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Selective Expression of Heteromeric AMPA Receptors Driven by Flip–Flop Differences

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Initial models of AMPA receptor assembly postulated the unrestricted stochastic association of individual subunits. The low Ca^{2+} permeability and nonrectified current–voltage relationship of most native AMPA receptors were ascribed to dominant effects of the glutamate receptor 2 (GluR2) subunit. A recent model, however, proposes instead the preferred assembly of GluR1 and GluR2 subunits into tetrameric complexes as pairs of identical heteromeric dimers. To compare unrestricted versus selective models of GluR1 and GluR2 assembly, these subunits, in both flip and flop isoforms, were expressed in varying ratios in human embryonic kidney 293 cells.

Coexpression of pairs of wild-type subunits produced expression of a predominance of heteromeric over homomeric receptors. Only a single functional type of heteromeric receptor was observed, indicating a pattern of apparent dominance not only of GluR2 for ion selectivity, but also of the flip isoform for receptor desensitization. Expression of wild-type GluR1 flip, however, with a mutant form of the same subunit carrying an arginine residue at the glutamine/arginine site ($GluR1^R$ flip) demonstrated a lack of dominance of $GluR1^R$ in determination of ion selectivity, whereas expression of $GluR1^R$ flip with GluR1 flop reproduced the pattern of apparent complete dominance. Together, the data support the selective expression of heteromeric receptors and are compatible with an equilibrium model of assembly of tetramers as pairs of identical heteromeric dimers. Expression of co-assemblies of the flip and flop isoforms, like that of the GluR1 and GluR2 subunits, is strongly favored over that of homomeric assemblies.

Key words: **AMPA; assembly; desensitization; permeability; receptor; glutamate; calcium; Ca**

Introduction

The properties of AMPA receptors have important implications for synaptic physiology and for pathophysiological roles played by these ligand-gated ion channels. Early studies of cloned AMPA receptor subunits revealed the key role played by the glutamate receptor 2 (GluR2) subunit, and by a single arginine residue at the "glutamine/arginine (Q/R) site," in determining ion selectivity and rectification of the channel (Hollmann et al., 1991; Hume et al., 1991; Verdoorn et al., 1991). Most evidence was interpreted as favoring a dominant role for GluR2 in the properties of AMPA receptors, with a single copy of GluR2 sufficient to produce receptor assemblies with low divalent cation permeability and near-linear rectification (Burnashev et al., 1992; Washburn et al., 1997). Furthermore, AMPA receptor subunits expressed in heterologous systems appeared to be indiscriminate in their co-assembly, with each of the subunits capable of forming homomeric assemblies and seemingly any pairing of subunits compatible with the formation of functional heteromeric receptors (Keinanen et al., 1990; Hollmann et al., 1991). Several electrophysiological studies failed to find evidence for selective assembly of AMPA receptors, instead reporting findings compatible with an unrestricted stochastic association of individual subunits producing AMPA receptors of variable stoichiometry (Geiger et al., 1995; Washburn et al., 1997).

In contrast to this support for a seemingly indeterminate assembly process, physiological evidence suggests the importance of the specific subunit composition of individual receptors. For example, in hippocampal interneurons, AMPA receptors of high or low Ca^{2+} permeability, and thus of different subunit composition, segregate to different synapses (Tóth and McBain, 1998). Also, GluR1 mediates insertion of heteromeric GluR1–GluR2 AMPA receptors into excitatory synapses during potentiation, whereas GluR2–GluR3 assemblies serve to maintain synaptic strength (Hayashi et al., 2000; Shi et al., 2001). Thus questions have persisted about whether all combinatorial possibilities of subunit stoichiometries contribute functional AMPA receptor assemblies and whether receptors with intermediate physiological properties of ion selectivity or rectification might be found.

Simplified systems of heterologous expression of AMPA subunits can help answer such questions. The tetrameric structure of AMPA receptors was established by expression in oocytes (Mano and Teichberg, 1998) and in human embryonic kidney 293 (HEK293) cells (Rosenmund et al., 1998). More recently, elegant work from the Rosenmund laboratory using coexpression of native and mutant versions of GluR1 and GluR2 has presented evidence for selective expression of certain heteromeric assemblies

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of these two subunits (Mansour et al., 2001). The evidence supported preferential formation of heteromeric tetramers as dimers of identical heteromeric dimers, each dimer containing one GluR1 and one GluR2 subunit. Functional complexes with odd numbers of GluR2 subunits were excluded in this model, and homomeric assemblies were relatively underrepresented. In addition, inclusion of a single subunit bearing the arginine ("R") residue at the Q/R site did not appear to suppress current rectification, contrasting with the dominant effect inferred previously. Ion selectivity was not examined directly but is expected to parallel rectification properties.

In native neurons expressing mixtures of Ca^{2+}-impermeable and Ca^{2+}-permeable AMPA subunits, we and others have presented evidence apparently consistent with stochastic subunit assembly and with a strictly dominant role for GluR2 in suppressing divalent cation permeability (Geiger et al., 1995; Washburn et al., 1997; Brorson et al., 1999). In light of the results cited above, however, it is worthwhile to reexamine the determination of ion selectivity of AMPA receptors by GluR2 expression. Are there multiple types of heteromeric receptor assemblies, some with intermediate phenotypes of divalent cation permeability, or instead are AMPA receptors expressed only as heteromers of specific subunit stoichiometry and order? We sought to examine these questions using expression of GluR1 and GluR2 in HEK293 cells.

Materials and Methods

Plasmids. Plasmids containing the GluR1 flop (GluR-A flop) and GluR2 flip (GluR-B flip) cDNAs in the pRK5 expression vector under the cytomegalovirus (CMV) promoter, used in the first set of experiments, were kind gifts from Dr. Doris Patneau (Oklahoma State University, Tulsa, OK) and used with the permission of Dr. Peter Seeburg (Max-Planck-Institute for Medical Research, Heidelberg, Germany). Plasmids containing cDNAs for GluR1 flop and GluR1 flip contained in the pcDNA 1 expression vector under the CMV promoter, used in all subsequent experiments, were kind gifts of Dr. Yael Stern-Bach (Hebrew University-Hadassah School of Dental Medicine, Jerusalem, Israel). The mutant subunit GluR1 flip Q600R ($GluR1^R$ flip) was produced using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), using the following oligonucleotides directed at the Q/R site: forward, 5′-CCCTGGGGGCCTTCATGCGGCAAGGATGTGAC-3′; and reverse, 5′-GTCACATCCTTGCCGCATGAAGGCCCCCAGGG-3′. The mutant plasmid as well as wild-type GluR1 flip and flop plasmids were sequenced by the University of Chicago Cancer Research Center DNA sequencing facility to ensure correctness, using sequencing primers spanning the flip–flop region as well as the Q/R site: forward 5′-AGG GAC GAG ACC AGA CAA CCA G-3′ and reverse 5′-TCG TAC CAC CAT TTG TTT TTC A-3′. Plasmid DNA was amplified in DH5α-competent cells, purified using the Qiagen Plasmid Maxi Kit (Qiagen Sciences, Valencia, CA), and quantified by absorption spectrophotometry. Restriction endonuclease digestion was used to confirm identification of plasmids.

