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Evidence for Multiple AMPA Receptor Complexes in Hippocampal CA1/CA2 Neurons

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The AMPA receptor, which is involved in most fast glutamatergic transmission in the mammalian brain and is expressed in most neurons, is made up of four subunits, GluR1–4. *In situ* hybridization, immunocytochemistry studies, and single-cell PCR analyses show that the number and type of AMPA receptor subunits expressed vary among neuronal populations and that two to four subunits usually are expressed in each neuron. Neurons that express two or more subunits theoretically could produce multiple pentameric receptor complexes that differ in their subunit compositions, and these complexes could be targeted to different synaptic populations. To determine whether a single neuronal population produces multiple AMPA receptor complexes, we used a preparation of CA1/CA2 hippocampal pyramidal neurons and immunoprecipitation with subunit-specific antibodies to characterize the receptor complexes. The CA1/CA2 pyramidal neurons express high levels of GluR1–3 and receive multiple excitatory inputs, offering the possibility that distinct receptor complexes may be assembled and expressed selectively at different synaptic populations. Our results suggest the presence of two major populations of AMPA receptor complexes: those made up of GluR1 and GluR2 and those made up of GluR2 and GluR3. Very few complexes contained both GluR1 and GluR3, whereas ~8% of the total AMPA receptor complexes was homomeric GluR1. The integrity of the receptor complex was verified by measuring [^{3}H]AMPA binding activity in the immunoprecipitated fractions. These results show that AMPA receptor complexes with different subunit compositions are present in CA1/CA2 pyramidal neurons and suggest an additional mechanism to regulate receptor expression in neurons.

Key words: glutamate receptor; excitatory amino acids; AMPA; hippocampus pyramidal neurons; immunoprecipitation; receptor subunit

Most neurons express multiple subtypes of both ionotropic and metabotropic glutamate receptors, and for the ionotropic receptors, several subunits of any particular receptor. The mechanisms by which neurons regulate the assembly of subunits into functional receptor complexes and the targeting of these complexes to the appropriate synaptic locations are poorly understood. Because glutamate receptor complexes are thought to be pentamers, neurons that express several subunits of a receptor theoretically could form multiple distinct receptor complexes with different subunit compositions and different functional characteristics. Such a capability would allow neurons that receive several different synaptic inputs to target specific receptor complexes to individual postsynaptic sites to fit particular functional requirements.

The AMPA receptor, which is involved in most fast glutamatergic transmission in the mammalian brain and is expressed in most neurons, is made up of four subunits, GluR1–4 (or GluR A–D), each of which contains two major splice variants, flip and flop (Hollmann et al., 1989; Keinänen et al., 1990; Sommer et al., 1990) (for review, see Hollmann and Heinemann, 1994). Each subunit can form a functional homomeric receptor when expressed in oocytes or transfected cells, although generally it is thought that in neurons, AMPA receptors are heteromeric and made up of at least two different subunits based on physiological properties of the cloned and native receptors. *In situ* hybridization and immunocytochemistry studies and single-cell PCR analyses show that the number and type of AMPA receptor subunits expressed vary among neuronal populations and that two to four subunits usually are expressed in each neuron (Keinänen et al., 1990; Lambolez et al., 1992; Petralia and Wenthold, 1992; Martin et al., 1993; Tölle et al., 1993; Bochet et al., 1994; Jonas et al., 1994). Populations of neurons expressing primarily GluR1 have been identified in cortex, striatum, and spinal cord, and such neurons may form predominantly homomeric receptors (Petralia and Wenthold, 1992; Furuyama et al., 1993; Martin et al., 1993; Tachibana et al., 1994). GluR2 is a key subunit in that its presence in the receptor complex produces a channel that is calcium impermeable, whereas those formed by the other subunits, either alone or in combination, are calcium-permeable (Hollmann et al., 1991; Verdoorn et al., 1991). Most neurons express GluR2 and form calcium-impermeable channels, although populations of neurons that lack GluR2 have been identified in both hippocampus and cortex (Ozawa and Iino, 1993; Bochet et al., 1994; Jonas et al., 1994). Bergmann glia also lack GluR2 and have calcium-permeable channels (Burnashev et al., 1992; Müller et al., 1992). Although the influence of the other subunits is less dramatic, the properties of the receptor are dependent on the subunit and splice variant composition (Mosbacher et al., 1994; Geiger et al., 1995). For example, receptors containing GluR3 or GluR4 flop subunits have desensitization time constants lower than those containing GluR3 or GluR4 flip subunits or GluR1 flip or flop subunits (Mosbacher et al., 1994).

Functional data supporting the existence of multiple AMPA receptor complexes in a single neuron are limited. Lerma et al. (1994) used cultured or acutely dissociated hippocampal neurons

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and found receptor complexes containing or missing GluR2, as determined by calcium permeability and rectification properties of the neuron. These studies suggest that GluR2-containing and GluR2-lacking receptor complexes coexist in the same neuron and that they may differ in their subcellular distribution, with receptors lacking GluR2 being enriched in dendrites. A similar study on rat retinal ganglion cells showed that channels with high and low calcium permeability were found on a single neuron (Zhang et al., 1995). In cultured cortical neurons, receptor populations with different sensitivities to the potentiator cyclothiazide were observed in the same outside-out macropatch, suggesting the presence of different AMPA receptors (T. Verdoorn, personal communication).

To study the properties of AMPA receptor complexes in neurons, we have used a preparation of CA1/CA2 hippocampal pyramidal neurons and immunoprecipitation with subunit-specific antibodies to determine the subunit composition of detergent-solubilized AMPA receptors. The CA1/CA2 pyramidal neurons express high levels of GluR1–3 in both flip and flop splice variants (Keinänen et al., 1990; Sommer et al., 1990; Pellegrini-Giampietro et al., 1992; Sato et al., 1993; Bochet et al., 1994) and receive multiple excitatory inputs, offering the possibility that distinct receptor complexes may be assembled and expressed selectively at different synapse populations. The CA1/CA2 region (Boss et al., 1987) can be dissected to yield a preparation highly enriched in pyramidal neurons of sufficient quantity to permit biochemical and immunoprecipitation analyses. It has been estimated that >95% of the neurons in the CA1/CA2 region of the rat are pyramidal (Bernard and Wheal, 1994). Our results show that at least three populations of AMPA receptor complexes with different subunit compositions are present in CA1/CA2 pyramidal neurons and suggest an additional mechanism to regulate receptor expression in neurons.

