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QUANTITATION OF AMPA RECEPTOR SURFACE EXPRESSION IN CULTURED HIPPOCAMPAL NEURONS

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Abstract—Protein and messenger RNA levels of the AMPA-type glutamate receptor subunits 1–3 are high in many brain regions, but it is not known how much of the glutamate receptor protein is expressed on the surface of neurons in the form of functional receptors. To provide insight into this matter, western blot immunoreactivities for glutamate receptors 1 and 2/3, as well as binding of the specific ligand [^{3}H]AMPA, were quantified following three independent treatments modifying surface receptors in intact primary hippocampal cultures: (i) proteolysis of surface receptors by chymotrypsin, (ii) cross-linking of surface receptors with the membrane-impermeant reagent bis(sulfosuccinimidyl)suberate, and (iii) biotinylation of surface receptors with the membrane-impermeant reagent sulfosuccinimidyl-2(biotinamido)ethyl-1,3-dithiopropionate. All three of these methods demonstrated that 60–70% of total glutamate receptor subunit 1 protein and 40–50% of total glutamate receptor 2/3 protein are expressed on the surface of hippocampal neurons. Parallel studies revealed that 52% of total [^{3}H]AMPA binding sites could be precipitated with avidin beads following biotinylation of intact cultures, providing an estimate of [^{3}H]AMPA binding site surface expression in accord with the estimates of the surface expression of glutamate receptor subunits 1–3. Experiments examining the surface expression of ^{32}P-labeled glutamate receptor subunit 1 demonstrated that approximately 65% of the phosphorylated form of the subunit is located in the plasma membrane, an estimate similar to the that derived via western blot for the entire glutamate receptor subunit 1 population in the same samples. Moreover, no significant change in the surface expression profile of the glutamate receptor subunits 1–3 was observed following stimulatory treatments known to increase glutamate receptor phosphorylation.

These data indicate that slightly more than half of the AMPA receptors in cultured hippocampal neurons are located in the plasma membrane, and that AMPA receptor surface expression is not rapidly altered by glutamate receptor phosphorylation. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: glutamate receptor, non-*N*-methyl-D-aspartate receptor, trafficking, biotinylation, proteolysis, cross-linking.

Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors, which are comprised of heteromeric combinations of the glutamate receptor subunits (GluR)1–4, mediate rapid excitatory transmission at most synapses in the mammalian brain.[35] Like all neurotransmitter receptors, AMPA receptors can perform their role in synaptic transmission only when they are inserted into a neuronal plasma membrane and exposed extracellularly. Little is known, however, about the trafficking and surface expression of AMPA receptors. Immunohistochemical experiments using subunit-specific antibodies.[2,6,8,27,29,34] have demonstrated staining for AMPA receptor subunits both in synaptic membrane regions and in apparently intracellular compartments. These studies represent an initial description of AMPA receptor intracellular distribution, but they are limited by several considerations: (i) since these studies were performed on permeabilized neurons, they cannot distinguish between receptors subunits which are *in* the plasma membrane and subunits which are localized *near* the plasma membrane; (ii) they cannot distinguish functional receptors from unassembled subunits; (iii) they do not allow for quantitative comparisons between samples; and (iv) they cannot provide any information concerning the localization of the phosphorylated vs non-phosphorylated forms of the receptor.

We have attempted to address these points by performing analyses of AMPA receptor surface expression in cultured rat hippocampal neurons. Immunohistochemical studies have revealed that the subcellular distribution of AMPA receptor subunits in cultured hippocampal neurons[6,8] is qualitatively similar to that observed in brain slices,[2,27,29,34] indicating that primary hippocampal cultures represent a

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Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BS^3, bis(sulfosuccinimidyl) suberate; CaM kinase II, calcium/calmodulin-dependent protein kinase II; EDTA, ethylenediaminetetra-acetate; GluR1, 2, 3, glutamate receptor subunits 1, 2, 3; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; LTP, long-term potentiation; MEM, minimum essential medium; NHS-ss-biotin, sulfosuccinimidyl-2(biotinamido)ethyl-1,3-dithiopropionate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TPA, *O*-tetradecanoylphorbol 13-acetate.

good model system for studying the trafficking of AMPA receptors in neurons. In the present work, AMPA receptors were quantified by two methods, GluR1–3 immunoreactivity on western blots and [^{3}H]AMPA binding; incorporation of ^{32}P into immunoprecipitated GluR1 was also examined. Surface receptors were distinguished from intracellular receptors by three independent methods: (i) susceptibility to extracellular chymotryptic cleavage, (ii) cross-linking by a membrane-impermeant reagent, bis(sulfosuccinimidyl)suberate (BS^3), and (iii) biotinylation by a membrane-impermeant biotinylating reagent, sulfosuccinimidyl-2(biotinamido)ethyl-1,3-dithiopropionate (NHS-ss-biotin).

EXPERIMENTAL PROCEDURES

Animals and supplies

Timed-pregnant Sprague–Dawley rats were obtained from Bantin and Kingman. Culture plates were from Falcon. Minimum essential medium (MEM; without glutamine) and heat-inactivated fetal calf serum were from Gibco. Mito+serum extender was from Collaborative Research. Chymotrypsin and papain were from Worthington. NHS-ss-biotin, BS^3, dimethyl pimelimidate and neutravidin linked to Ultralink™ beads were from Pierce. Anti-GluR1 and anti-GluR2/3 antibodies were a gift from Robert J. Wenthold (NIH). Anti-actin monoclonal antibodies and okadaic acid were from Boehringer Mannheim. Anti-β-tubulin monoclonal antibodies were from Sigma. Goat anti-rat brain calcium/calmodulin-dependent (CaM) protein kinase II (alpha subunit) antibodies were developed by Bethyl Laboratories. Donkey anti-rabbit and donkey anti-mouse horseradish peroxidase-linked secondary antibodies were from Amersham; swine anti-goat was from Boehringer Mannheim. ReadySafe™ liquid scintillation fluid was obtained from Beckman. *O*-Tetradecanoylphorbol 13-acetate (TPA) was obtained from Calbiochem. GF/C filters were from Whatman. [^{3}H]AMPA and the Renaissance™ chemiluminescence kit were obtained from DuPont/NEN. Radiolabeled orthophosphate was from ICN. All other reagents were from Sigma.