Transfection of AMPA receptor subunits. HEK293 cells (American Type Culture Collection CRL-1573) were grown in DMEM with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin at 37°C in 5% CO_2 (all from Invitrogen, Carlsbad, CA). The growth medium was replenished every 3 d. Before transfection, HEK293 cells were trypsinized and replated into 100 mm culture dishes at a density of $\sim 6–7 \times 10^5$ cells per dish. Transient transfection used a polycationic detergent (Lipofectamine/Plus; Invitrogen), according to the manufacturer's protocol. When nearly confluent, cells were washed twice with DMEM without FBS to completely remove serum. Each transfected plate received a total of 4 μg of the plasmid DNA carrying glutamate receptor cDNAs, divided between two subunits in varying ratios (1:0, 4:1, 1:1, 1:4, and 0:1), as well as 2 μg per plate of the green fluorescent protein (GFP) expression plasmid pEGFP-C1. Under those conditions, subconfluent HEK293 cells were transfected with an efficiency of 40%, as revealed by green fluorescence. After transfection, transfected cells were washed with DMEM, trypsinized, and replated on 15 mm glass coverslips (1×10^4 cells per coverslip) for electrophysiology and on 100 mm dishes for protein harvesting. The cells were grown in DMEM with 10% FBS and treated with 10 μM 6,7-dinitroquinoxaline-2,3-dione. After 1–4 d of transfection, cells were selected for patch clamping on the basis of a healthy appearance, physical separation from neighboring cells, and detection of green fluorescence under illumination at 480 nm.

Western blotting. Transfected cells were harvested with harvest buffer (1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 100 μg/ml aprotinin in PBS), spun at 13,000 rpm for 33 min at 4°C, and sonicated for 5 min. The pellets were resuspended in solubilization buffer (harvest buffer with 1% Triton X-100), mixed for 30 min, and repelleted by centrifugation at 13,000 rpm for 33 min at 4°C. The supernatants were transferred to Eppendorf tubes. Protein concentrations were measured by DC protein assay (Bio-Rad, Hercules, CA). From each sample, 5 μg of protein was separated by 8% SDS-PAGE and transferred to an Immobilon-P polyvinylidene fluoride microporous membrane (Amersham Biosciences, Arlington Heights, IL). The membrane was blocked with Tris-buffered saline (TBS-T; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6, 0.1% Tween 20) containing 5% defatted milk powder at 4°C for 24 hr, followed by overnight incubation at 4°C with a polyclonal primary antibody raised against the C-terminal peptide of GluR1 (Upstate Biotechnology, Lake Placid, NY) diluted 1:2000 in TBS-T with 3% defatted milk powder. Blots were washed three times with TBS-T, incubated with peroxidase-conjugated anti-rabbit secondary antibody in TBS-T for 2 hr at room temperature, rinsed three times with TBS-T, and developed using ECL Western blotting reagents (Amersham Biosciences). Blots were digitally imaged by the Fluorchem 8800 Digital Imaging System (Alpha Innotech, San Leandro, CA), with adjustment of exposure time to strictly avoid digital signal saturation. Band intensity was quantified as peak area (net of background signal) in line scans of each lane, using ImageQuant software (Molecular Dynamics, Eugene, OR).

Whole-cell patch-clamp recordings. Whole-cell voltage-clamp recordings of ligand-gated currents were performed as described previously (Brorson et al., 1999). Borosilicate glass pipettes were of a resistance of 1.8–5 MΩ. Two intracellular solutions were used. The first, used for the initial studies of coexpression of GluR1 flop and GluR2 flip, consisted of 120 mM CsF, 3 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (pH adjusted to 7.25 with 12 mM CsOH); for all subsequent recordings, a solution consisting of CsCl 120 mM, EGTA 11 mM, $CaCl_2$ 1 mM, $MgCl_2$ 2 mM, HEPES 10 mM, pH adjusted to 7.2 with CsOH (total $[Cs^+] \sim 155$ mM) was used. Seal formation was performed in a buffer containing 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH = 7.40 with NaOH. Cells were accepted for study if a stable seal formed with a whole-cell resistance of at least 120 MΩ and access resistance of <10 MΩ.

For determination of AMPA receptor Ca^{2+} permeability, glutamate was applied via a solenoid valve-controlled theta tube applicator, in Na^+-free extracellular solutions containing either 15 or 50 mM Ca^{2+}, to which cyclothiazide (50–100 μM) was added to inhibit desensitization. The 15 mM Ca^{2+} solution consisted of 12.8 mM $CaCl_2$, 2.2 mM $Ca(OH)_2$, 10 mM glucose, 10 mM HEPES, and 240 mM sucrose; pH was adjusted to 7.4. The 50 mM Ca^{2+} solution consisted of 47.8 mM $CaCl_2$, 2.2 mM $Ca(OH)_2$, 10 mM glucose, 10 mM HEPES, and 147 mM sucrose; pH was adjusted to 7.4. Extracellular solutions were supplemented with Cd^{2+} (100 μM) to ensure that no contribution was possible from voltage-gated Ca^{2+} channels. For measures of current rectification, glutamate was applied in a 20 mM Na^+ solution consisting of 15.3 mM NaCl, 4.7 mM NaOH, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 228 mM sucrose, with pH adjusted to 7.4 with HCl, and with tetrodotoxin (0.5 μM) added.

Cells were held at a membrane potential of -50 or -80 mV, and current–voltage (I–V) relationships were recorded. Only cells with detectable transient glutamate-evoked currents were included. Leak current before agonist application was subtracted from agonist-evoked peak or steady-state currents at each potential. Reversal potentials in 15 or 50

mM Ca^{2+} solutions were determined from fit of net peak currents plotted against holding potential. The permeability ratio P_{Ca2+}/P_{Cs+} was estimated as described previously (Brorson et al., 1999). Desensitization was calculated as the difference between the peak current and the current remaining at 800 msec after peak, divided by the peak current. In receptors including flip isoforms, cyclothiazide-resistant desensitization was found to decrease to a low steady-state value over the first several minutes of whole-cell recordings, so only late measures of desensitization, after at least 4 min in the whole-cell mode, were included for analysis. Rectification was expressed as the ratio of currents at +10 mV to those at −80 mV, measured in 20 mM Na^+ solution.

Data analysis. *I–V* relationships for each cell were normalized by dividing by the difference between evoked currents at 0 and −60 mV, thus producing a unitary chord conductance between these potentials. Mean *I–V* curves, averaged from these normalized data to eliminate differences in current magnitude attributable to cell size or transfection efficiency, were calculated for each subunit transfection ratio. In each data set, a prototype "type I" *I–V* curve was taken as the average of normalized measured *I–V* curves in cells transfected with a 4:1 predominance of GluR2 (or GluR1^R), and a "type II" curve was similarly taken from cells transfected with wild-type GluR1 alone. These type I and type II curves were then added in various proportions to generate look-up tables relating net reversal potential to fraction type II current, thus allowing conversion of the measured reversal potential in each cell to the fractions of type I and type II currents required to produce such a composite reversal potential. For the data of Figure 2, in each cell the desensitizing current at −60 mV was used to independently estimate the contribution of type II current. This amplitude was applied as a multiplier to the normalized type II *I–V* curve and subtracted from the total *I–V* curve in each 4:1 cell to produce the residual *I–V* curves displayed in Figure 2*C*. For desensitization data (see Fig. 3), measured desensitization was converted to type I and type II fractions by assigning the nondesensitizing, flip-dominated phenotype to type I receptors and the desensitizing phenotype of flop homomers to type II receptors. Equal mean unitary conductances for all GluR1 flip–GluR1 flop assemblies were assumed.

Predictions of the various assembly models were compared with fractional type II current contributions in each data set. The predicted proportions of each specific receptor assembly were weighted by the estimated relative time-averaged unitary conductance appropriate for such a channel and summed to either the type I or type II contributions, as assigned by the model assumptions. The relative time-averaged unitary conductances of GluR1–GluR2 or of GluR1–GluR1^R heteromers, compared with GluR1 homomers, were assumed to be 0.8, and those of GluR2 homomers or GluR1^R homomers were assumed to be 0.05, as in Mansour et al. (2001). Greater differences in unitary mean conductance, as may be suggested by the reported large single-channel conductance of type II receptors (Swanson et al., 1997), would lead to even greater relative contributions from any GluR1 homomers present and thus would require even greater selectivity for heteromeric assembly to account for the data.