MATERIALS AND METHODS

Tissue preparation. Brains were obtained from 30- to 40-d-old male Sprague–Dawley rats and frozen immediately on dry ice. Brains were thawed partially, and 1-mm-thick coronal sections of the forebrain were made with a razor blade using a metal template. Sections containing the entire hippocampus were placed immediately in cold PBS containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 2.5 mM EDTA. Subdivisions of the hippocampus were identified as described by Paxinos and Watson (1986). Visible landmarks, as seen with a dissecting microscope, were useful in subdividing the hippocampus and included the alveus, pyramidal cell body layer, and the row of blood vessels at the level of the hippocampal fissure that separates the CA1/CA2 regions from the dentate gyrus. Dissected pieces were placed in PBS containing protease inhibitors on ice. Detergent solubilization was performed as described previously (Hunter et al., 1990; Wenthold et al., 1992). Tissue was homogenized at 30–60 mg wet weight/ml in 0.5 M potassium phosphate, pH 7.4, containing 20% (w/v) glycerol, 0.1 mM PMSF, and 1 mM EDTA. Triton X-100 was added to a final concentration of 1% (w/v), and the solution was incubated with mixing at 37°C for 30 min. After centrifugation at 100,000 × *g* for 1 hr, samples were dialyzed against 0.1 M Tris, pH 7.5, containing 0.1% Triton X-100 and 10% glycerol. The preparation then was centrifuged at 100,000 × *g* for 1 hr, and the supernatant was stored at −80°C. Using this protocol, ~85% of [^{3}H]AMPA binding activity is in the detergent-soluble fraction (Hunter et al., 1990).

Immunoprecipitation. The antibodies to AMPA receptor subunits used in this study were characterized previously (Wenthold et al., 1992) and include antibodies specific for GluR1 (Ab 7), GluR2/3 (Ab 25), GluR4 (Ab 22), GluR2 (Ab 33), and GluR3 (Ab 35). The specificities of the antibodies were verified using human embryonic kidney cells (HEK-293) transfected with cDNAs for GluR1, -2, -3, and -4, as described previously (Wenthold et al., 1994). cDNAs were kindly provided by Drs. S. Heinemann (GluR1–3) and P. Seeburg (GluR4). The antibody to GluR2/3 also recognizes the GluR4c splice variant (Gallo et al., 1992). These antibodies were made to peptides corresponding to sequences at or near the C terminus of the subunits. All antibodies were affinity purified with the same peptide used for immunization and have been shown to be selective for their respective subunits using Western blots of transfected cell membranes (Wenthold et al., 1992). All antibodies were made in rabbits. For immunoprecipitation, 10 μg of antibody was incubated with 25 μl (volume of packed resin) of protein-A agarose (Pierce, Rockford, IL) in PBS, pH 7.5, containing 0.1% Triton X-100 for at least 4 hr. When immunoprecipitation was performed with two antibodies, 10 μg of each antibody was used with a total of 25 μl of protein-A agarose. The resin was washed three times with Tris-buffered saline, pH 7.5, and mixed overnight with 500 μl of detergent-solubilized CA1/CA2 preparation. The mixture was centrifuged briefly in a microcentrifuge, and the unbound fraction was removed. The resin was washed three times with Tris-buffered saline containing 0.1% Triton X-100 and was boiled in SDS-PAGE sample buffer (2× concentration). The resin was removed by centrifugation. Controls were performed using resin without primary antibody.

AMPA binding activity to the solubilized receptor was measured at 10 nM [^{3}H]AMPA and 100 mM KSCN using polyethylene glycol precipitation as described previously (Hunter et al., 1990). The amount of [^{3}H]AMPA was expressed as the percent of that in controls in which no primary antibody was used in the immunoprecipitation step.

Electrophoresis. Analyses were performed on both the unbound and the bound fractions of the immunoprecipitations. The unbound fractions, as well as the untreated solubilized fractions that served as controls, were mixed with an equal volume of SDS-PAGE sample buffer (2× concentration) and boiled for 3 min. Ten microliters were applied to a 4–20% acrylamide minigel (Novex, San Diego, CA). Transfer to nitrocellulose and developing of Western blots were performed as described previously (Wenthold et al., 1992), with the exception that detection was performed with chemiluminescence (DuPont NEN, Boston, MA). Primary antibodies were used at the following concentrations (in μg/ml): GluR1 0.5, GluR2/3 0.15, GluR4 0.75, GluR2 2.0, and GluR3 2.5.

To estimate the amount of immunoreactivity in the unbound fractions, films from the chemiluminescence detection were scanned using a Molecular Dynamics densitometer (Sunnyvale, CA). The immunoreactivity in the unbound fractions was compared with that in corresponding nonimmunoprecipitated samples. Different amounts of the nonimmunoprecipitated samples ranging from 5 to 100% of the total were used as standards and were run with each gel. Because the volumes of the unbound fractions did not change appreciably with immunoprecipitation, the same volumes of the unbound and nonimmunoprecipitated samples could be compared directly. In comparing control samples (incubated with resin but no primary antibody) with nonimmunoprecipitated samples, no differences were observed; therefore, nonimmunoprecipitated detergent-solubilized samples were used as standards.

Immunocytochemistry. Immunocytochemical analyses were performed with antibodies to GluR1, GluR2/3, and GluR4 as described previously (Petralia and Wenthold, 1992). Briefly, adult male rats were perfused with 4% paraformaldehyde, and brains were sectioned at 50 μm with a vibratome. Tissue sections were incubated overnight at 4°C with antibodies at the following concentrations (in μg/ml): GluR1 2.4, GluR2/3 2.8, and GluR4 1.4. The ABC reagent (Vectastain kit, Vector Laboratories, Burlingame, CA) was used to localize bound antibodies using 3′,3-diaminobenzidine tetrahydrochloride.

RESULTS

Immunocytochemical distribution of AMPA receptor subunits

Immunocytochemical analysis of AMPA receptor subunits was performed using antibodies selective for GluR1, GluR2/3, and GluR4 (Fig. 1). Intense staining of pyramidal cell bodies and dendrites is seen with anti-GluR1 and anti-GluR2/3 antibodies, whereas light staining is seen with anti-GluR4 antibodies. Staining of nonpyramidal neurons is relatively light for antibodies to GluR1 and GluR2/3, but staining for GluR4 is apparent in nonpyramidal neurons as well as in glia.