Cell cultures

Primary cultures of rat hippocampal neurons were prepared from rat pups 24–48 h postnatal. Hippocampi were dissected out into room temperature osmotically balanced saline solution (137 mM NaCl, 5.3 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 10 mM HEPES, 33 mM glucose, 44 mM sucrose, 0.024 g/l Phenol Red, pH 7.3, 325 mosm), cut into five or six pieces and incubated for 1 h with 10 ml of a 20 units/ml papain solution in saline solution. The papain solution was removed and the hippocampal fragments washed once in complete medium (MEM, 10% heat-inactivated fetal calf serum, 1 µl/ml serum extender, 21 mM glucose, 10 µg/ml 5-fluoro-2′-deoxyuridine, 25 µg/ml uridine). Cells were then dissociated into fresh complete medium via 15–20 passes through a Pasteur pipette and plated into sterile, poly-L-lysine-coated 35 mm culture plates at an approximate density of two hippocampi per dish. Cultures were fed with almost complete changes of medium on postculture days 1, 4 and 7 and typically used for experiments on culture day 9 or 10 (except where indicated). Cultures prepared via this method are of extremely high density (roughly 1–2 million cells per plate), and have a very high neuron-to-glia ratio since they are cultured in the presence of a mitotic inhibitor (5-fluoro-2′-deoxyuridine) from the very first day. Before use in experiments, cultures were typically washed with room-temperature saline solution three times, incubated for 20 min in saline solution following the third wash, washed once more and then exposed to the various treatments described below.

Proteolysis

Following a wash incubation of 20 min at 37°C, cultures were incubated with 1 mg/ml chymotrypsin in saline solution for 10 min with agitation at 37°C. The saline solution was then aspirated and the plates were washed three times in ice-cold harvest buffer [saline solution containing 2 mM (PMSF), 50 mM ethanolamine, 50 mM NaF, and 1 mM EDTA]. Preliminary experiments demonstrated that PMSF has a rapid and irreversible inhibitory effect on chymotrypsin activity. After the third wash, 1.0 ml of fresh, ice-cold harvest buffer was added and the cells were scraped up, transferred into a 1.5-ml snap-cap vial and placed on ice.

Cross-linking and biotinylation

Following a wash incubation of 20 min at 37°C, cultures were incubated with either 1 mg/ml BS^3 or 1 mg/ml NHS-ss-biotin in saline solution for 10 min with agitation at 37°C (these conditions were optimized in preliminary experiments). The saline solution was then aspirated and the plates were washed three times in ice-cold harvest buffer (ethanolamine is present in the harvest buffer to quench any unreacted BS^3 or NHS-ss-biotin). After the third wash, 1.0 ml of fresh, ice-cold harvest buffer was added and the cells were scraped up, transferred into a 1.5-ml snap-cap vial and placed on ice.

Preparation of lysed membranes

Following harvest of the cells, samples were homogenized via trituration. Aliquots of 100 µl were then removed and used for western blotting (see below). The remainder of the samples were frozen at −70°C, thawed and centrifuged for 20 min at 10,000 *g* at 4°C. The supernatant was removed and the pellets were washed twice with 1 ml of ice-cold assay buffer (50 mM HEPES, 100 µM EDTA, pH 7.4). The pellets were then resuspended in 425 µl assay buffer via sonication for 8–10 s using a Branson Sonifier 250 at 25% of maximum power and assayed for [^{3}H]AMPA binding activity. These samples are referred to as either ''lysed membranes prepared from intact cultures'' or ''lysed cultures''.

Samples referred to in the Results section as lysed membranes, which were used in some preliminary experiments, were prepared from postnatal day 2 rat telencephalon. Briefly, the telencephalic material was harvested following decapitation, placed in ice-cold saline solution (with 1 mM EDTA), homogenized, centrifuged at 800 *g* (4°C) for 10 min and the P1 pellet was discarded. The supernatant was recentrifuged for 20 min at 10,000 *g*, the resuming supernatant removed, and the membranes lysed via suspension in ice-cold water (with 1 mM EDTA present). The samples were then centrifuged again, the supernatant discarded, the samples resuspended in assay buffer and frozen at −70°C until use. On the day of experiments, samples were typically thawed and centrifuged 10 min at 10,000 *g*. The supernatants were then discarded and the pellets were washed twice in ice-cold assay buffer before the samples were resuspended via sonication in ice-cold assay buffer to an approximate protein concentration of 0.5 mg/ml.

Precipitation of biotinylated samples

Pellets of lysed membranes prepared from control intact cultures and intact cultures which had been biotinylated with NHS-ss-biotin were resuspended via 8–10 s sonication in 0.5 ml 500 mM HEPES, 100 µM EDTA, 1% Triton X-100 (solubilization buffer) and incubated with agitation

for 30 min at 4°C. The samples were then centrifuged for 30 min at 10,000 *g* (4°C); the resulting supernatants were defined as the soluble fraction and were incubated for 2 h at 4°C with 100 µl neutravidin-linked beads which had been preincubated with 1 mg/ml BSA in solubilization buffer to reduce non-specific interactions. The bead–soluble fraction mixtures were then spun for 1 min at 10,000 *g* and the supernatants were removed and saved; these are the "supernatant" fractions referred to in the Results section. The beads were then washed five times in solubilization buffer, and finally resuspended in a volume of sample buffer [2% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM β-mercaptoethanol, 1 mg/ml Bromophenol Blue] which was half the concentration of the supernatants (typically 250 µl). The beads were incubated in the sample buffer with agitation at room temperature for 15 min, and then centrifuged at 10,000 *g*. The supernatants were removed and used for western blotting; these samples are referred to in the Results section as the "precipitate" fractions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

Lysate samples were incubated with sample buffer for 5 min in boiling water, then loaded into 9% acrylamide gels and run at 150 V for 1 h. The dye front was not allowed to exit the bottom of the gel. Proteins were then blotted on to nitrocellulose for 4 h at 60 V. Blots were blocked for 30 min with 5% milk in Tris-buffered saline (TBS) (50 mM NaCl, 10 mM Tris, pH 7.4), then incubated with primary antibodies in 2% milk–TBS with 0.1% Tween-20 (wash buffer) for either 2 h at room temperature or overnight at 4°C. Blots were then washed three times (10 min each) in wash buffer and incubated for 1 h at room temperature with an appropriate horseradish peroxidase-linked secondary antibody at a dilution of 1 : 2000. Following three more washes with wash buffer, blots were developed via a 1 min incubation with the Renaissance chemiluminescence reagent (luminol in an oxidizing solution) followed by exposure to sheets of Kodak BioMax film for varied lengths of time. Films were developed such that all bands resulting from a given blot exposure were in the most linear range of intensity, as determined from preliminary experiments in which standard curves were constructed by plotting the relative OD of the immunoreactivities for increasing concentrations of lysed membranes vs the amount of membranes loaded per lane. Reductions in immunoreactivity in experimental samples were determined from these standard curves.

[^{3}H]*AMPA binding assay*

[^{3}H]AMPA binding was assayed via the filtration method as previously described,[12,31] with modifications. Samples were incubated in borosilicate test tubes in a final volume of 100 µl with the indicated concentration of [^{3}H]AMPA in the presence of 50 mM KSCN; incubations were for 40–60 min in an ice bath. The assay was terminated via the addition of 4 ml of ice-cold assay buffer with 50 mM KSCN followed rapidly by filtration through GF/C filters which had been soaked in 0.03% polyethylenimine for at least 30 min. The filters were rapidly washed twice with the assay buffer (with 50 mM KSCN) and then placed into scintillation vials to which 4 ml of scintillation fluid was added. Results were expressed as the percentage of specific binding (in c.p.m.) in treated samples relative to control samples. Non-specific binding was defined as that which occurred in the presence of 2.5 mM L-glutamate; non-specific binding was approximately 10% of the total binding at 2 nM [^{3}H]AMPA, 20% of total at 20 nM [^{3}H]AMPA, and 40% of total at 200 nM.