The stochastic model of subunit assembly has been described previously (Brorson et al., 1999). Predicted proportions of receptor assemblies were weighted by mean unitary conductances and summed according to the assumption that only GluR1(Q) homomers produce type II current ("dominance" of the R form). "Model 2" of Mansour et al. (2001) assumed the same fourth-order binomial distribution for receptor assemblies but assumed that both assemblies with zero or one R form subunit (homomeric and 1:3 stoichiometries) had type II properties. For application of "Model 4" of Mansour et al. (2001), which postulates selective heteromeric assembly restricted to symmetric 2:2 heteromers, GluR1 homomers, GluR1–GluR2 heteromers, and GluR2 homomers were presumed to be represented in numerical fractions predicted by p^3, $3p^2(1-p) + 3p(1-p)^2$, and $(1-p)^3$, respectively, with p defined as fraction of GluR1 transfected (see Fig. 6*B*). The equilibrium models presented here were applied similarly. Numerical fractions of receptor assemblies of each stoichiometry, as predicted by the model, were weighted by appropriate mean unitary conductances and summed to the total type I or type II current according to the assumptions of the individual model (see supplemental data, available at www.jneurosci.org). For fitting of the data according to Equation 2 or 3, the resulting predicted type II fractions were fit for each data set by a nonlinear least-squares regression protocol (SigmaPlot 5.0, Jandel Scientific Software, San Rafael, CA). In the case of GluR1 flip–GluR1 flop coexpression, identical mean unitary conductances were assumed, and equal formation of flip and flop homomers was assumed ($T = 1$), applying Equation 3 with one free parameter to fit the data. A similar fit was obtained if T was allowed to vary as a second free parameter (data not shown). Statistical testing was performed with SigmaStat (Jandel Scientific Software). Curves of data versus fraction of subunit were compared by two-way ANOVA, followed by Student–Newman–Keuls *post hoc* testing. Data variances were noticeably less when a single subunit was transfected alone (1:0 cells), so these values were excluded from the statistical evaluation, which assumed equal variance.

Materials. Cyclothiazide was the gift of Eli Lilly Corporation (Indianapolis, IN). Other chemicals were obtained from Sigma (St. Louis, MO).

Results

Coexpression of GluR1 flop and GluR2 flip produces only two types of receptors

We examined whole-cell AMPA receptor responses in HEK293 cells transfected with the flop isoform of GluR1 and the flip isoform of GluR2 in varying proportions from 1:0 to 4:1. GFP expression was used to identify successfully transfected cells. Glutamate (500 μM) was applied rapidly in the presence of cyclothiazide, which prevents or slows desensitization of AMPA receptors (Partin et al., 1994), allowing detection of peak evoked currents at the whole-cell level. To sensitively detect changes in divalent cation permeability, currents were measured in Ca^{2+}-based, Na^+-free external solutions.

In receptors forming from this combination of subunits, three physiological features, divalent cation permeability, rectification, and desensitization, each clearly distinguished AMPA receptors formed from homomeric GluR1 flop from those receptors in which GluR2 flip predominated (Fig. 1). Homomeric GluR1 flop receptors showed strong inward current rectification and relatively positive reversal potentials, indicating high Ca^{2+} permeability. The inward currents completely desensitized during 1 sec applications of glutamate, consistent with previous reports of the effects of cyclothiazide on pure flop isoforms (Partin et al., 1994). Inward rectification and high Ca^{2+} permeability are typical features of type II AMPA receptors, as described originally by Iino et al. (1990). In contrast, when GluR1 flop and GluR2 flip cDNAs were expressed in a 1:4 transfection ratio, the currents evoked by glutamate and cyclothiazide showed very little desensitization. The currents were slightly outwardly rectifying (because of the high internal Cs^+ concentration) and had uniform left-shifted reversal potentials consistent with a low channel divalent cation permeability, features typical of type I AMPA receptors (Iino et al., 1990; Ozawa et al., 1991). Expression of GluR2 flip alone produced only very small evoked currents (data not shown), making comparable analysis impossible.

GluR1 flop–GluR2 flip cDNAs expressed in a 1:1 ratio produced current traces and current–voltage curves that were indistinguishable from those in cells transfected with 1:4 ratios of subunit cDNAs, with low reversal potentials and low estimated Ca^{2+} permeability (Fig. 1). Thus, these cells had no detectable contributions of Ca^{2+}-permeable, type II currents, despite presumably expressing all possible receptor assemblies containing both GluR1 and GluR2 subunits. This implies that if heteromeric assemblies of varying stoichiometry (i.e., 1:3 and 3:1 as well as 2:2) are readily expressed, they all must have very similar type I properties. Similar observations have been taken as evidence for a strongly dominant effect of GluR2 subunits in controlling ion

permeability (Burnashev et al., 1992), in which inclusion of a single GluR2 subunit is sufficient to fully suppress AMPA receptor Ca^{2+} permeability and inward rectification.

When GluR1 and GluR2 were cotransfected in a 4:1 ratio, the resulting whole-cell current traces showed properties intermediate between those of type I and type II receptors. Partial desensitization of inward currents could be seen at negative potentials, with outwardly directed nondesensitizing currents at higher membrane potentials. Current reversal potentials were intermediate between those of type I and type II receptors, consistent with a modest average channel permeability to Ca^{2+}. Interestingly, biphasic currents, with separate contributions of desensitizing inwardly rectified currents and outward nondesensitizing currents, could be seen within individual traces at the intermediate potentials, directly demonstrating the presence of at least two channel types with different reversal potentials.

In these composite current traces, from cells transfected with a 4:1 ratio of subunit cDNA ("4:1 cells"), the desensitizing feature of GluR1 flop homomers provided an independent marker of their contributions. This afforded the opportunity to ask whether the whole-cell currents produced when multiple receptor assemblies are expressed could be accounted for simply as the sum of type I and type II AMPA receptor currents. Individual current traces from 4:1 cells clearly showed time-dependent features that could be generated by linear combination of the type I currents (i.e., those from 1:4 cells) and the desensitizing type II currents from 1:0 cells at the same potentials (Fig. 2*A*). More generally, the shape of the entire current–voltage relationships could be produced if desensitizing type II receptors and nondesensitizing type I receptors were the only channel types present (Fig. 2*B*). For each cell expressing 4:1 mixtures of GluR1 flop–GluR2 flip, the contribution of type II channels to the whole-cell currents was taken as the magnitude of the desensitizing current evoked in 50 mM Ca^{2+} at −60 mV, a potential at which type I currents were minimal. This magnitude was used to scale an average normalized current–voltage relationship for type II currents generated by GluR1 flop homomers (Fig. 1*B*). When this contribution of type II current was subtracted from the total current at each potential, the resulting current–voltage relationships were indistinguishable in reversal potential and rectification from those found in the 1:4 and 1:1 cells, in which GluR2 effects dominated (Fig. 2*C*,*D*). Thus only two functional types of AMPA receptors were detectable in these cells, with desensitization properties segregating closely with the ion selectivity properties of type I and type II receptors. This suggests that only a single functional type of heteromeric AMPA receptors contributes measurably to whole-cell currents. If only a single functional heteromeric receptor is present, one interpretation could be that GluR2 and flip forms are both fully dominant in their determination of receptor properties, so that heteromeric receptors of all stoichiometries are functionally similar. Alternatively, it may be that only one type of heteromeric assembly is expressed.