Immunoprecipitation of AMPA receptor subunits

The specificities of the antibodies used in these studies were demonstrated using membranes of cells transfected with AMPA

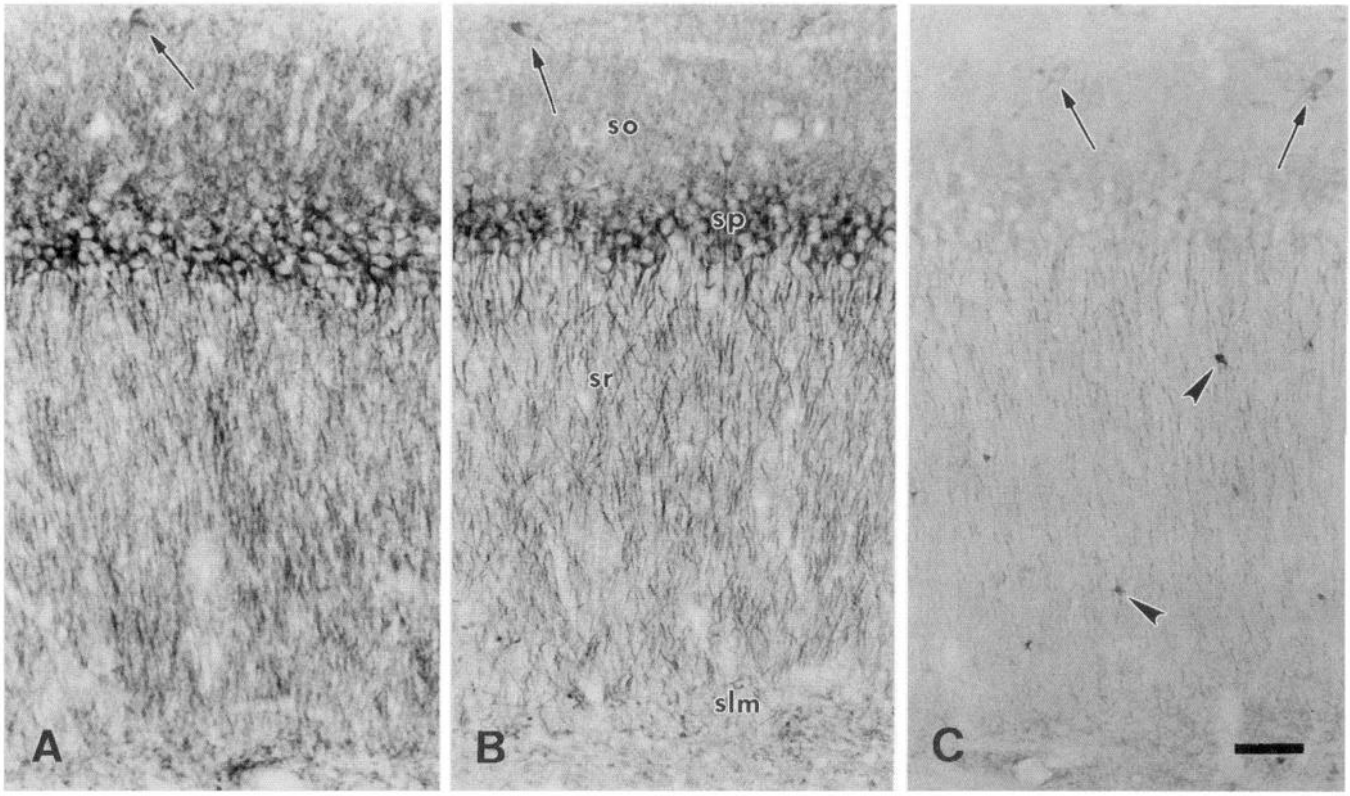

Figure 1. Sagittal sections of rat hippocampus immunostained with antibodies to GluR1 (*A*), GluR2/3 (*B*), and GluR4 (*C*). *so*, Stratum oriens; *sp*, stratum pyramidale (pyramidal neuron somata); *sr*, stratum radiatum; *slm*, stratum lacunosum-moleculare. *Arrow* indicates nonpyramidal neuron; *arrowhead*, glial cell. Scale bar, 50 μm.

receptor subunit cDNAs (Fig. 2). Immunoprecipitation was used to determine the subunit composition of detergent-solubilized receptor complexes. Triton X-100 has been shown previously to solubilize AMPA receptors effectively and retain [^{3}H]AMPA binding activity (Hunter et al., 1990). With the hippocampal CA1/CA2 preparation, all subunits were solubilized equally well with 75–85% in the soluble fraction (Fig. 3). Under the conditions used in this study, immunoprecipitation was nearly 100% for GluR1, GluR2/3, and GluR4 using antibodies selective for those particular subunits. Anti-GluR2 and anti-GluR3 only poorly immunoprecipitated receptor subunits and, therefore, were used only for Western blotting. The degree of immunoprecipitation of the target subunit and the coimmunoprecipitating subunits was quantified by measuring the amount of subunit remaining in the unbound fraction compared with controls or nonimmunoprecipitated solubilized receptor. Analyses of the unbound fractions are shown in Figure 4. Immunoprecipitation with anti-GluR1 or anti-GluR2/3 antibodies removed essentially all of the target subunits with no detectable GluR1 or GluR2/3 immunostaining, respectively, remaining in the unbound fraction. Immunoprecipitation with anti-GluR2/3 antibodies, although removing all GluR2/3 immunoreactivity, left ~19% of the GluR1 in the unbound fraction. Immunoprecipitation with both anti-GluR2/3 and GluR4 antibodies did not reduce additionally the amount of GluR1 in the unbound fraction. However, if both anti-GluR1 and anti-GluR2/3 antibodies were used, GluR1 immunostaining was removed completely from the unbound fraction. These results show that the nonimmunoprecipitated GluR1 is not associated with GluR2, -3, or -4 and represents either homomeric receptor complexes or single subunits that may have originated from disrupting a receptor complex; ~15–20% of the total GluR1 is present in this fraction.