Metabolic labeling and immunoprecipitation

Hippocampal cultures were preincubated for 2 h in phosphate-free MEM and then labeled with [^{32}P]orthophosphate (1 mCi) in 1.0 ml phosphate-free MEM for 1 h at 37°C. The cultures were washed and incubated for 10 min more at 37°C with either saline solution (for control cultures) or 1 mg/ml chymotrypsin in saline solution. Following three washes with cold saline solution containing PMSF, the cells were harvested into a 0.5 ml of a solubilization buffer containing 50 mM HEPES, pH 7.4, 0.5 M NaCl, 0.5% Triton X-100, 0.1% deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 10 mM NaF and 1 µM microcystin-LR. The harvested cells were then briefly sonicated and incubated end-over-end at room temperatures for 15 min to achieve maximal solubilization. Each solubilized sample was then incubated for 2 h at 4°C with 10 mg of protein A-Sepharose beads which had been cross-linked with dimethyl pimelimidate to 2 µl of anti-GluR1 antibody.[44] Following this incubation, the supernatants were removed and the beads were washed six times in solubilization buffer. After the final wash, the beads were resuspended in 100 µl SDS sample buffer, boiled for 5 min, and briefly centrifuged; the supernatants were taken and loaded on 9% SDS–polyacrylamide gel electrophoresis (PAGE) gels as described. Incorporation of ^{32}P was quantified by exposing dried gels to film for varying lengths of time and then performing densitometric scanning as described.

RESULTS

Western blot analyses of lysed hippocampal cultures probed with antibodies against GluR1 and GluR2/3 revealed major immunoreactive bands in the range of 105,000 mol. wt, as previously described.[44] GluR4 immunostaining was also examined and found to be extremely low in the hippocampal culture samples relative to staining for other samples such as adult brain cerebellum. This observation that there is little GluR4 in primary hippocampal cultures is consistent with previous work,[6,8] and thus GluR4 surface expression was not examined in the present analysis.

Estimation of glutamate receptor subunits 1–3 *surface expression via proteolysis*

Preliminary experiments with lysed brain membranes demonstrated that an incubation for 10 min at 37°C with 1 mg/ml chymotrypsin was sufficient to obviate almost all GluR1 and GluR2/3 105,000 mol. wt immunostaining as well as [^{3}H]AMPA binding. The same treatment performed on intact primary cultures of rat hippocampal neurons yielded a consistently large decrease in GluR1 and GluR2/3 105,000 mol. wt immunostaining as well as the appearance of breakdown products in the range of 65,000–75,000 mol. wt (Fig. 1); quantitation of the blots yielded estimates of a $64 \pm 2\%$ and a $46 \pm 3\%$ reduction (mean ± S.E.M.; n=12) in the intensity of the 105,000 mol. wt bands for GluR1 and GluR2/3, respectively (Fig. 2). The top band of the GluR2/3 doublet appeared to be markedly more affected by the chymotrypsin treatments than the bottom band, but the resolution level of the blots did not allow for individual quantitation of these two bands. No significant differences were observed in the percentage reduction in GluR1 or GluR2/3 105,000 mol. wt immunostaining at 5 min, 15 min, and 45 min

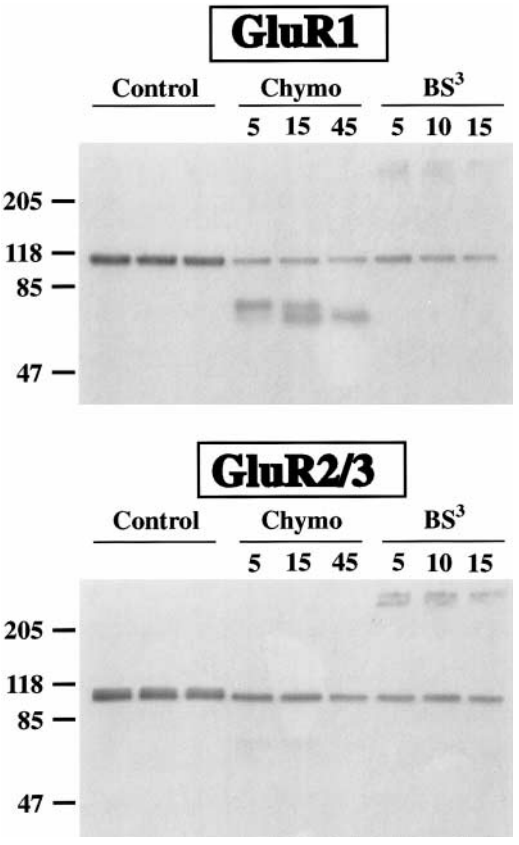

Fig. 1. Reduction of GluR1 (top) and GluR2/3 (bottom) western blot immunoreactivity by proteolysis and cross-linking of surface receptors. The first three lanes of each blot were loaded with lysate from individual untreated primary hippocampal cultures, the next three lanes with lysate from cultures treated with 1 mg/ml chymotrypsin for either 5, 15 or 45 min at 37°C, and the last three lanes with lysate from cultures treated with 1 mg/ml BS^3 for 5, 10 or 15 min at 37°C. The migration of mol. wt markers is shown at the left of the blots (values $\times 1000$). The two autoradiograms of western blots shown here were slightly over-exposed to show more clearly the banding pattern of the chymotrypsin-induced breakdown products and BS^3-induced aggregated products; autoradiograms of these and other blots which were quantified via densitometric scanning were developed into the most linear range of the film and analysed via comparison to standard curves.

of chymotrypsin application (Fig. 1). The immunoreactivity of the 65,000–75,000 mol. wt breakdown products decreased and changed pattern with extended exposure to chymotrypsin (Fig. 1), while an increasing amount of immunoreactivity was found migrating with the dye front (not shown). These data suggest that brief periods of chymotrypsin treatment are sufficient to proteolyse all surface AMPA receptors, and that extended treatments lead to further degradation of breakdown products.

Blots of the control and proteolysed samples were also probed for immunoreactivity to three intracellular proteins, the alpha subunit of the abundant enzyme calcium/calmodulin-dependent (CaM) protein kinase II and the cytoskeletal components actin and tubulin. Staining with the CaM kinase II antibody yielded a doublet in the range of 50,000–52,000 mol. wt, while the actin and tubulin monoclonal antibodies recognized single bands on our blots of approximately 43,000 mol. wt and 61,000 mol. wt, respectively. Immunoreactivity for all three of these proteins was not significantly different in the 10 min proteolysed samples relative to the control samples (n=12; data are shown in Fig. 2), suggesting that only transmembrane proteins were susceptible to cleavage by chymotrypsin.