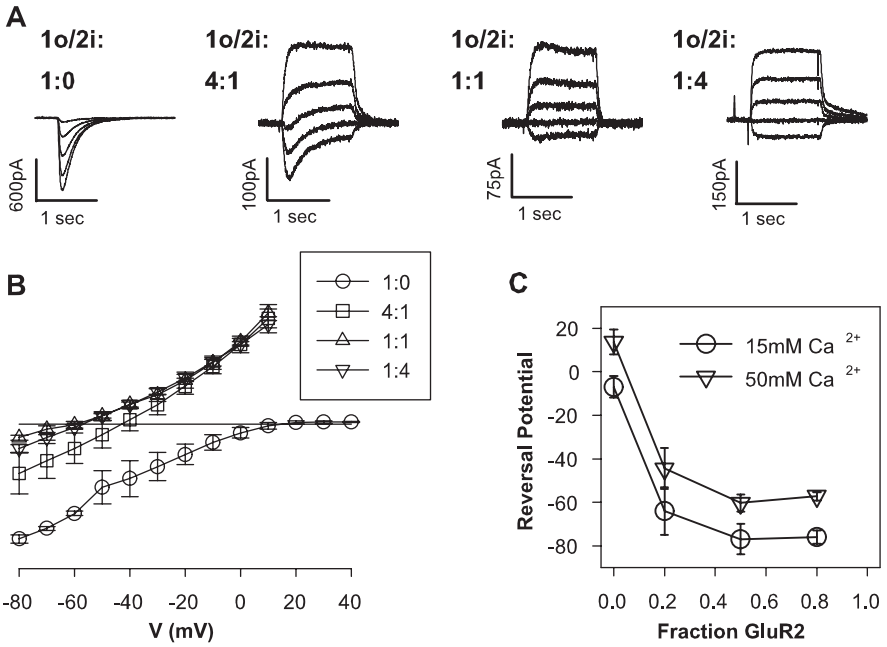

Figure 1. AMPA receptor responses in GluR1 flop–GluR2 flip transfected cells. In HEK293 cells transfected with cDNAs encoding the GluR1 flop (1o) and GluR2 flip (2i) subunits, whole-cell current responses to 1 sec applications of glutamate (500 μM) plus cyclothiazide (50 μM) were recorded at membrane potentials ranging from −110 to +40 mV. *A*, Current traces at −80–0 mV (20 mV intervals) in 50 mM Ca^{2+}, Na^{+}-free solutions, from representative cells for each cDNA ratio. *B*, Current–voltage curves, averaged from normalized data to eliminate cell-to-cell differences in current magnitudes attributable to cell size or transfection efficiency, produced in cells transfected with 1o/2i cDNAs in varying ratios. *C*, Reversal potentials versus fractional transfection of GluR2 cDNA. The overall P_{Ca2+}/P_{Cs+} permeability ratios, estimated from reversal potentials in 15 mM Ca^{2+}, were 4.7 ± 1.5, 0.29 ± 0.12, 0.19 ± 0.08, and 0.16 ± 0.02, respectively, in cells transfected with 1:0, 4:1, 1:1, and 1:4 ratios (mean ± SD; n = 6–7).

Is flip dominant?

The possibility that flip isoforms generally dominate in determining receptor desensitization properties could be quickly dismissed if it could be shown that flip isoforms fail to dominate over flop isoforms in determining desensitization properties of pure GluR1 assemblies. We coexpressed the flip and flop isoforms of GluR1 in varying ratios and measured the desensitization in the presence of cyclothiazide (Fig. 3). As expected, at all subunit isoform ratios the current–voltage relationships were inwardly rectifying and showed evidence of high Ca^{2+} permeability (data not shown). Desensitization varied from being nearly complete in pure GluR1 flop receptors to none in 100% GluR1 flip receptors. Surprisingly, rather than a gradual decrease in cyclothiazide-resistant desensitization with increasing fractions of the flip isoform, a pattern of apparent dominance of flip isoforms appeared. The alternative hypothesis offered above, that of restricted stoichiometry, could also explain these results only if flip and flop isoform differences, like the GluR1 and GluR2 subunit differences, are sufficient to drive preferential subunit association.

Is Ca^{2+} permeability dominantly determined in homomeric assemblies?

A direct test was needed of the dominance of the arginine (R) residue at the Q/R site of the GluR2 subunit in determining channel ion selectivity and rectification. We used GluR1 flip mutated at the Q/R site to encode for arginine ($GluR1^{R}$ flip). If assembly

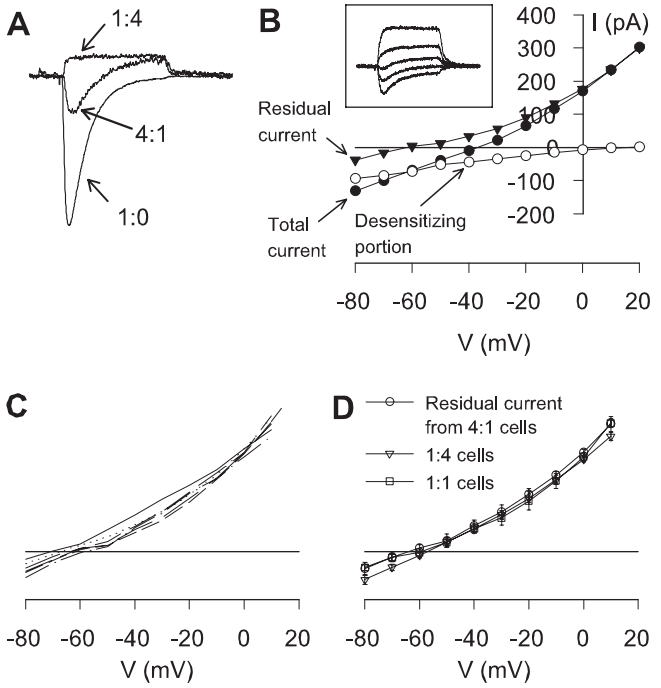

Figure 2. Analysis of mixed whole-cell currents into type I and type II contributions. *A*, Representative current traces at −50 mV from cells transfected with 1:0, 4:1, and 1:4 ratios of GluR1 flop–GluR2 flip cDNAs. The current trace from the 4:1 cell suggests components of both the desensitizing inward current seen in 1:0 cells and the nondesensitizing outward current typical of 1:4 cells. *B*, In a representative example of a cell transfected with GluR1 flop–GluR2 flip in a 4:1 ratio, the magnitude of desensitizing current was measured as 67 pA. Scaling the normalized type II current–voltage curve by this factor, and subtracting it from total peak currents, produced a residual current–voltage curve with features typical of type I AMPA receptors. *C*, Applying this analysis to all cells transfected with a 4:1 ratio of GluR1 flop–GluR2 flip resulted in residual currents with quite uniform rectification and ion selectivity properties. *D*, The mean residual currents from 4:1 cells after removal of desensitizing current portions were indistinguishable from the type I currents produced in cells transfected in 1:1 or 1:4 ratios, indicating that a simple sum of type I and type II currents could describe the composite currents observed in cells expressing a mosaic of receptor types (mean ± SD).