Immunoprecipitation with anti-GluR1 antibodies removed all GluR1 immunostaining but less than half of the GluR2/3 immunostaining (Fig. 4). Again, immunoprecipitation with a combination of antibodies to GluR1 and GluR4 did not decrease additionally the amount of GluR2/3 immunostaining in the depleted fraction, whereas a combination of antibodies to GluR1 and GluR2/3 removed all GluR2/3 immunostaining. These results suggest that about half of the AMPA receptor complexes contain no GluR1 or GluR4, but only GluR2 and/or GluR3. To determine the nature of this population, the fractions were stained with antibodies selective for GluR2 or GluR3 (Fig. 4). Immunoprecipitation with anti-GluR2/3 antibodies removed all GluR2 immunoreactivity, whereas immunoprecipitation with anti-GluR1 antibodies removed ~70% of the GluR2 immunoreactivity. This indicates that ~70% of the GluR2 is associated with GluR1. Immunoprecipitating with anti-GluR2/3 antibodies removed all GluR3 immunoreactivity in the depleted fraction. However, immunoprecipitation with anti-GluR1 antibodies left ~90% of the GluR3 in the depleted fraction. These results suggest that only a small population of receptor complexes contains both GluR1 and GluR3 and that most complexes are comprised of GluR1 and GluR2 or GluR3 and GluR2.

Consistent results were obtained by analyzing the bound fractions from the immunoprecipitations (Fig. 5). After immunoprecipitation with anti-GluR2/3 antibodies, the bound fraction contained immunoreactivity for GluR1, GluR2/3, GluR2, and GluR3. After immunoprecipitation with anti-GluR1 antibodies, intense immunoreactivity for all the subunits was in the bound fraction

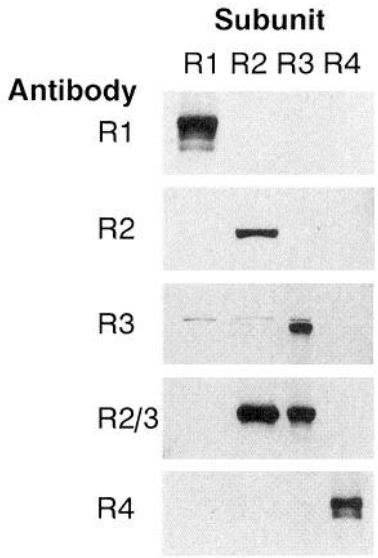

Figure 2. Subunit-specific antibody staining of Western blots of membranes of HEK-293 cells transfected with AMPA receptor cDNAs. Blots were stained with antibodies to GluR1 (*R1*), GluR2 (*R2*), GluR3 (*R3*), GluR2/3 (*R2/3*), and GluR4 (*R4*). The same amount of transfected cell membrane for any one subunit was used for all five antibodies. In all cases, the antibodies label a major band corresponding to the appropriate subunit. Anti-GluR2/3 antibodies label both GluR2 and GluR3. For GluR1 and GluR4, slightly lower molecular weight bands also are present, and these are likely to be degradation products and/or nonglycosylated forms of the receptor. A minor band, stained with the anti-GluR3 antibody, is seen above the GluR3 band; this band also is seen in cells transfected with GluR1 and GluR2 and represents a cross-reacting protein expressed by HEK-293 cells (see also Wenthold et al., 1992). Because of the high expression levels of GluR4, it is usually not detected in the GluR4 lane. However, with prolonged staining of the GluR4 lane, this band is visible in the GluR4 lane (data not shown).

except GluR3, which showed only a very minor band. This is consistent with results of analysis of the unbound fraction showing that antibodies to GluR1 do not coimmunoprecipitate GluR3 to a significant degree.

GluR4 is a minor subunit in CA1/CA2 based on both immunostaining (Fig. 1) and Western blots (Fig. 3) and its lack of immunoprecipitation of other AMPA receptor subunits (Fig. 4). However, it is important to verify that antibodies to GluR4 can immunoprecipitate the GluR4 subunit and coimmunoprecipitate other subunits. As shown in Figure 6, antibodies to GluR4 removed immunostaining of GluR4 in the unbound fraction, whereas antibodies to GluR1 and GluR2/3 reduced GluR4 staining in the unbound fraction. These results show that antibodies to GluR4 immunoprecipitate the GluR4 subunit and that at least part of the GluR4 is present in complexes containing GluR1 and/or GluR2/3. The apparent discrepancy between these results and those of Figure 4, in which immunoprecipitation with antibodies to GluR4 did not deplete the other subunits significantly, can be explained by the relatively low abundance of GluR4 in this preparation.

Assessing the intactness of the AMPA receptors in the detergent-solubilized preparation

One explanation for the above results showing the presence of multiple AMPA receptor complexes is that the immunoprecipitated complexes are fragments of the original receptor complex that was disrupted during the detergent solubilization or immunoprecipitation procedures or complexes that are not assembled

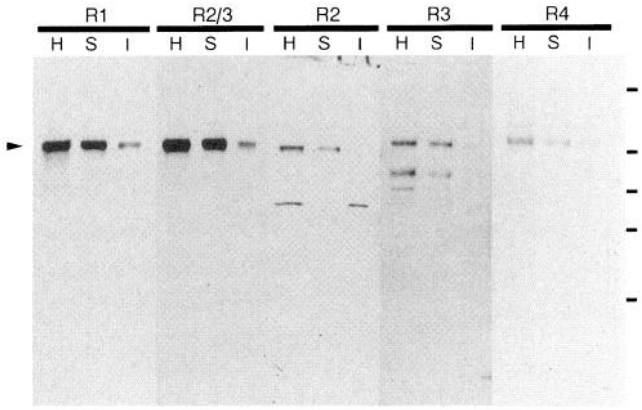

Figure 3. Western blot analysis of hippocampal fractions. *H*, Total homogenate; *S*, Triton X-100-soluble; *I*, Triton X-100-insoluble. Antibodies selective for GluR1, GluR2/3, GluR2, GluR3, and GluR4 were used as described (see Fig. 2 for conventions). Samples were adjusted such that equal amounts were applied for H, S, and I. Therefore, if 100% of the staining was recovered in the S fraction, the staining intensity would equal that of H. Quantitation of one experiment showed that 85, 82, 79, and 75% of the immunostaining was recovered in the S fraction for *R1*, *R2/3*, *R2*, and *R3*, respectively. *Arrowhead* indicates position of the receptor subunit. *Bars* show the position of prestained molecular weight standards myosin, phosphorylase B, bovine serum albumin, ovalbumin, and chymotrypsinogen migrating at M_r = 210, 103, 71, 46, and 25 kDa.