Estimation of glutamate receptor subunits 1–3 *surface expression via cross-linking*

Incubation of lysed brain membranes for 10 min at 37°C with 1 mg/ml BS^3, a membrane-impermeable, irreversible, amine-reactive cross-linking reagent, yielded a nearly 100% reduction in GluR1 and GluR2/3 105,000 mol. wt immunostaining as well as a near total inactivation of [^{3}H]AMPA binding (data not shown). The probable explanation for the former observation is that completely cross-linked AMPA receptors are so large that they are barely able to penetrate SDS–PAGE gels, while the loss of [^{3}H]AMPA binding might be explained by the fact that a lysine residue (Lys445 in the GluR1 subunit) has been shown to be essential for the formation of the AMPA receptor agonist binding site.[20,41] Cross-linking of this key lysine by BS^3 might be expected to disrupt the structure of the binding site and prevent [^{3}H]AMPA from binding. Incubation of intact cultures with 1 mg/ml BS^3 for 10 min at 37°C resulted in a large reduction in the staining of the 105,000 mol. wt GluR band and the appearance of high mol. wt aggregates which barely entered the gel (Fig. 1). The cross-linking treatment removed $61 \pm 2\%$ and $43 \pm 2\%$ of the 105,000 mol. wt GluR1 and GluR2/3 immunoreactive bands, respectively (n=12; data are shown in Fig. 2). As with the chymotrypsin experiments, the top band of the GluR2/3 doublet seemed to be decreased by the cross-linking treatment more than the bottom band, and brief cross-linking treatments were sufficient to achieve essentially maximal reduction in 105,000 mol. wt GluR immunostaining (Fig. 1); more extended treatments did not significantly reduce the intensity of the 105,000 mol. wt band but did reduce the staining of the higher mol. wt aggregates at the top of the gel, presumably because extremely cross-linked species become too large to penetrate the gel matrix. Immunoreactivities for CaM kinase II, actin and tubulin in the cross-linked samples were not significantly different from control (n=12; Fig. 2), despite the observations made in preliminary studies with lysed brain membranes that immunoreactivities for all three of the intracellular markers could be markedly reduced by cross-linking with BS^3.

The cross-linking method was also used in two experiments to study the time-course of AMPA

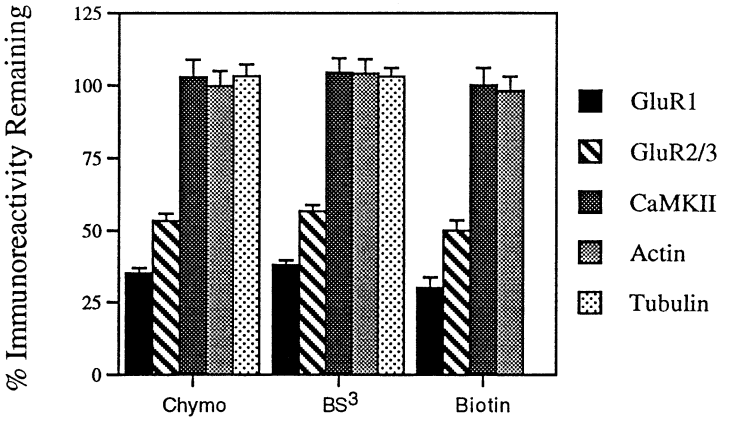

Fig. 2. Reduction of western blot immunoreactivities for GluR1 and GluR2/3, but not for the intracellular proteins CaM kinase II, actin and tubulin, by extracellular manipulations. Autoradiograms of western blots visualized via chemiluminescence were quantified via densitometric scanning and averaged. The blots contained lysate samples derived from intact cultures which had been treated with 1 mg/ml chymotrypsin (Chymo), BS^3 (BS^3) or NHS-ss-biotin (Biotin) for 10 min at 37°C. The biotinylated samples were solubilized and precipitated with neutravidin beads, with the control vs biotinylated supernatants being loaded on to the gels. Blots were probed separately with antibodies to GluR1, GluR2/3, CaM kinase II, actin and tubulin. Quantification of tubulin was not possible for biotinylated/precipitated samples since tubulin does not solubilize well under the conditions used. The values shown for GluR1 and GluR2/3 staining represent immunoreactivity of the 105,000 mol. wt major bands only. Error bars represent the S.E.M. values for each condition (n values are given in the text).

receptor surface expression across days in culture. At culture day 3, the density of GluR immunostaining per unit protein was quite low, but the surface cross-linking treatments still substantially reduced immunostaining of the 105,000 mol. wt band and produced an aggregate at the top of the gel (not shown). At culture days 6, 9 and 12, GluR1–3 immunostaining was of similar high intensity, and a similar proportion of immunostaining was removed by surface cross-linking. Thus, there were no significant differences in surface expression after six days in culture, and surface GluR expression was evident as early as culture day 3.

Estimation of glutamate receptor subunits 1–3 *surface expression via biotinylation and precipitation*

Intact cultures were incubated for 10 min at 37°C with 1 mg/ml NHS-ss-biotin, a cleavable, amine-reactive, membrane-impermeant biotinylation reagent. This reagent has been used for biotinylation of surface AMPA receptors in studies performed on GluR3-expressing oocytes.[37] Following biotinylation of the intact hippocampal neurons with NHS-ss-biotin, cultures were harvested, solubilized with a buffer containing 1% Triton X-100 and precipitated with neutravidin beads. No immunoreactivity for either GluR1 or GluR2/3 was precipitated by the neutravidin beads from solubilized control cultures which had not been biotinylated. A large amount of GluR subunit protein was precipitated from biotinylated cultures, however, and quantification of the removal of GluR1 and GluR2/3 immunoreactivity from the solubilized supernatant fractions yielded estimates of $69 \pm 4\%$ and $50 \pm 3\%$ removal, respectively (n=5; Fig. 2). Longer incubations of the cultures with NHS-ss-biotin or higher concentrations of the reagent did not yield significantly different results. Immunoreactivity for actin and CaM kinase II was not significantly altered in the control vs biotinylated supernatants (n=5; Fig. 2); tubulin immunoreactivity could not be quantified in these samples because tubulin does not solubilize well under the conditions tested.

Estimation of the surface expression of [^{3}H]*AMPA binding sites*

The biotinylation/precipitation procedure was also used to estimate the surface expression of [^{3}H]AMPA binding sites. Precipitation with neutravidin beads removed $52 \pm 4\%$ (n=12) of specific 20 nM [^{3}H]AMPA binding from solubilized samples prepared from intact biotinylated cultures (Fig. 3, right panel). This estimated reduction is relative to the amount of [^{3}H]AMPA binding found in solubilized control culture samples which had also been incubated with neutravidin beads. When different concentrations of agonist were used in the binding assay (2 nM or 200 nM), the percentage reduction induced by the biotinylation/precipitation was similar to the reduction observed at 20 nM (53% and 55% reductions, respectively; n=2).

