, arrow) or with no detectable delivery to spines (Fig. 5A, arrowhead). Clustering was quantified by computing an index of an autocorrelation function (R50%) calculated over a region of interest before and after tetanus (Fig. 5B) (25). In the absence of stimulation, this index changed little over time, on average increasing 5.4 ± 6.6% (mean ± SD, N = 20, randomly chosen dendrites) between two observation periods separated by 15 min. However, upon tetanic stimulation, 27 dendritic segments from 18 experiments became clustered (R50% decreased by 17.8 ± 1.6%, mean ± SD) (26). Dendritic regions showing spine delivery of GluR1-GFP generally showed clustering of receptor (R50% decreased by 18.3 ± 2.6% at the 10 dendrites analyzed above, showing delivery of GluR1-GFP to spines after tetanus).

We wished to determine if tetanus-induced redistribution of GluR1-GFP included delivery to the surface. We first established a method using TPLSM to image surface recombinant receptor in fixed slices (Fig. 3G) (14). The distribution and quantification of GluR1-GFP with these methods (13.3 ± 0.9% on surface) generally agree with values obtained with immuno-gold electron microscopy (9% on surface, Fig. 3F). Regions examined in live tissue during stimulation were analyzed for surface distribution after fixation. Regions in which GluR1-GFP had undergone clustering with tetanic stimulation showed a greater amount of receptor at the surface (Figs. 4A, column 4, and 5C; 18.6 ± 0.2 % on surface), although most of the receptor still remained intracellular. At spines that showed GluR1-GFP delivery after a tetanus (including previously “empty spines”), surface GluR1-GFP could also be detected (Fig. 4A). This indicates that some of the GluR1-GFP delivered into spines after a tetanus reached the spine surface, suggesting their contribution to an increase in synaptic transmission.

To determine whether the redistribution of GluR1-GFP by tetanic stimulation requires synaptic activation of NMDA receptors, we conducted experiments with (D,L)-2-amino-5-phosphono valeric acid (APV), a reversible NMDA receptor antagonist (Fig. 6). With APV in the bath, tetanic stimulation produced no clear redistribution of GluR1-GFP (neither spine delivery nor clustering; Fig. 6A). After washing APV for 45 min, another tetanus was delivered at the same site. Now spine delivery and clustering could be detected (see Fig. 6, no APV, −7 and 15 min). Ensemble averages from several experiments in which spine delivery and clustering were monitored in the presence and subsequent absence of APV are shown in Fig. 6B. These results show that both clustering and spine delivery of GluR1-GFP require synaptic activation of NMDA receptors. These experiments also demonstrate that the effect of tetanic stimulation is not due to direct depolarization of dendrites by the current passed through the stimulating electrode, because such effects would not be blocked by APV.

In this study, we showed that the GFP-tagged GluR1 receptor is electrophysiologically functional and mimics a number of cell-targeting properties of endogenous receptors. In dissociated neurons, the protein is delivered to synapses in the absence of evoked activity. In contrast, in slices given no stimulation, a large fraction of the recombinant GluR1-GFP, as well as endogenous GluR1, is found in the intracellular dendritic compartment and excluded from synapses. This difference may explain the observed difficulty with which LTP is generated in dissociated neurons (27). This intracellular pool is within 1 to 2 μm of synapses and thus could be rapidly delivered to synaptic sites during plasticity.

Indeed, we found that GFP-tagged receptors in hippocampal slice neurons were rapidly recruited to dendritic spines after a tetanic stimulus (Figs. 4 and 6). Immunostaining indicated that at least some of the recruited GluR1-GFP reached the spine surface. The delivery to the dendritic shaft surface may also represent synaptic delivery, as shaft synapses (or short “stubby” spines) are more common in young tissue (28). The spine delivery of the tagged AMPA receptor required synaptic NMDA receptor activation, providing a strong link between receptor recruitment and activity-induced forms of plasticity. These results provide direct evidence showing rapid effects of synaptic activity on postsynaptic membrane trafficking.

In about half of the spines detected with GluR1-GFP after tetanus, there was no fluorescence at the corresponding region before tetanic stimulation. On the basis of their length (0.95 ± 0.17 μm) and a previous study (29), these spines are not likely to have been generated after tetanic stimulation. Our previous study indicated that tetanic stimuli do not generate short spines, but rather such stimuli generate filopodial structures that are typically >3 μm in length (29). In view of these observations, it is likely that GluR1-GFP was delivered to existing spines and not newly formed spines, although such a possibility cannot be excluded by our results. If receptors were delivered to existing “empty” spines, these could represent “silent synapses”: synapses with only NMDA receptors that gain AMPA receptors during LTP (3).

Our results build on a number of studies suggesting that the delivery of AMPA receptors to synapses contributes to activity-dependent plasticity. Inhibition of membrane fusion processes in the postsynaptic cell blocks LTP (30). Furthermore, the COOH-termini of AMPA receptor subunits GluR2 and GluR4c
bind N-ethylmaleimide–sensitive fusion protein, a protein involved in membrane fusion processes (31). Vesicular organelles, possibly undergoing exocytosis and endocytosis, have been detected with electron microscopy in spines (32). And last, dendrites can display a calcium-evoked exocytosis of trans-Golgi–derived organelles that is thought to mediate LTP (33). Other postsynaptic mechanisms, such as an increase in conductance of AMPA receptors (4, 5), may also occur in parallel. Our results also do not rule out a contribution by presynaptic modifications.