completely. To address this, we measured [^{3}H]AMPA binding to the immunoprecipitated fractions, presuming that only intact receptor complexes are capable of binding the ligand. Quantifying binding activity in the unbound fractions showed that both anti-GluR1 and anti-GluR2/3 immunoprecipitated [^{3}H]AMPA binding, whereas anti-GluR4 antibodies had no significant effect (Fig. 7). After immunoprecipitation with anti-GluR2/3 antibodies, 12% of the [^{3}H]AMPA binding remained in the depleted fraction; including anti-GluR4 antibodies did not increase the amount of immunoprecipitation. However, when anti-GluR1 antibodies were included, the amount of [^{3}H]AMPA binding in the depleted fraction was reduced to 3.7%, indicating that ~8% of the [^{3}H] binding in the preparation is attributable to homomeric GluR1. This is consistent with the estimate of 15–20% for the proportion of GluR1 subunits that exist as homomeric complexes (Fig. 4), because GluR1 is responsible for only part of the AMPA binding activity. Furthermore, ligand affinities and binding capacities may vary among subunits and subunit combinations, making an exact correlation impossible. Immunoprecipitation with anti-GluR1 antibodies left 45% of the [^{3}H]AMPA binding activity in the depleted fraction and indicates that about half of the [^{3}H]AMPA binding activity is associated with receptor complexes that do not contain GluR1. This is consistent with the results showing that 53% of the GluR2/3 immunostaining remains in the depleted fraction after immunoprecipitation with anti-GluR1 antibodies.

DISCUSSION

The objective of this study was to analyze AMPA receptors of CA1/CA2 hippocampal pyramidal neurons and to determine the subunit composition of the receptor complexes formed. These neurons express high levels of GluR1, 2, and 3 and receive multiple different synaptic inputs on their dendrites. Our results show that two major complexes are present: those containing GluR1 and GluR2 and those containing GluR2 and GluR3. Complexes containing both GluR1 and GluR3 are minor, and we

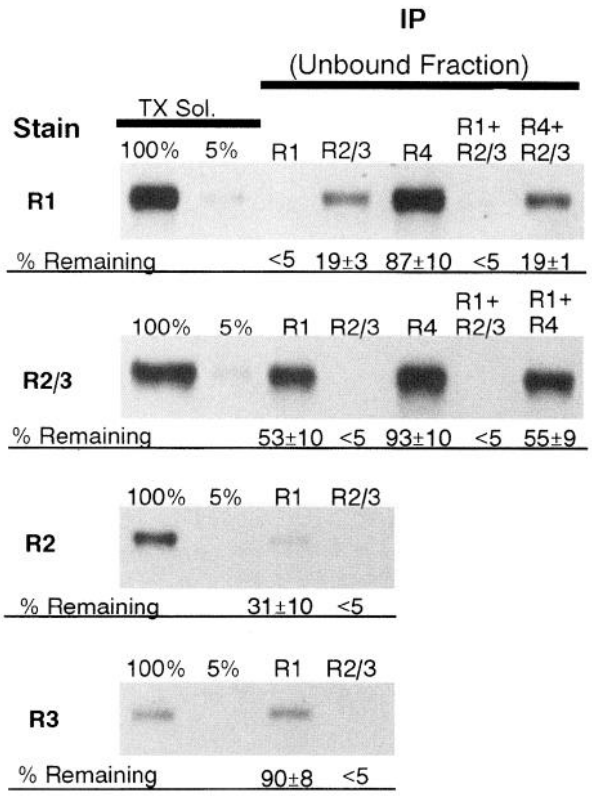

Figure 4. AMPA receptor subunits remaining (unbound fraction) after immunoprecipitation of detergent-solubilized CA1/CA2 region. The *left two lanes* of each panel show staining of the nonimmunoprecipitated detergent-solubilized fraction (*TX Sol.*). For the *100% lane*, 10 μl of sample was applied, whereas for the *5% lane*, 0.5 μl (diluted to 10 μl with sample buffer) was applied; these lanes represent the range of labeling for quantitation of the immunoreactivity in the depleted fractions. In addition, for each gel, standards of 7.5, 5.0, 2.5, and 1.0 μl of the solubilized fraction also were analyzed (data not shown). To determine the amount of immunoprecipitation, 10 μl of the depleted fraction was applied; this is equivalent to an equal volume of the solubilized fraction (100%). The subunit-specific antibody used for immunoprecipitation is listed *above the immunostained band*, and the antibody used for staining the Western blot is listed on the *far left* (see Fig. 2 for conventions). The percent of immunostaining remaining in the depleted fractions, relative to the nonimmunoprecipitated detergent-solubilized fractions, is listed *below each band*. These are the mean ± SEM of three separate experiments. Although in some cases immunostaining was not detected, the lowest standard used was 5% of the detergent-solubilized fraction; therefore, the amount of immunoreactivity remaining was noted as <5%. *IP*, Immunoprecipitated.

find that ~8% of the total [^{3}H]AMPA binding activity can be attributed to homomeric GluR1.

The results of the present study rely on two critical factors: the specificity of the antibodies and the validity of the immunoprecipitation procedure. The antibodies were made to peptides corresponding to the C terminus (GluR1, GluR2/3, and GluR4) or near the C terminus (GluR2 and GluR3) and included sequences not shared with other subunits. The specificity of the antibodies was established by analysis of membranes of cells transfected with cDNAs of the four subunits (Fig. 2) (Wenthold et al., 1992). The fact that the antibody to GluR2/3 also recognizes the GluR4c splice variant should not affect the interpretation of the results, but actually strengthens our results. For example, immunoprecipitation with anti-GluR2/3 and GluR4 antibodies identified a population of homomeric GluR1 receptors. Because the antibody to GluR2/3 also recognizes GluR4c, it is expected that GluR4c is immunoprecipitated in these fractions and would not be present in the unbound fraction in a complex with GluR1. In measuring the unbound fraction with anti-GluR2/3 antibodies, results were substantiated using selective antibodies to GluR2 and GluR3.