[^{3}H]AMPA binding to lysed membranes prepared from intact cultures which had been incubated with chymotrypsin, BS^3 or NHS-ss-biotin under the conditions previously specified was also quantified relative to binding to lysed membranes prepared from untreated cultures. Treatment of intact cultures with chymotrypsin seemed to have little effect on binding;

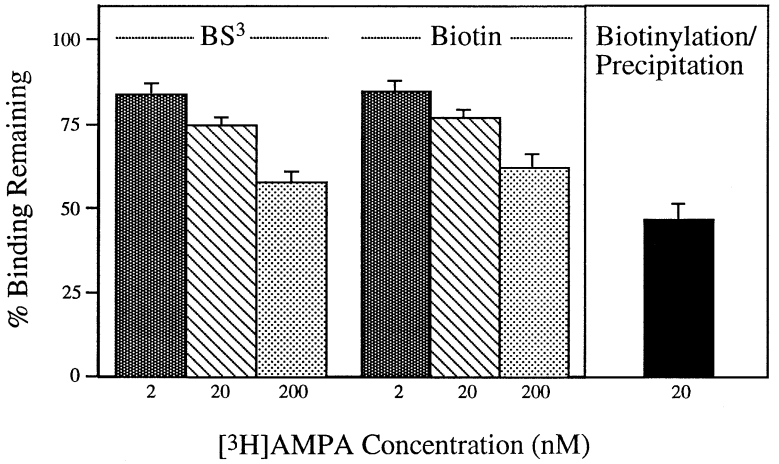

Fig. 3. Effects of amine-reactive treatments and biotinylation/precipitation on [^{3}H]AMPA binding. Primary hippocampal cultures were incubated with 1 mg/ml BS^3 or NHS-ss-biotin for 10 min at 37°C and then harvested, frozen and prepared into membranes. The two sets of bars in the left panel represent the percentage of [^{3}H]AMPA binding to membranes remaining following the two treatments (BS^3 and Biotin) relative to binding to membranes prepared from untreated cultures within each experiment. The dark bars indicate binding at 2 nM [^{3}H]AMPA, the striped bars represent binding at 20 nM [^{3}H]AMPA and the light bars represent binding at 200 nM [^{3}H]AMPA. The panel to the right demonstrates the percentage of binding remaining after treatment of intact cultures with NHS-ss-biotin followed by solubilization and precipitation with neutravidin beads. Error bars represent the S.E.M. values for each condition (n values are given in the text).

20 nM [^{3}H]AMPA binding to membranes prepared from cultures which had been treated with chymotrypsin for 10 min was 105 ± 3% of control (n=12). Two experiments performed using a 10-fold lower and a 10-fold higher concentration of [^{3}H]AMPA in the binding assay also revealed no apparent effect of the chymotrypsin treatment on binding: at 2 nM and 200 nM [^{3}H]AMPA, binding was 107% and 104% of control, respectively.

Treatment of intact cultures with the two amine-reactive reagents, BS^3 and NHS-ss-biotin, resulted in reductions in [^{3}H]AMPA binding: 25 ± 2% for BS^3 (n=14) and 23 ± 2% for NHS-ss-biotin (n=14) (Fig. 3, left panel). As with the western blot results, longer incubations with the reagents or higher concentrations did not yield different results. Two additional experiments were conducted examining whether the effects of the two treatments were additive. This did not seem to be the case, as treatment of intact cultures with BS^3 followed by NHS-ss-biotin or NHS-ss-biotin followed by BS^3 resulted in estimated reductions in binding of 20% and 21%, respectively (n=2). Further experiments examined the binding of a 10-fold lower concentration of [^{3}H]AMPA (2 nM) as well as the binding of a 10-fold higher concentration (200 nM). The reductions in binding induced by the two amine-reactive treatments were significantly different at the different concentrations of [^{3}H]AMPA tested; at 2 nM radiolabeled agonist, the average reductions were 16 ± 3% and 15 ± 3% for BS^3 and NHS-ss-biotin, respectively (n=6), while at 200 nM [^{3}H]AMPA the average reductions were 42 ± 3% and 38 ± 4% (n=6) (Fig. 3, left panel). Thus, the percentage decrease in membrane [^{3}H]AMPA binding observed following treatment of intact cultures with the amine-reactive reagents was dependent on the concentration of radiolabeled agonist used in the binding assay.

Estimation of the surface expression of phosphorylated glutamate receptor subunit 1

In order to examine the subcellular localization of phosphorylated AMPA receptors, hippocampal cultures were metabolically labeled with [^{32}P]orthophosphate and then harvested after incubation with either control buffer or buffer containing 1 mg/ml chymotrypsin. GluR1 from these samples was then immunoprecipitated and the label incorporated into both the 105,000 mol. wt main band and the 65,000–75,000 mol. wt breakdown product was quantified. Preliminary experiments with this immunoprecipitation protocol demonstrated that almost all GluR1 staining, as well as a significant fraction of GluR2/3 staining, is immunoprecipitated from the solubilized samples under these conditions. This is expected since the GluR1–4 subunits are known to assemble into functional multimers which can be immunoprecipitated intact.[44] Thus, the 105,000 mol. wt radiolabeled band quantitated on the autoradiograms for these experiments probably consists of mostly GluR1, but also with some GluR2 and GluR3. As shown in Fig. 4B, a large amount of radiolabel is removed from the 105,000 mol. wt main band by treatment of the labeled cultures with chymotrypsin before harvest, and novel radiolabeled bands of 65,000–75,000 mol. wt appear which correspond in size and relative intensity to the GluR-immunoreactive break-