In addition to the spine delivery of GluR1-GFP, tetanic stimulation induced the formation of clusters of the tagged receptor within dendrites. These structures may be related to the spine apparatus, membranous structures at the base of spines (32) that appear to contain AMPA receptors (34). The entry of calcium through synaptic NMDA receptors may cause nucleation of AMPA receptor–containing membranes close to active synapses. Once formed, such sites may serve several functions. These sites may replenish those receptors delivered to spines during plasticity. Additionally, they may serve as a “synaptic tag” (35), providing a docking site for AMPA receptors synthesized at distant sites. Last, they could provide a site for local AMPA receptor synthesis (36). In these capacities, such clusters could represent a structural modification serving as a long-lasting memory mechanism.

References and Notes

9. GF (enhanced GFP; Clontech) was inserted between the third and fourth amino acids after the predicted signal peptide cleavage site of rat GluR1. The cDNA was synthesized with standard molecular biology techniques. HEK cells were transfected with plasmid-based mammalian expression vector with lipofectin (Gibco-BRL Life Technologies). GluR1-GFP and GluR2 were cotransfected in a 1:1 ratio. Protein immunoblotting was carried out with antibodies to GluR1 (Chemicon International). Whole-cell recordings were obtained 2 to 5 days after transfection in HEPES (10 mM)–buffered Hanks’ solution in the presence of 100 µM APV (tetanus) (0.1 µg/ml; Chemicon International). For details of quantification, see (25).
11. Whole-cell recordings from neurons infected with Sindbis virus for 1 to 4 days show normal passive membrane properties (for example, input resistance: uninfected, 276 ± 64, N = 13; GluR1-GFP infected, 302 ± 51, N = 8; P = 0.78) (S. Shi, Y. Hayashi, R. Malinow, unpublished results).
12. Dissociated cultured neurons were prepared as previously described (33).
13. To estimate the level of recombinant GluR1-GFP expression, relative to endogenous GluR1 expression, we performed immunohistochemistry on fixed (14) dissociated hippocampal neurons (33) with a GluR1 COOH-terminal antibody (1 µg/ml; Chemicon International) that recognizes both proteins (Fig. 1B) as primary and Texas Red coupled as secondary (which does not overlap in fluorescence with GFP). In a field of infected and noninfected cells, dendrites (80 µm in diameter) were visualized in 2.7 ± 0.2 fold (mean ± SEM, N = 8) immunolabel compared with similar regions of uninfected cells.
14. Tissue was fixed with freshly made 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) (dissociated neurons: 4°C for 30 min; organotypic slices: 16°C for 1 h). With such fixation, immunohistochemistry (see below) detected only surface epitopes (dissociated cells; Fig. 2C, organotypic slices; Fig. 3C). To detect intracellular epitopes, tissue was further treated with 0.3% Triton X-100 in PBS (dissociated cells: 4°C for 10 min; organotypic slices: 4°C for 30 min). Immunohistochemistry: Cells were blocked in blocking solution (10% horse serum in PBS, 60 min) and then incubated with primary antibody (4°C overnight) in blocking solution. All primary rabbit polyclonal antibodies were visualized with biotin–conjugated antibody to rabbit immunoglobulin G (Jackson Immunoresearch). Images of dissociated neurons were collected with a cooled charge-coupled device (Photometrics) and analyzed with NIH Image or Jandel Micropublisher (Scientific Imaging). For whole-cell recordings from dissociated neurons, internal solution consisted of (in mM) 115 cesium methanesulfonate, 20 CsCl, 10 Hepes, 2.5 MgCl2, 4 NaN3, adenosine triphosphate, 0.4 Na2-guanosine triphos-
R E S E A R C H A R T I C L E S

Reconstructing Phylogeny with and without Temporal Data

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Conventional cladistic methods of inferring evolutionary relationships exclude temporal data from the initial search for optimal hypotheses, but stratocladistics includes such data. A comparison of the ability of these methods to recover known, simulated evolutionary histories given the same, evolved character data shows that stratocladistics recovers the true phylogeny in over twice as many cases as cladistics (42 versus 18 percent). This comparison involved 550 unique taxon-by-character matrices, representing 15 evolutionary models and fossil records ranging from 100 to 10 percent complete.

Phylogenetic analysis seeks to identify the pattern of historical relationships among organisms. However, controversy persists over not only what constitutes an appropriate inference strategy, but also what categories of data are acceptable as evidence (1). One recent debate focuses on the use of stratigraphic data, or the temporal order of specimens in the fossil record, as evidence for inferring relationships (2). Proponents of cladistic methods often argue that temporal data may be misleading as indicators of relationship (3). Arguments supporting this position are essentially a priori and do not address the relative efficacy of methods that do and do not use temporal data. We explore the efficacy of using temporal data through simulations of evolutionary histories and associated character data, assessing the relative performance of two phylogenetic methods, cladistics and stratocladistics.

Both conventional cladistic analysis and stratocladistics rely on parsimony, in the sense of minimizing ad hoc auxiliary hypotheses, to evaluate alternative interpretations of relationship. Cladistic analysis selects interpretations that minimize hypotheses of homoplasy, or shared traits that do not result from common ancestry (4, 5). Hypotheses of homoplasy are counted individually and, lacking contravening evidence, equally. Stratocladistics (6) incorporates stratigraphic data into the logic of cladistic hypothesis choice by selecting interpretations of relationship that minimize hypotheses of homoplasy and nonpreservation of lineages through intervals that preserve other lineages under analysis, giving neither category of