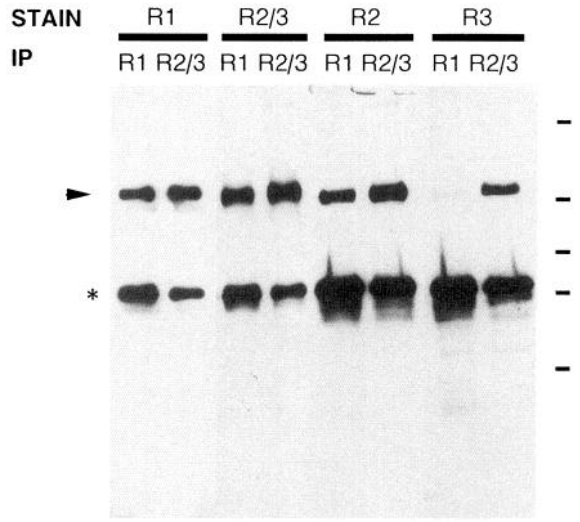

Figure 5. Immunoprecipitation of AMPA receptor subunits (bound fraction). Detergent-solubilized CA1/CA2 region samples were immunoprecipitated with anti-GluR1 or anti-GluR2/3 antibodies, and the bound fractions were analyzed with the same antibodies as well as those selective for GluR2 and GluR3 (see Fig. 2 for conventions). The subunit-specific antibody used for immunoprecipitation is noted directly *above the stained band*, and the antibody used for immunostaining is listed in the *top row*, *above the bar*. Little GluR3 is coimmunoprecipitated with GluR1. The *arrowhead* indicates the position of the receptor subunit, and the *asterisk* shows immunoglobulin heavy chain. *Bars* show the position of prestained standards as described in the legend of Figure 3.

Three potential problems associated with immunoprecipitation include selective immunoprecipitation of subpopulations of the target protein, nonspecific aggregation leading to false-positives in coimmunoprecipitation, and generation of fragments of the receptor complex. Selective immunoprecipitation of subpopulations of receptor subunits may occur if the epitope, which is the target of the immunoprecipitating antibody, is masked under some conditions, such as when the complex contains one particular subunit. In the present study, this concern was eliminated by developing an immunoprecipitation procedure in which the major antibodies used in the immunoprecipitation, anti-GluR1 and anti-GluR2/3,

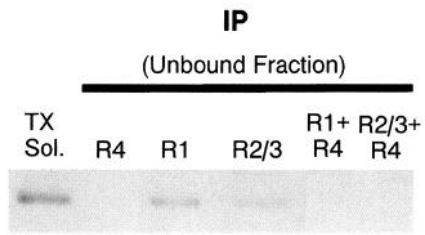

Figure 6. Immunoprecipitation of GluR4. Immunoprecipitation was performed with antibodies listed above stained band, and all were stained with antibodies to GluR4 (see Fig. 2 for conventions). Antibodies to GluR4 removed GluR4 staining completely from unbound fraction, whereas antibodies to GluR1 and GluR2/3 decreased staining partially, compared with control (*TX Sol.*), which is staining of the nonimmunoprecipitated detergent-solubilized fraction.

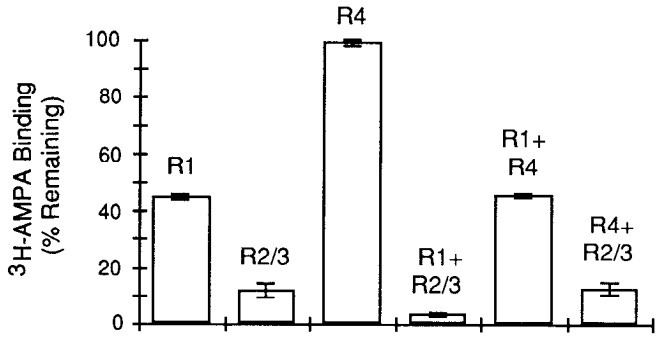

Figure 7. Immunoprecipitation of [^{3}H]AMPA binding activity from detergent-solubilized CA1/CA2 preparation. Immunoprecipitation was performed using the antibody noted *above the bars*, and [^{3}H]AMPA binding was determined in the unbound fraction. Results are expressed as percent of binding remaining compared with controls in which no primary antibody was used in the immunoprecipitation. Data are expressed as mean ± SEM of three experiments and are as follows: *R1*, 45.3 ± 1.9; *R2/3*, 12.0 ± 3.5; *R4*, 98.8 ± 1.7; *R1 + R2/3*, 3.7 ± 1.1; *R1 + R4*, 46.2 ± 1.2; *R4 + R2/3*, 12.3 ± 3.0.

immunoprecipitated essentially all, greater than 95%, of the target subunit. GluR4, which is present in low levels in this preparation, also was immunoprecipitated quantitatively with anti-GluR4 antibodies. Therefore, all populations of receptors containing the respective subunits were immunoprecipitated. However, we cannot rule out the possible existence of novel AMPA receptor subunits, splice variants, or subunits with modified C termini that may coimmunoprecipitate with the other subunits, but would not be recognized by the antibodies used in this study. The experimental design also eliminated concern for nonspecific aggregation; the key results were obtained from analysis of the unbound fraction. In the analysis of the bound fractions, the critical finding was that GluR3 did not coimmunoprecipitate with GluR1, so that aggregation is not a concern in this result. The final possibility that fragments of the receptor complex are present and are responsible for the immunoprecipitation results must be considered. Such fragments could represent assembly intermediates or originate from disruption of the receptor complex at some point during the solubilization or immunoprecipitation. The major evidence arguing against this is the correlation between subunit immunoprecipitation and [^{3}H]AMPA binding, in which both analyses were done on the same samples. Immunoprecipitation with anti-GluR1 antibodies left 53% of the GluR2/3 immunoreactivity and 45% of the [^{3}H]AMPA binding in the unbound fraction. This shows that about half of the AMPA binding is to complexes that do not contain GluR1, which fits with the finding that about half of the GluR2/3 immunoreactivity is not immunoprecipitated with anti-GluR1 antibodies. Immunoprecipitation with anti-GluR2/3 antibodies left 12% of the AMPA binding and 19% of the GluR1 immunostaining in the unbound fraction. In both cases, the remaining fractions were nearly completely eliminated by also immunoprecipitating with anti-GluR1 antibodies. This shows that the apparent homomeric GluR1 receptor complexes do bind [^{3}H]AMPA. The difference in percent remaining, 12% versus 19%, is consistent with the fact that 12% refers to the amount of [^{3}H]AMPA binding, which would include all subunits, whereas 19% refers to GluR1 immunostaining only. We cannot rule out the possibility that receptor fragments may bind [^{3}H]AMPA. However, AMPA binding is a measure of a receptor complex capable of binding agonist, and it seems likely that disruption of the quaternary structure to the point of removing subunits would be sufficient to destroy ligand binding. Gel exclusion chromatography of detergent solubilized AMPA receptors shows a broad symmetrical peak of [^{3}H]AMPA binding (Hunter et al., 1990; Blackstone et al., 1992), the size of which is consistent with an intact pentameric complex, but leaving open the possibility that smaller, or larger, complexes also may contribute to AMPA binding.