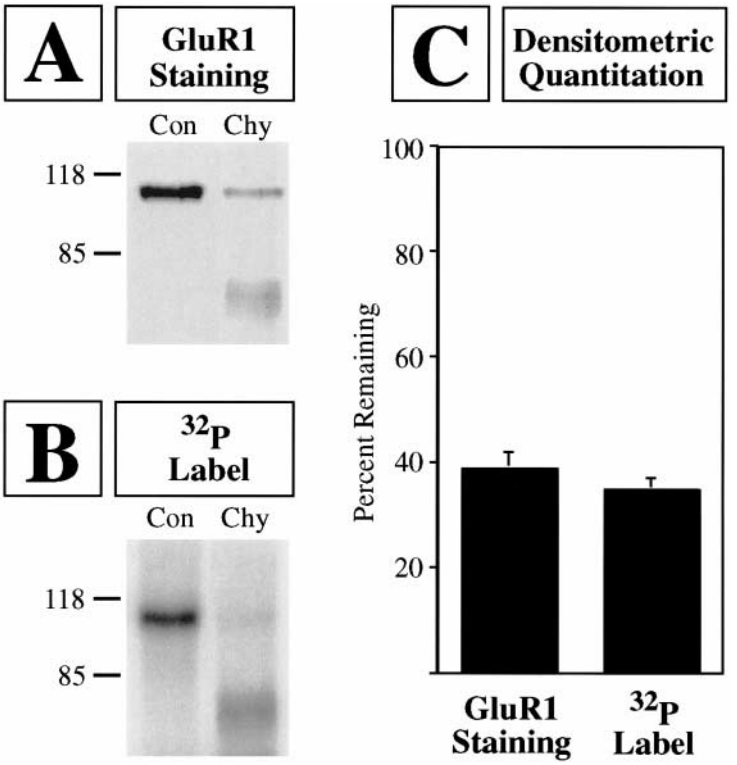

Fig. 4. The surface expression of phosphorylated GluR1 is similar to the surface expression of total GluR1. (A) Anti-GluR1 western blot of two cultures which had been labeled with ^{32}P and then immunoprecipitated with an anti-GluR1 antibody. (B) Autoradiogram of the same two samples. The lane on the left is from a control culture (Con), while the lane on the right is from a culture that was treated for 10 min with 1 mg/ml chymotrypsin (Chy) in order to cleave surface receptors. (C) Percentage of both anti-GluR1 staining and ^{32}P label incorporation remaining following the chymotrypsin treatments. Bars and error bars represent the mean and S.E.M. for six experiments.

down products in the same samples (Fig. 4A). The quantitation of the amount of radiolabel removed by the chymotrypsin treatments from the 105,000 mol. wt main band is shown in Fig. 4C; the values are similar for quantitation of both incorporated radiolabel and total western blot anti-GluR1 staining. Thus, the percentage of phosphorylated GluR1 on the surface does not differ significantly from the percentage of total GluR1 on the surface.

Estimation of glutamate receptor subunits 1–3 *surface expression following stimulation*

To investigate further the potential effects of phosphorylation on the trafficking of AMPA receptors, the surface expression of the GluR subunits in the hippocampal cultures was examined following stimulatory treatments known to increase GluR phosphorylation: activation of *N*-methyl-D-aspartate-type glutamate receptors with 10 μM glutamate and 1 μM glycine for 5 min, activation of protein kinase C with 2 μM TPA for 15 min, and inhibition of protein phosphatases with 1 μM okadaic acid for 15 min. Metabolic labeling studies demonstrated that all three of these treatments significantly increased phosphorylation of the GluR subunits, consistent with previous results.[40] Western blots of samples derived from stimulated vs unstimulated samples revealed that total anti-GluR1 and anti-GluR2/3 staining was unaltered following stimulation, suggesting that the affinity of the antibodies for the GluR subunits was unaltered by receptor phosphorylation and that the total amount of GluR protein was not changed. The surface expression of GluR1 was examined via the cross-linking method in control cultures and in matched cultures which had been stimulated with the aforementioned agents. No significant differences relative to the unstimulated controls were observed for GluR1 surface expression following the stimulatory treatments; the percentages of immunoreactivity removed by surface cross-linking were $63 \pm 3\%$, $65 \pm 3\%$, $60 \pm 5\%$ and $63 \pm 2\%$ for control, glutamate/glycine-stimulated, TPA-stimulated and okadaic acid-stimulated cultures, respectively (n=4). A similar lack of effect of stimulation was observed for the surface expression of GluR2/3 immunoreactivity (not shown).

DISCUSSION

The present study examines the surface expression of AMPA receptors via a combination of

three techniques: cleavage of surface receptors by chymotrypsin, and modification of surface receptors with the amine-reactive chemicals BS^3 and NHS-ss-biotin. Since both chymotrypsin recognition sites and free amine groups are abundant in most proteins, the set of techniques described in the present work represents a general approach to studying the surface expression of transmembrane proteins. For studies such as these, it is desirable to employ several different techniques in quantitating surface expression since any one technique alone could be subject to artifact. One specific concern in studying synaptic transmembrane proteins is the accessibility of reagents to the synaptic cleft, particularly for larger molecules such as chymotrypsin. This did not seem to be a problem in the present study, since the surface expression estimates derived from the proteolysis technique were quite similar to those derived from the cross-linking and biotinylation techniques. Since BS^3 and NHS-ss-biotin are similar in size to many glutamate receptor antagonists and modulatory reagents which are known to have access to glutamatergic synapses, it is likely that all three of the techniques described in the present report were able to affect both synaptic and non-synaptic surface receptors.

Approximately half of the total GluR1–3 subunits in cultured rat hippocampal neurons are inserted into the plasma membrane. Quantitation from proteolysis, cross-linking and biotinylation/precipitation studies yielded surface estimates for GluR1 of 64%, 61% and 69%, respectively, and surface estimates for GluR2/3 of 46%, 43% and 50%. The average of these six values is 55%; this may be considered to be a rough estimate of AMPA receptor surface expression in these cultured neurons, although the relative ratios of the subunits are not known. The biotinylation/precipitation studies revealed that 52% of total [^{3}H]AMPA binding sites are in the plasma membrane, an estimate similar to the western blot estimate. These estimates provide a quantitative complement to immunohistochemical analyses[2,6,8,27,29,34] and fractionation studies[13] which have revealed that staining for the GluR1–3 subunits is found both in the synaptic plasma membrane and in apparently intracellular areas.

Estimates of the surface expression of GluR1 and GluR2/3 in the present study are similar but not identical. Roughly two-thirds of total GluR1 protein seems to be located in the plasma membrane, as compared to slightly less than half of total GluR2/3 protein. It is possible, however, that there are more profound surface expression differences between subunits. This possibility is raised by the apparent difference in susceptibility to extracellular treatments exhibited by the two bands of the GluR2/3 doublet (Fig. 1, bottom panel). While it is not known whether these two bands correspond differentially to GluR2 and GluR3, it would clearly be of interest to examine the surface expression of individual recombinantly expressed GluR subunits. Heinemann and colleagues[37] used a biotinylation/precipitation method similar to the one described here to determine that roughly 30% of total GluR3 protein expressed in oocytes is located in the plasma membrane; other AMPA receptor subunits were not examined. This value for GluR3 is considerably lower than the estimates of GluR1–3 surface expression derived from the present study. This discrepancy could be indicative of differences in GluR trafficking between neurons and oocytes, differences in trafficking of the various subunits, or differences in the trafficking of homomeric vs heteromeric receptors.