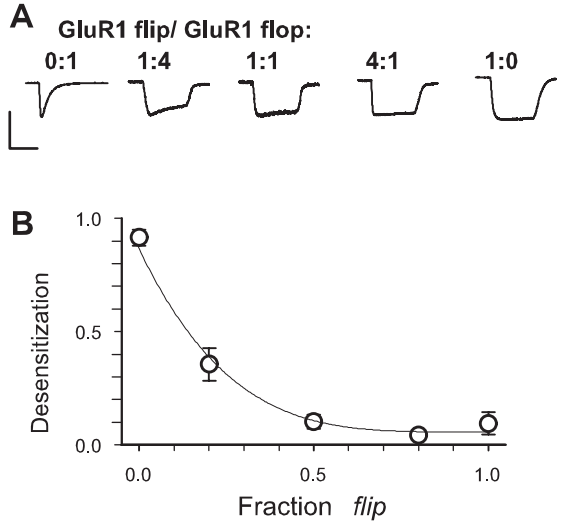

Figure 3. AMPA receptor responses in GluR1 flip–GluR1 flop cotransfected cells. Whole-cell current responses to 1 sec applications of glutamate (500 μM) plus cyclothiazide (100 μM) in cells transfected with cDNA encoding the GluR1 flop (1o) and GluR1 flip (1i) subunits, measured in 15 mM Ca^{2+} at −80 mV. *A*, Representative traces in cells transfected with various ratios of 1i/1o (calibration: horizontal, 1 sec; vertical, 800, 400, 200, 800, and 800 pA). *B*, Cyclothiazide-resistant desensitization occurring during the 1 sec glutamate applications for cells transfected with each cDNA ratio (mean ± SEM; $n = 3–8$). The curve displays the predictions of a stochastic assembly model, assuming dominance of the flip isoform.

and trafficking properties are unaltered by this point mutation in a single internal residue (but see Discussion), the homomeric association of Q and R forms of GluR1 flip will then occur on a stochastic basis, producing expression of all possible stoichiometries. If R forms completely dominate in determining ion selectivity so that only assemblies with all four subunits in the Q form have high Ca^{2+} permeability, such stochastic assembly predicts a sharply curved pattern of dependence of current reversal potential on the fraction of the R form (see below). We expressed the mutant GluR1^{R} flip subunit in varying ratios with the wild-type (GluR1^{Q} flip) subunit, using the functional effects as a marker for incorporation of GluR1^{R} flip subunits (Fig. 4). Reversal potentials were measured in Na^{+} free, 15 mM Ca^{2+}-based extracellular solutions to assess ion selectivity and rectification in a 20 mM Na^{+} solution. As expected, expression of wild-type GluR1$^{(Q)}$ flip alone produced inwardly rectifying currents with high reversal potential, indicating high Ca^{2+} permeability. Coexpression of GluR1^{R} flip in increasing fractions produced current–voltage relationships with gradually decreasing reversal potentials and rectification. In fact, a nearly linear dependence of reversal potential or rectification on the fraction of the R form was found. This gradual dependence of ion selectivity on the proportion of the R form that was expressed is inconsistent with stochastic assembly with a dominant effect of GluR1^{R} on ion selectivity. Instead, incomplete suppression of divalent cation permeability by a single copy of GluR1^{R} is suggested.

Previous work has suggested that the same holds for GluR2^{R} flip in homomeric combination with mutant GluR2^{Q} flip: a graded decrease in current rectification is seen with increasing fraction of GluR2^{R} (Mansour et al., 2001). These results imply that if assembly and trafficking of homomeric receptors occur on a stochastic basis, then the R form, carried by either GluR1 or GluR2, is not completely dominant in determining ion selectivity or rectification properties. Consequently, the results of Figures 1 and 2, preferential expression of a single functional type of heteromeric receptor, cannot result from stochastic assembly with dominant effects of GluR2 flip, but instead point to the preferential expression of GluR1–GluR2 heteromeric channels, all nondesensitizing and all of low Ca^{2+} permeability. The simple explanation may best be found in a model of restricted assembly of heteromers of a single stoichiometry, like that proposed by Mansour et al. (2001).

The observed apparent dominance of the flip isoform in determining the desensitization properties of GluR1 flip–GluR1 flop combinations remains to be explained. To test whether this pattern of "dominance" may also be caused by preferential assembly, in this case between flip and flop isoforms, we coexpressed GluR1^{R} flip with GluR1 flop. In direct contrast to the homomeric combination of pure flip forms of GluR1 subunits, the combination of flip with flop isoforms of GluR1 reproduced the apparent dominance of the R-carrying version, both in determining ion selectivity (reversal potential) and in rectification (Fig. 4). Thus, comparing these results, differences in the flip–flop region, located in an extracellular loop of AMPA subunits, are sufficient to produce the preferential expression of type I heteromeric channels. If not to be explained by a dominant effect of R subunit forms, this again may suggest a preferential formation of heteromeric assemblies of a single functional type, when flip and flop subunits are coexpressed. Both the apparent com-

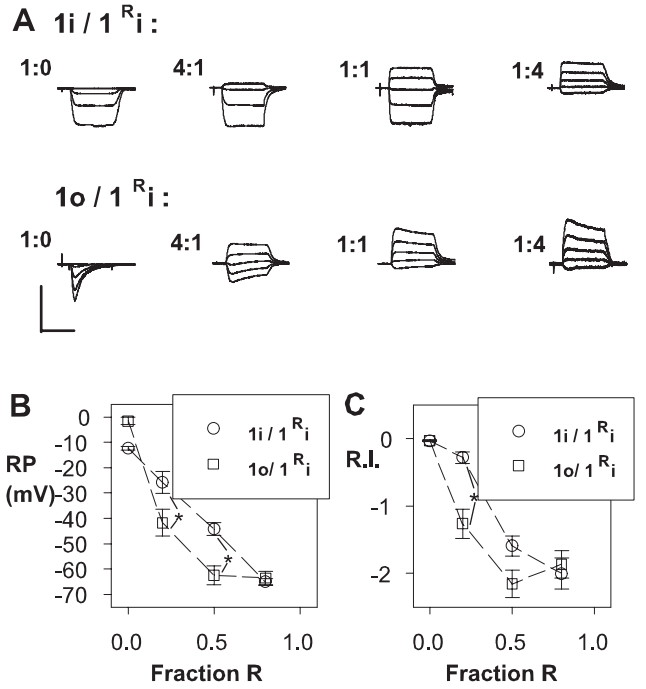

Figure 4. Cotransfection of GluR1^R flip with flip or flop GluR1 isoforms. The GluR1 flip subunit, mutated to carry the arginine residue at the Q/R site (1^{R}i) was coexpressed with wild-type versions of either the GluR1 flip or GluR1 flop subunits. *A*, Representative current–voltage sets (at membrane potentials of −80 – 0 mV in 20 mV steps) in 15 mM Ca^{2+}, Na^+-free solutions for each subunit combination. Increasing fractions of the GluR1^R flip subunit produced progressive suppression of Ca^{2+} permeability and inward rectification. *B*, Relationships of average reversal potential to fraction of GluR1^R flip for each ratio were significantly different when coexpressed with GluR1 flip or GluR1 flop (mean ± SEM; $n = 6–7$). *C*, Rectification (current at +10 mV divided by that at −80 mV) versus fraction of GluR1^R flip subunit transfected (mean ± SEM; $n = 3–8$). Curves in *B* and *C* were significantly different by two-way ANOVA; $p < 0.05$. Asterisks mark points significantly different in *post hoc* analysis.

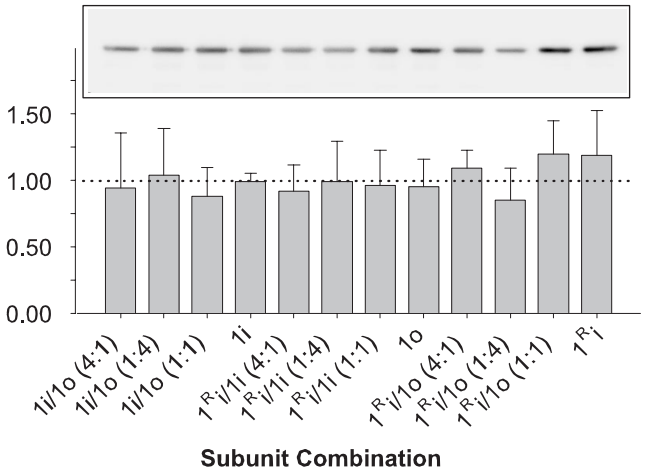

Figure 5. Western blotting for GluR1. The various GluR1 constructs, at all subunit ratios used, were transfected in parallel in HEK293 cells, and membrane proteins were harvested. Western blots for GluR1 revealed a single band of ~100 kDa in each case (inset). Normalized immunoreactivity (mean ± SD; $n = 3$) did not differ significantly among subunit combinations ($p = 0.896$; repeated-measures ANOVA).

plete dominance of flip for desensitization and the apparent complete dominance of arginine at the Q/R site for ion selectivity and rectification then may be explained by selective assembly and expression of subunits as heteromeric complexes.