The 8% of the [^{3}H]AMPA binding, which we attribute to homomeric GluR1, could originate from the relatively small population of interneurons in this preparation, but this appears unlikely based on the low number of such neurons and their minimal expression of GluR1. In Wistar rats, nonpyramidal neurons make up fewer than 5% of the total neurons in the CA1/CA2 region of the hippocampus (Bernard and Wheal, 1994). We do not find heavy expression of GluR1 in CA1/CA2 interneurons based on antibody staining, which is consistent with previous studies on this area (Keinänen et al., 1990; Sommer et al., 1990; Monyer et al., 1991; Sato et al., 1993). Calcium-permeable AMPA receptors on hippocampal pyramidal neurons have been reported (Lerma et al., 1994), and these could be homomeric GluR1.

Studies on other receptors have provided strong evidence that neurons can produce several different receptor complexes and probably target them to different cellular locations. The subunit composition of the nicotinic acetylcholine receptor complex has been studied in the chick ciliary ganglion neurons (Conroy et al., 1992; Vernallis et al., 1993; Conroy and Berg, 1995), which express five subunits, α3, β4, α5, β2, and α7. At least three complexes, which differ in their subunit composition, are present in these neurons. Synaptic receptors are made up of α3, β4, and α5, but ~20% of these complexes also contain β2. The α7 subunit forms a third complex that does not contain other subunits and appears to have a nonsynaptic localization. There is evidence that the GABA receptor also may exist in some neurons as multiple complexes with different subunit compositions. Cerebellar granule cells express significant amounts of mRNA for six subunits and lower amounts for three additional subunits (Laurie et al., 1992). Because functional GABA receptors appear to be made up of five subunits, the number of subunits alone would indicate that at least two different complexes are present in these neurons. In Purkinje neurons, the distribution of α1 and α3 subunits differed between the soma and the dendrites, suggesting that at least two GABA receptor complexes are present (Fritschy et al., 1992).

A number of mechanisms could play a role in generating multiple receptor complexes in a neuron. In the initial events of the assembly process, if the affinity of GluR1 and GluR3 is greater for GluR2 than for each other, complexes that do not contain both GluR1 and GluR3 would be favored. In fact, a high affinity of the other subunits for GluR2 may provide a mechanism to ensure that most complexes would contain GluR2 and, thus, would be calcium impermeable. GluR1 and GluR3 can form complexes with each other in transfected cells and oocytes (Boulter et al., 1990; Nakanishi et al., 1990; Sekiguchi et al., 1994), and immunoprecipitation studies on whole brain show GluR1/GluR3- containing complexes. Three or four AMPA receptor subunits have been coexpressed in oocytes (Boulter et al., 1990; Nakanishi et al., 1990; Sekiguchi et al., 1994) and transfected cells (Sommer et al., 1990) and form complexes with physiological properties different from those formed from two subunits. Other mechanisms to affect the number and composition of receptor complexes would include factors that affect the assembly process or the stability of the complex once it is formed. Several proteins

of the endoplasmic reticulum function as molecular chaperones and influence the folding and assembly of complex proteins (Hurtley and Helenius, 1989; Hammond and Helenius, 1993; Frydman et al., 1994). Two such proteins, immunoglobulin-binding protein and calnexin, have been shown to associate with the nicotinic acetylcholine receptor (Forsayeth et al., 1992; Gelman et al., 1995).

The present results suggest that assembly of AMPA receptors may be regulated in neurons such that multiple complexes that differ in their subunit compositions are generated. Because hippocampal pyramidal neurons receive multiple excitatory inputs, one could speculate that the different AMPA receptor complexes are targeted to different synaptic populations. In support of this, Lerma et al. (1994) reported a greater density of calcium-permeable AMPA receptors on dendrites of hippocampal pyramidal neurons compared with cell bodies, suggesting a differential targeting of receptor complexes. However, a study by Spruston et al. (1995) on the same type of neuron did not show differences between receptors on the soma and on dendrites. In addition, if neurons contain multiple different receptor complexes, it is possible that some populations of receptors are not expressed at the synapse and remain intracellular; a large pool of intracellular AMPA receptors is found through immunocytochemical studies, and this may represent receptor complexes being transported to and from the synapse (Petralia and Wenthold, 1992; Martin et al., 1993; Vickers et al., 1993). The mechanism of regulation of this intracellular pool of receptors and many additional aspects concerning the regulation of glutamate receptors at the cellular level, including their assembly, intracellular targeting, and synaptic expression, remain to be studied.

REFERENCES

Bernard C, Wheal HV (1994) Model of local connectivity patterns in CA3 and CA1 areas of the hippocampus. Hippocampus 4:497–529.

Blackstone CD, Moss SJ, Martin LJ, Levey AI, Price DL, Huganir RL (1992) Biochemical characterization and localization of a non-NMDA glutamate receptor in rat brain. J Neurochem 58:1118–1126.

Bochet P, Audinat E, Lambolez B, Crépel F, Rossier J, Iino M, Tsuzuki K, Ozawa S (1994) Subunit composition at the single-cell level explains functional properties of a glutamate-gated channel. Neuron 12:383–388.

Boss BD, Turlejski K, Stanfield BB, Cowan WM (1987) On the numbers of neurons in fields CA1 and CA3 of the hippocampus of Sprague–Dawley and Wistar rats. Brain Res 406:280–287.

Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S (1990) Molecular cloning and functional expression of glutamate receptor genes. Science 249:1033–1037.

Burnashev N, Khodorova A, Jonas P, Helm PJ, Wisden W, Monyer H, Seeburg PH, Sakmann B (1992) Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. Science 256:1566–1570.

Conroy WG, Berg DK (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. J Biol Chem 270:4424–4431.

Conroy WG, Vernallis AB, Berg DK (1992) The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive subtypes in brain. Neuron 9:679–691.

Forsayeth JR, Gu Y, Hall ZW (1992) BiP forms stable complexes with unassembled subunits of the acetylcholine receptor in transfected COS cells and in C2 muscle cells. J Cell Biol 117:841–847.

Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Möhler H (1992) Five subtypes of type A γ-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. Proc Natl Acad Sci USA 89:6726–6730.

Frydman J, Nimmesgern E, Ohtsuka K, Hartl FU (1994) Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. Nature 370:111–117.

Furuyama T, Kiyama H, Sato K, Park HT, Maeno H, Takagi H, Tohyama M (1993) Region-specific expression of subunits of ionotropic glutamate receptors (AMPA-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociception. Mol Brain Res 18:141–151.

Gallo V, Upson JM, Hayes WP, Vyklicky LJ, Winters CA, Buonanno A (1992) Molecular cloning and developmental analysis of a new glutamate receptor subunit isoform in cerebellum. J Neurosci 12:1010–1023.

Geiger JRP, Melcher T, Koh D-S, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron 15:193–204.

Gelman MS, Chang W, Thomas DY, Bergeron JJM, Prives JM (1995) Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of nicotinic acetylcholine receptors. J Biol Chem 270:15085–15092.

Hammond C, Helenius A (1993) A chaperone with a sweet tooth. Curr Biol 3:884–886.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31–108.

Hollmann M, O'Shea-Greenfield A, Rogers SW, Heinemann S (1989) Cloning by functional expression of a member of the glutamate receptor family. Nature 342:643–648.

Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} channels of KA-AMPA-gated glutamate receptor channels depends on subunit composition. Science 252:851–853.

Hunter C, Wheaton KD, Wenthold RJ (1990) Solubilization and partial purification of an AMPA binding protein from rat brain. J Neurochem 54:118–125.

Hurtley SM, Helenius A (1989) Protein oligomerization in the endoplasmic reticulum. Annu Rev Cell Biol 5:277–307.

Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H (1994) Differences in Ca^{2+} permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. Neuron 12:1281–1289.

Keinänen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. Science 249:556–560.

Lambolez B, Audinat E, Bochet P, Crépel F, Rossier J (1992) AMPA receptor subunits expressed by single Purkinje cells. Neuron 9:247–258.

Laurie DJ, Seeburg PH, Wisden W (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J Neurosci 12:1063–1076.

Lerma J, Morales M, Ibarz JM, Somohano F (1994) Rectification properties and Ca^{2+} permeability of glutamate receptor channels in hippocampal cells. Eur J Neurosci 6:1080–1088.

Martin LJ, Blackstone CD, Levey AI, Huganir RL, Price DL (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. Neuroscience 53:327–358.

Monyer H, Seeburg PH, Wisden W (1991) Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. Neuron 6:799–810.

Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppersberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. Science 266:1059–1062.

Müller T, Möller T, Berger T, Schnitzer J, Kettenmann H (1992) Calcium entry through kainate receptors and resulting potassium-channel blockage in Bergmann glial cells. Science 256:1563–1566.

Nakanishi N, Shneider NA, Axel R (1990) A family of glutamate receptor genes: evidence for the formation of heteromultimeric receptors with distinct channel properties. Neuron 5:569–581.

Ozawa S, Iino M (1993) Two distinct types of AMPA responses in cultured rat hippocampal neurons. Neurosci Lett 155:187–190.

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates, 2nd Ed. New York: Academic.

Pellegrini-Giampietro DE, Zukin RS, Bennett MVL, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. Proc Natl Acad Sci USA 89:10499–10503.

Petralia RS, Wenthold RJ (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. J Comp Neurol 318:329–354.

Sato K, Kiyama H, Tohyama M (1993) The differential expression patterns of messenger RNAs encoding non-*N*-methyl-D-aspartate glutamate receptor subunits (GluR1–4) in the rat brain. Neuroscience 52:515–539.

Sekiguchi M, Doi K, Zhu W-S, Watase K, Yokotani N, Wada K, Wenthold RJ (1994) A deletion in the second cytoplasmic loop of GluR3 pro-

duces a dominant negative mutant of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor. J Biol Chem 269:14559–14565.
Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Köhler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science 249:1580–1585.
Spruston N, Jonas P, Sakmann B (1995) Dendritic glutamate receptor channels in rat hippocampal CA3 and CA1 pyramidal neurons. J Physiol (Lond) 482:325–352.
Tachibana M, Wenthold RJ, Morioka H, Petralia RS (1994) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat spinal cord. J Comp Neurol 344:431–454.
Tölle TR, Berthele A, Zieglgänsberger W, Seeburg PH, Wisden W (1993) The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. J Neurosci 13:5009–5028.
Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. Science 252:1715–1718.
Vernallis AB, Conroy WG, Berg DK (1993) Neurons assemble acetylcholine receptor with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. Neuron 10:451–464.
Vickers JC, Huntley GW, Edwards AM, Moran T, Rogers SW, Heinemann SF, Morrison JH (1993) Quantitative localization of AMPA/kainate and kainate glutamate receptor subunit immunoreactivity in neurochemically identified subpopulations of neurons in the prefrontal cortex of the macaque monkey. J Neurosci 13:2982–2992.
Wenthold RJ, Yokotani N, Doi K, Wada K (1992) Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies: evidence for a hetero-oligomeric structure in rat brain. J Biol Chem 267:501–507.
Wenthold RJ, Trumpy VA, Zhu W-S, Petralia RS (1994) Biochemical and assembly properties of GluR6 and KA2, two members of the kainate receptor family, determined with subunit-specific antibodies. J Biol Chem 269:1332–1339.
Zhang D, Sucher NJ, Lipton SA (1995) Co-expression of AMPA/kainate receptor-operated channels with high and low Ca^{2+} permeability in single rat retinal ganglion cells. Neuroscience 67:177–188.