Whether or not there are subunit differences in GluR trafficking, or differences between cell types, it can be estimated from the current results that 50–60% of the total AMPA receptor population in cultured hippocampal neurons is located in the plasma membrane. Binding of [^{3}H]AMPA was not reduced at all by brief treatments with chymotrypsin, suggesting that chymotrypsin cleaves the receptor amino-terminal of the S1 agonist binding determinants. The proteolytic product of 65,000–75,000 mol. wt (Figs 1, 4) would be consistent with this interpretation as the S1 domain is approximately 40,000 mol. wt from the amino terminus.[37] Also consistent with our data is the observation that proteolytic cleavage of AMPA receptors by trypsin does not seem to affect physiological responses to AMPA receptor agonists.[1] Binding of 20 nM [^{3}H]AMPA was reduced 20–25% by two amine-reactive treatments (BS^3 and NHS-ss-biotin) which probably exert this effect by reacting with a lysine residue (Lys445 for GluR1) known to be essential for agonist binding to AMPA receptors.[20,41] This estimate of binding site surface expression would seem to be incongruous with the estimates of receptor surface expression derived from the western blot studies. However, further binding studies revealed that binding of 2 nM [^{3}H]AMPA was reduced by only approximately 15%, while binding of 200 nM [^{3}H]AMPA was reduced by approximately 40%; thus, the apparent reduction in [^{3}H]AMPA binding induced by the amine-reactive treatments is dependent on the agonist concentration used in the binding assay.

The most likely explanation for this dependence on agonist concentration is that surface-exposed and intracellular AMPA receptors exist in different conformations, with the surface-exposed conformation having a lower affinity for agonists. It is well known that there are both high-affinity (K_D=15 nM under the conditions used in the present experiments) and low-affinity (K_D=500 nM) [^{3}H]AMPA binding sites in mammalian brain membranes.[12,31,32] The low-affinity sites are predominant in adult brain membranes, and the idea has been put forward that these sites correlate with binding to synaptic receptors.[12,36] If surface receptors bind with lower average affinity than intracellular receptors, then [^{3}H]AMPA binding assays performed at subsaturating ligand

concentrations are bound to underestimate the percentage of surface-exposed binding sites, with lower agonist concentrations resulting in more grossly distorted estimates. The highest concentration examined in the present studies (200 nM) is still below the K_D of the low-affinity binding sites, and it is therefore likely that the estimates of binding site surface expression derived at this concentration (38–42%) are still underestimates. Accurate binding studies cannot readily be performed at saturating [^{3}H]AMPA concentrations, however, since such studies would incur a very unfavorable ratio of specific to non-specific binding.

The estimated percentage of surface binding sites derived from the biotinylation/precipitation experiments (52%) matches the estimates for surface GluR protein. The biotinylation/precipitation procedure is different from the other techniques examining surface binding sites in that it involves a solubilization step. Solubilization might be expected to place all AMPA receptors in a uniform conformation, and indeed it has been reported that solubilization of AMPA receptors results in a homogeneous population of high-affinity [^{3}H]AMPA binding sites.[12] This explains why the estimates of binding site surface expression derived from the biotinylation/precipitation studies are: (i) not dependent on the concentration of agonist used in the binding assay; and (ii) considerably higher than the estimates derived from the membrane binding studies, since the latter are complicated by a heterogeneous mixture of binding site affinities.

The nicotinic acetylcholine receptor (nAChR) is the most intensively studied neurotransmitter receptor, and much is known about its subcellular distribution. It has been shown in both skeletal muscle cells[9,33] and ciliary ganglion neurons[15] that a significant proportion (20–50%) of the total nAChR population resides intracellularly. These results are similar to those reported here for AMPA receptor subcellular distribution in hippocampal neurons. It is not definitively known, in the case of the nAChR, whether the observed intracellular population represents receptors in the process of being synthesized and degraded or, alternatively, whether there is a pool of functional receptors available at all times. Similarly, these two possibilities cannot be distinguished from our studies on AMPA receptors.

The possibility that there is a pool of intracellular AMPA receptors available as a functional reserve is of interest with regard to potential mechanisms of synaptic plasticity. There is considerable evidence that the AMPA receptor-mediated component of glutamatergic synaptic transmission is selectively enhanced following long-term potentiation (LTP) of hippocampal synaptic connections.[16,21,25,30] Furthermore, it has been reported that AMPA receptor-mediated synaptic responses tend to be considerably more variable than responses mediated by *N*-methyl-D-aspartate receptors,[18] and that this variability of AMPA receptor-mediated responses decreases significantly following induction of LTP.[18,38] It has been widely proposed[7,14,18,19,22–24,26] that such results might be explained by a rapid increase in the number of available cell surface AMPA receptors at synapses following LTP, with this increase being perhaps induced by changes in AMPA receptor phosphorylation state.[24]

The surface expression of many receptors and transporters, including glucose transporters,[4,39] GABA transporters[5] and nicotinic acetylcholine receptors,[10] is regulated by protein phosphorylation. AMPA receptors are known to be basally phosphorylated by endogenous kinases[3,28,40] and activation of kinases has been shown to enhance AMPA receptor-mediated currents.[11,17,28,42,43,45] This enhancement of currents, as well as the enhancement of currents observed following LTP, could plausibly result from either a rapid change in the total number of surface receptors or a change in the efficacy of receptors already on the surface. Our data suggest that the former mechanism is unlikely, based on two pieces of evidence from the present study: (i) phosphorylated GluR1 is found on the surface of hippocampal neurons to about the same extent as total GluR1; and (ii) the surface expression of the GluR subunits is not acutely altered following stimulatory treatments known to increase GluR phosphorylation.

CONCLUSIONS

Our results demonstrate that slightly more than half of the total AMPA receptor population in cultured hippocampal neurons is found at the cell surface, and that this subcellular distribution of AMPA receptors is not rapidly altered by receptor phosphorylation. These data offer a more quantitative description of AMPA receptor subcellular localization than has previously been available and also serve to constrain and refine hypotheses concerning the mechanisms of synaptic plasticity.

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REFERENCES

1. Allen C. N., Brady R., Swann J., Hori N. and Carpenter D. O. (1988) *N*-Methyl-D-aspartate (NMDA) receptors are inactivated by trypsin. *Brain Res.* **458,** 147–150.