One of the important assumptions required in interpreting the foregoing experiments is the presumption that proportional expression of subunit proteins approximates the cDNA transfection ratios. Western blots of samples from coexpression of the GluR1 flop and GluR2 flip constructs used for the first data set (Figs. 1, 2) indeed showed that relative GluR1 and GluR2 integrated band densities scaled with transfection ratios (data not shown). Inference of the actual ratios of protein expression from relative immunoreactivity using two different antibodies, however, is not reliable. An advantage of the subsequent data sets involving only GluR1 cDNA constructs was that the same antibody could be used to quantify total GluR1 protein expression from cells transfected with all subunit combinations and ratios (Fig. 5). Integrated band density from digitally imaged Western blots revealed no significant differences in average protein expression levels from any of the individual constructs or coexpression ratios, indicating that all GluR1 cDNA constructs produced protein expression with similar efficiency.

Comparing models of assembly

Although these results argue for preferential assembly of a single functional type of heteromeric receptor, they do not limit the stoichiometry of this receptor, nor do they quantify the degree of selectivity for expression of heteromers. Further insight requires quantitative comparison of such data with the possible models of assembly and expression.

An unrestricted, stochastic assembly process has been modeled in previous reports (Geiger et al., 1995; Washburn et al., 1997; Brorson et al., 1999). This model describes expression of assemblies of all stoichiometries, in proportions determined by the binomial distribution (Fig. 6*A*). If complete dominance of GluR2 for ion selectivity is also assumed, then the fraction of type II, Ca^{2+}-permeable current is simply the fourth power of the fraction of non-GluR2 subunits, weighted by the time-averaged relative unitary conductance of the type II channels (the "stochastic/dominant" model) (Fig. 6*C*). In contrast, the arguments above and the results of Mansour et al. (2001) suggest that in homomeric assembly, the R forms might not be dominant, but rather that two R subunits are required to suppress Ca^{2+} permeability and rectification. Mansour et al. (2001) postulated that receptors containing either one or two R subunits might have type II properties (Model 2) (Mansour et al.). This stochastic/nondominant model of homomeric assembly might apply to coexpression of nearly identical subunits like GluR1 flip with GluR1^R flip, with the mutant and wild-type subunits indistinguishable by the assembly process. In fact, the results of GluR1 flip–GluR1^R flip coexpression are fit fairly well by this stoichastic/nondominant model of homomeric assembly, but clearly are not fit by the dominant model (Fig. 6*C*). In contrast, the stochastic/dominant model approximates the results found with coexpression of GluR1 flop with GluR1^R flip, but substantially overestimates the reversal potentials produced from coexpression of GluR1 flop and GluR2 flip at intermediate transfection ratios (Fig. 6*C*). Finally, in describing restricted heteromeric assembly, Model 4 of Mansour et al. (2001) postulated formation of only specific heteromeric receptors, in relative proportions with homomeric assemblies as determined by a third-order polynomial (Fig. 6*B*). This model, with current contributions again weighted according to mean single-channel conductances, fits the data from GluR1 flop–GluR1^R flip coexpression moderately well but leads to a still higher overestimation of the fractional type II currents from coexpression of GluR1 flop and GluR2 flip at 4:1 and

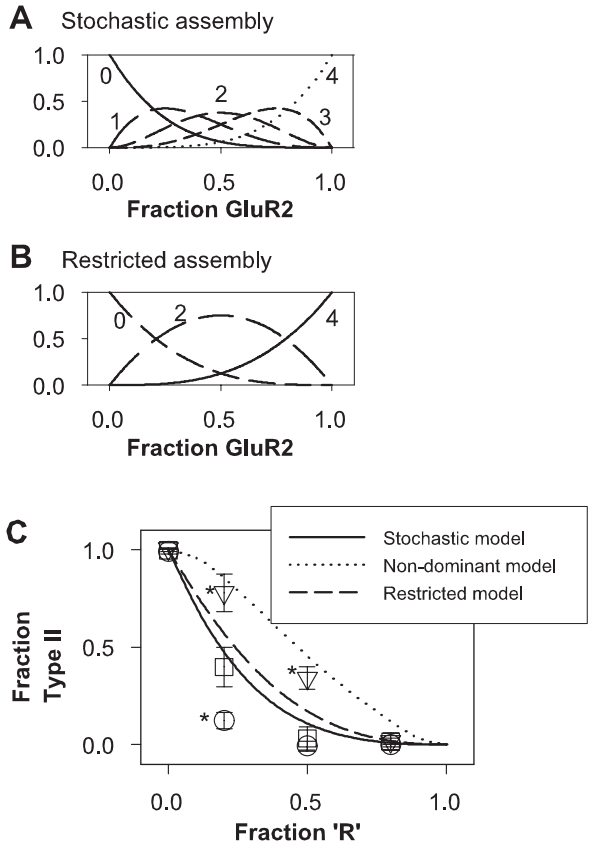

Figure 6. Comparison of stochastic and restricted assembly models. *A*, Fractions of receptors having zero, one, two, three, or four GluR2 subunits in a stochastic model of assembly, after the binomial distribution. *B*, Predicted fractions of receptors having zero, two, or four GluR2 subunits according to the restricted assembly model (Model 4) of the Rosenmund group (Mansour et al., 2001), with assembly of only symmetric 2:2 heteromers allowed. *C*, Comparisons of model predictions with data. Mean (± SEM) fractions of type II current were computed for each value of *q*, the fraction of transfected R form subunits from each data set. For cotransfections of GluR1 flop and GluR2 flip subunits (circles), GluR1 flop and GluR1^{R} flip subunits (squares), and GluR1 flip and GluR1^{R} flip subunits (triangles), fractional type II currents were derived from measured reversal potentials in 15 mM Ca^{2+} solutions (asterisk indicates significantly different from corresponding 1o/1^{R}i point in two-way ANOVA analysis; $p < 0.05$). Predictions of the stochastic– dominant assembly model, a stochastic, nondominant model (Model 2 of the Rosenmund group), and the restricted assembly model (Model 4) are shown.

1:1 transfection ratios (Fig. 6*C*). Thus, either the stochastic/dominant model or the restricted model for heteromeric receptor assembly may account for the pattern of receptor expression from coexpressions of GluR1 flop with GluR1^{R} flip. Neither model, however, appears to adequately account for the significantly greater degree of apparent dominance of type I receptor properties found when GluR1 flop and GluR2 flip, differing in both subunit and splice variant sequences, were coexpressed.