2. Baude A., Molnar E., Latawiec D., McIlhinney R. A. J. and Somogyi P. (1994) Synaptic and nonsynaptic localization of the GluR1 subunit of the AMPA-type excitatory amino acid receptor in the rat cerebellum. *J. Neurosci.* **14,** 2830–2843.
3. Blackstone C. D., Murphy T. H., Moss S. J., Baraban J. M. and Huganir R. L. (1994) Cyclic AMP and synaptic activity-dependent phosphorylation of AMPA-preferring glutamate receptors. *J. Neurosci.* **14,** 7585–7593.
4. Calderhead D. M., Kitagawa K., Tanner L. I., Holman G. D. and Lienhard G. E. (1990) Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. *J. biol. Chem.* **265,** 13,800–13,808.
5. Corey J. L., Davidson N., Lester H. A., Brecha N. and Quick M. W. (1994) Protein kinase C modulates the activity of a cloned gamma-aminobutyric acid transporter expressed in *Xenopus* oocytes via regulated subcellular redistribution of the transporter. *J. biol. Chem.* **269,** 14,759–14,767.
6. Craig A. M., Blackstone C. D., Huganir R. L. and Banker G. (1993) The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. *Neuron* **10,** 1055–1068.
7. Edwards F. A. (1991) LTP is a long-term problem. *Nature* **350,** 271–272.
8. Eshhar N., Petralia R. S., Winters C. A., Niedzielski A. S. and Wenthold R. J. (1993) The segregation and expression of glutamate receptor subunits in cultured hippocampal neurons. *Neuroscience* **57,** 943–964.
9. Fambrough D. and Devreotes P. N. (1978) Newly synthesized acetylcholine receptors are located in the Golgi apparatus. *J. Cell Biol.* **76,** 237–244.
10. Green W. N., Ross A. F. and Claudio T. (1991) Acetylcholine receptor assembly is stimulated by phosphorylation of its gamma subunit. *Neuron* **7,** 659–666.
11. Greengard P., Jen J., Nairn A. and Stevens C. F. (1991) Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science* **253,** 1135–1138.
12. Hall R. A., Kessler M. and Lynch G. (1992) Evidence that high- and low-affinity DL-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) binding sites reflect membrane-dependent states of a single receptor. *J. Neurochem.* **59,** 1997–2004.
13. Henley J. M. (1995) Subcellular localization and molecular pharmacology of distinct populations of [^{3}H]-AMPA binding sites in rat hippocampus. *Br. J. Pharmac.* **115,** 295–301.
14. Isaac J. T. R., Nicoll R. A. and Malenka R. C. (1995) Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15,** 427–434.
15. Jacob M. H., Lindstrom J. M. and Berg D. K. (1986) Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. *J. Cell Biol.* **103,** 205–214.
16. Kauer J. A., Malenka R. C. and Nicoll R. A. (1988) A persistent postsynaptic modification mediates long-term potentiation in the hippocampus. *Neuron* **1,** 911–917.
17. Keller B. U., Hollmann M., Heinemann S. and Konnerth A. (1992) Calcium influx through subunits GluR1/GluR3 of kainate/AMPA receptor channels is regulated by cAMP dependent protein kinase. *Eur. molec. Biol. Org. J.* **11,** 891–896.
18. Kullmann D. M. (1994) Amplitude fluctuations of dual-component EPSCs in hippocampal pyramidal cells: implications for long-term potentiation. *Neuron* **12,** 1111–1120.
19. Kullmann D. M. and Nicoll R. A. (1992) Long-term potentiation is associated with increases in quantal content and quantal amplitude. *Nature* **357,** 240–244.
20. Li F., Owens N. and Verdoorn T. A. (1995) Functional effects of mutations in the putative agonist binding regions of recombinant alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Molec. Pharmac.* **47,** 148–154.
21. Liao D., Hessler N. A. and Malinow R. (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* **375,** 400–404.
22. Lisman J. E. and Harris K. M. (1993) Quantal analysis and synaptic anatomy—integrating two views on hippocampal plasticity. *Trends Neurosci.* **16,** 141–147.
23. Lynch G. and Baudry M. (1984) The biochemistry of memory: a new and specific hypothesis. *Science* **224,** 1057–1063.
24. Malinow R. (1994) LTP: desperately seeking resolution. *Science* **266,** 1195–1196.
25. Manabe T. and Nicoll R. A. (1994) Long-term potentiation: evidence against an increase in transmitter release probability in the CA1 region of hippocampus. *Science* **265,** 1888–1892.
26. Manabe T., Wyllie D. J. A., Perkel D. J. and Nicoll R. A. (1993) Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. *J. Neurophysiol.* **70,** 1451–1459.
27. Martin L. J., Blackstone C. D., Levey A. I., Huganir R. L. and Price D. L. (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. *Neuroscience* **53,** 327–358.
28. McGlade-McCulloh E., Yamamoto H., Tan S. E., Brickey D. A. and Soderling T. R. (1993) Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Nature* **362,** 640–642.
29. Molnar E., Baude A., Richmond S. A., Patel P. B., Somogyi P. and McIlhinney R. A. J. (1993) Biochemical and immunocytochemical characterization of antipeptide antibodies to a cloned GluR1 glutamate receptor subunit: cellular and subcellular distribution in the rat forebrain. *Neuroscience* **53,** 307–326.
30. Muller D., Joly M. and Lynch G. (1988) Contributions of quisqualate and NMDA receptors to the induction and expression of LTP. *Science* **242,** 1694–1697.
31. Murphy D. E., Snowhill E. W. and Williams M. (1987) Characterization of quisqualate recognition sites in rat brain tissue using DL-[^{3}H]-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and a filtration assay. *Neurochem. Res.* **12,** 775–782.
32. Olsen R. W., Szamraj O. and Houser C. R. (1987) [^{3}H]AMPA binding to glutamate receptor subpopulations in rat brain. *Brain Res.* **402,** 243–254.
33. Pestronk A. (1985) Intracellular acetylcholine receptors in skeletal muscle of the adult rat. *J. Neurosci.* **5,** 1111–1117.
34. Petralia R. S. and Wenthold R. J. (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J. comp. Neurol.* **318,** 329–354.
35. Seeburg P. (1993) The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* **16,** 359–365.

36. Standley S., Irvin N. and Baudry M. (1994) Differential subcellular localization of two populations of glutamate/AMPA receptors in the rat telencephalon. *Neurochem. Int.* **25,** 287–293.
37. Stern-Bach Y., Bettler B., Hartley M., Sheppard P. O., O'Hara P. J. and Heinemann S. F. (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron* **13,** 1345–1357.
38. Stevens C. F. and Wang Y. (1994) Changes in reliability of synaptic function as a mechanism for plasticity. *Nature* **371,** 704–707.
39. Suzuki K. and Kono T. (1980) Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. natn. Acad. Sci. U.S.A.* **77,** 2542–2545.
40. Tan S. E., Wenthold R. J. and Soderling T. R. (1994) Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. *J. Neurosci.* **14,** 1123–1129.
41. Uchino S., Sakimura K., Nagahari K. and Mishina M. (1992) Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor. *Fedn Eur. biochem. Socs Lett.* **308,** 253–257.
42. Wang L.-Y., Dudek E. M., Browning M. D. and MacDonald J. F. (1994) Modulation of AMPA/kainate receptors in cultured murine hippocampal neurones by protein kinase C. *J. Physiol.* **475,** 431–437.
43. Wang L.-Y., Salter M. W. and MacDonald J. F. (1991) Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science* **253,** 1132–1135.
44. Wenthold R. J., Yokotani N., Doi K. and Wada K. (1992) Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies. *J. biol. Chem.* **267,** 501–507.
45. Yakel J. L., Vissavajihala P., Derkach V. A., Brickey D. A. and Soderling T. R. (1995) Identification of a Ca^{2+}/calmodulin-dependent protein kinase II regulatory phosphorylation site in non-*N*-methyl-D-aspartate glutamate receptors. *Proc. natn. Acad. Sci. U.S.A.* **92,** 1376–1380.

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