An equilibrium model of receptor assembly as a dimer of subunit dimers

If instead of stochastic association of subunits there is preferential assembly of heteromeric subunit dimers followed by restricted pairing of dimers into selected tetramers, a different model is needed. In the assembly of two different subunits, the direction of coupling between subunits may be important. Then there are 4 distinct dimers, and 10 possible tetrameric assemblies of dimers of dimers that can form (Fig. 7). If for heuristic purposes the simple assumption is made that a near-equilibrium holds within the assembly compartments between monomeric subunits and different types of dimers, and also between dimers and tetrameric "dimers of dimers," then relative proportions of different tetrameric assemblies can be related to proportions of monomeric subunits by the products of individual equilibrium constants (for details, see supplemental data). For "homomeric" assembly, in which two subunits A and B are indistinguishable with respect to the assembly process, it can be shown that this equilibrium assumption then leads to a simple prediction for the proportions of each of the possible heteromeric stoichiometries. Then when B bears the R form at the Q/R site, following the assumption of Model 2 of Mansour et al. (2001), the fraction of type II receptors may be taken as the fraction of receptors containing either zero or one copy of B, given by:

$$F_{II} = \frac{(1-q)^4 + 2(1-q)^3 q}{(1-q)^4 + 2(1-q)^3 q + 4(1-q)^2 q^2 + 2(1-q)q^3 + q^4}, \quad (1)$$

where q represents the fractional expression of subunit B.

For assembly between subunits A and B that may differ in their association affinities, a complex model with multiple free parameters results; however, if restricted assembly is introduced, as in the model of Mansour et al. (2001), with only twofold axially symmetric heteromeric assemblies allowed, then only the two homomeric assemblies and the two axially symmetric heteromeric assemblies AB·AB and BA·BA contribute (Fig. 7). Then the fractional expression of homomeric A_4 receptors is given by:

$$F_{A_4} = \frac{(1-q)^4}{(1-q)^4 + 2S(1-q)^2 q^2 + Tq^4}, \quad (2)$$

where S represents the average preference for assembly of either of the symmetric heteromers compared with that of A_4 homomers, and T represents the relative assembly of B compared with A homomers. Finally, when contributions of one type of homomer, for example the homomeric B_4 receptor, can be neglected (i.e., if it has negligible conductance), this simplifies to:

$$F_{A_4} \cong \frac{(1-q)^2}{(1-q)^2 + 2Sq^2}. \quad (3)$$

Selectivity of heteromeric assembly

These "equilibrium" models of assembly can be tested against the present data. For GluR2 or GluR1^{R}, the small unitary currents of homomeric channels (Swanson et al., 1997; Mansour et al., 2001) allow their contributions to whole-cell currents to be neglected. For restricted heteromeric assembly the simple inverse quadratic Equation 3 then applies for describing the dependence of the fraction of homomeric type II receptors on the proportion of R-containing subunits. In the case of the combination of GluR1 flip and GluR1 flop, both homomeric and heteromeric assemblies are expected to contribute to whole-cell currents, with similar unitary conductances, so that Equation 2 applies, with type II receptors taken to be the desensitizing fraction. In either case the parameter S provides a measure of the degree of preference for formation of heteromeric assemblies. A value of $S = 1$ indicates equal preference for heteromeric or homomeric assembly, whereas higher values are required to produce the apparent dominance of heteromeric assemblies.

Comparing the data from coexpression of each pair of subunits with these models, the calculated proportions of type II

current were fit by Equation 2 or 3 using a nonlinear least-squares regression algorithm, with *S* as the single free parameter. The resulting predicted curves fit the data with highly significant correlations (Fig. 8*A*). The values of *S*, representing the selectivity for heteromeric assembly of the nonidentical subunits, were significantly >1 for each heteromeric subunit combination, but not when, for comparison, the model of restricted assembly was applied to the data from the homomeric combination of GluR1^{R} flip–GluR1 flip (Fig. 8*B*). An unrestricted assembly model, however, like that of Equation 1, is more likely applicable to such a homomeric combination, and this model, without free parameters, also adequately matched the data set. Among the heteromeric subunit combinations, the selectivity for heteromeric assembly was quite high for all subunit combinations, but apparently more so when the subunits differed throughout their sequences than when the only differences were in the flip–flop region. This suggests possible independent effects of GluR1–GluR2 and flip–flop sequence differences in promoting heteromeric assembly.

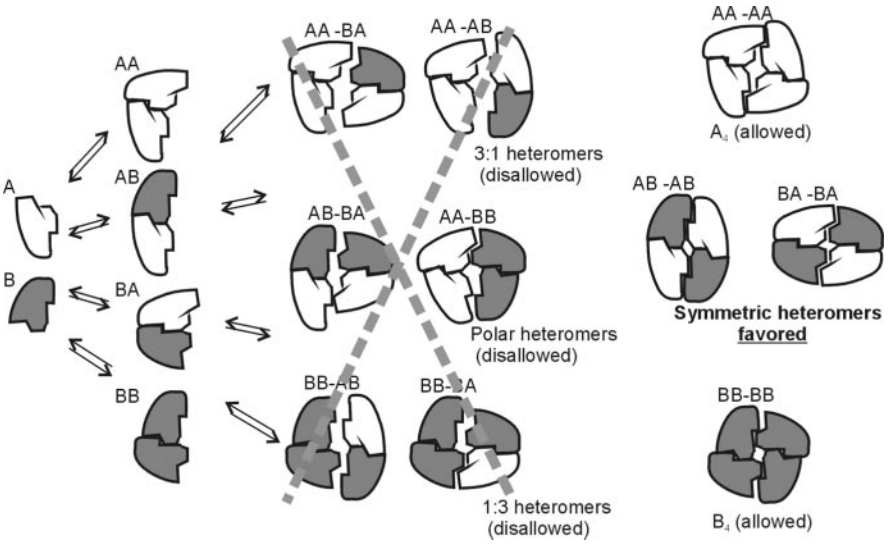

Figure 7. An equilibrium model of subunit assembly as a dimer of dimers. Any pair of different subunits can form 4 possible different dimers and 10 possible distinct tetrameric "dimers of dimers," assuming that bonding order is important and that subunits retain their dimeric bonds within the tetramers. Conformational features of subunit interfaces might promote formation of heteromeric dimers that selectively "fit" with identical dimers, but not with homomeric dimers or with dimers of reverse order.

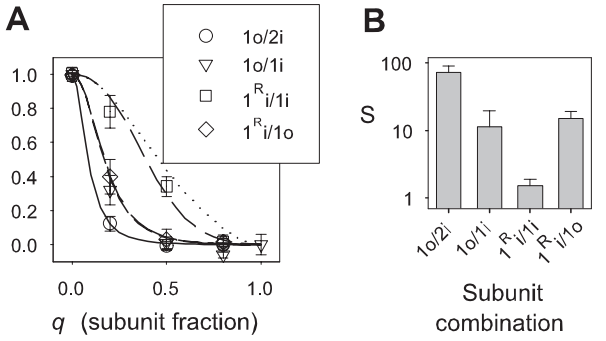

Figure 8. Testing the equilibrium model of subunit assembly. *A*, Fractions of type II currents as a function of subunit transfection fraction for each subunit combination (mean ± SEM). For cotransfection of GluR1 flip and GluR1 flop (1o/1i), the fraction of the nondesensitizing current phenotype is plotted. Results from each data set were fit by nonlinear regression according to an equilibrium model for subunit assembly (see text). All data sets were fit with highly significant correlation ($r = 0.90 - 0.99$; $p < 0.001$ in each case). Fitted curves are shown; results for the 1^{R}i/1o and 1o/1i data sets are indistinguishable. For comparison, the homomeric–nondominant equilibrium model of assembly (Eq. 1; see Results), applicable to the 1^{R}i/1i data, is also shown (dotted line). *B*, Estimates of the fitting parameter *S*, representing selectivity for expression of heteromeric over homomeric tetramers, for each subunit combination (mean ± SEM).

Discussion

Analysis of whole-cell currents generated from coexpression of GluR1 flop and GluR2 flip indicated that only two functionally distinct receptors are produced, those with strongly desensitizing, inwardly rectifying Ca^{2+}-permeable channels, attributable to GluR1 flop homomers, and nondesensitizing, Ca^{2+}-impermeable heteromeric channels. The dependence on a fraction of GluR2 flip of the relative proportion of these two channel types suggested an apparent dominant effect of GluR2 on ion selectivity and of the flip isoforms on cyclothiazide-resistant desensitization. Coexpression of GluR1 in flip and flop isoforms also suggested a possible dominant effect of flip on desensitization properties. It was only with expression of a purely homomeric combination of GluR1 flip, in two forms differing only in the single point mutation to arginine at the Q/R site, that this pattern of apparent dominance was absent. Substituting the flop isoforms for the flip isoforms of wild-type GluR1 in coexpression with GluR1^{R} flip was sufficient to restore the appearance of complete dominance of the R form for ion selectivity and rectification. Given that the R form is not actually dominant when unrestricted assembly occurs in expression of homomeric receptors, taken together these data strongly support the alternative possibility, the preferential expression of heteromeric receptors, with either the differences between GluR1 and GluR2 or those between flip and flop isoforms being sufficient to drive preferential assembly and surface expression.

In showing that expression of heteromeric assemblies of GluR1 and GluR2 is preferred, the present data confirm the findings of Mansour et al. (2001). They extend this finding by showing that flip and flop isoform differences are also sufficient to drive preferential expression of heteromeric receptors. The data are shown to be compatible with a novel quantitative model of assembly on the basis of the equilibrium association of subunits as dimers of dimers. This model incorporates the same assumption of restriction of heteromeric assembly to symmetric pairs of heteromeric dimers proposed by the Rosenmund group (Mansour et al., 2001). It differs, however, in mathematical form from the previous model, which involved a third-order polynomial derived from the binomial expansion. If assembly is not stochastic, it is not clear why the relative proportions of receptor assemblies should follow a distribution derived from a combinatorial analysis. Instead, subunit assembly will be determined by the relative affinities of protein–protein interactions in the assembly process in the ER, as well as by protein quality control and trafficking mechanisms and those determining receptor insertion and retention in the cell surface membrane. In the light of this complex cell biology of membrane protein processing, perhaps it is surprising that any simple quantitative model could be successful. The success of the model proposed here, derived from simple assumptions of equilibrium relationships between proteins and of surface expression paralleling assembly, clearly does not rule

out important roles for protein trafficking and receptor insertion–removal mechanisms in determining receptor expression patterns. Regardless, the qualitative analysis is strongly supportive of the selective expression of heteromers of restricted stoichiometry, and the parameter *S*, generated by the equilibrium model, provides a useful quantitative measure of this selectivity, whatever its biological basis.

The present model depicts restriction of heteromeric assembly to axially symmetric 2:2 heteromers (Fig. 7), following Mansour et al. (2001); however, nothing in the present data distinguishes between the formation of such "symmetric" tetramers and "polar" tetramers, with approximate mirror symmetry. Assembly restricted to either two symmetric tetramers or two polar tetramers would result in the same mathematical form of the relationship between proportional expression of heteromers and subunit proportion. Thus this model may be equally applicable to NMDA receptor subunit assembly, for which recent evidence from expression of tandem subunit constructs has supported the formation of polar tetramers (Schorge and Colquhoun, 2003) rather than ones with twofold rotational symmetry.

An implicit assumption in these studies is that monomeric subunit protein levels reliably reflect the cDNA transfection ratios. Care was taken to avoid differences in efficiency of transfection or translation, with coexpression of subunit pairs always undertaken with both cDNA constructs carried by the same plasmid under the same promoter. Western blots showed no signs of consistent protein overproduction from any particular cDNA constructs, as a possible alternative explanation for apparent dominance of R or flip subunit forms.

Another important consideration in interpreting these data is the possibility that certain monomeric subunits are selectively retained or exported from the endoplasmic reticulum (ER)-related compartments in which assembly occurs. Indeed, a recent report showed that GluR2 selectively accumulates in the ER and that this is dependent on the expression of arginine in the Q/R site (Greger et al., 2002). Furthermore, R subunits were shown to impede assembly and ER export at the level of tetramer formation, whereas the Q forms readily tetramerized and trafficked out of the ER (Greger et al., 2003). These findings possibly provide an alternative interpretation of the present data, explaining the preferential formation of GluR2-containing heteromers by the mass action of accumulated GluR2 protein even when small proportions of GluR2 cDNA are transfected. It is not clear, however, how this principle alone could explain the dearth of surface expression of GluR1 homomers, nor why preferential heteromeric expression occurred when GluR1 flop, but not GluR1 flip, was coexpressed with $GluR1^{R}$ flip (Fig. 4). Ascribing the latter to differential subunit trafficking on the basis of differences in flip–flop sequences would require new assumptions regarding the modulation of these steps. Nevertheless, selective transport, membrane insertion, and surface removal mechanisms, in addition to regulation of assembly, may all contribute to selectivity for expression of AMPA receptors as heteromers. It is possible that although tetramerization is controlled primarily by the state of the Q/R site (Greger et al., 2003), initial dimer formation may occur with strong preference for GluR1–GluR2 and flip–flop heteromers. The present results argue for a specific effect of differences in the flip–flop region in promoting surface expression of heteromers. Although this is most simply explained and modeled by differential affinities in initial assembly steps only, the reality is likely more complex, involving as well the subsequent steps of receptor expression.

It is somewhat surprising that the short amino acid sequences carried by the alternative flip and flop splice variant cassettes may be sufficient to strongly affect selective subunit assembly. The accepted topology of AMPA receptors places these sequences in the "S2" extracellular loop before the terminal membrane spanning domain, contributing to agonist binding site formation (Stern-Bach et al., 1994). Often the pore-forming helices have been considered to provide the subunit junctions; however, studies of chimeric molecules formed from AMPA and kainate receptor subunits have revealed that the C-terminal portion of the S2 region, containing the flip–flop sequences, is among the determinants of compatibility for assembly of functional heteromers (Ayalon and Stern-Bach, 2001). Furthermore, structural models derived from crystallographic studies of the extracellular agonist-binding domains have shown that residues included in the flip–flop cassette lie in the "J helix" at the dimer subunit interface in positions where they may be critical for subunit association as well as cyclothiazide binding (Sun et al., 2002). The promotion of heteromeric assembly by flip–flop differences may be the consequence of the small number of amino acid differences between flip and flop isoforms in this region.

Interestingly, the data suggest that differences both between GluR1 and GluR2 sequences and between the flip and flop sequences combine to drive heteromeric assembly and expression more strongly than do flip–flop differences alone (Fig. 8). Whether the principle of preferential heteromeric assembly also applies to other pairs of individual subunits has not been tested directly. The flip and flop region sequences, being highly conserved across all of the four AMPA receptor subunit genes (Sommer et al., 1990), might have the same effect on promoting heteromeric subunit assembly, whatever the subunit context. On the other hand, heteromer formation between different subunits may depend strongly on which subunits are involved. Recent data indicate that GluR1 and GluR3 do not readily co-associate when GluR2 is present and can compete for heteromer formation, but GluR1–GluR3 heteromers can form to some degree when GluR2 is absent (Sans et al., 2003). Thus it will be of interest to observe whether flip–flop isoform differences might promote heteromeric assembly of unfavored subunit combinations.

In native neurons, several subunits are expressed in some cases with significant contributions of both flip and flop isoforms (Brorson et al., 1999; Vandenberghe et al., 2000). If flip–flop isoform differences, as well as subunit differences, promote heteromeric assembly, the result may be a greater contribution from Ca^{2+}-permeable AMPA receptors in such neurons than would be predicted merely from the proportion of GluR2. This may have implications for disease processes such as global ischemia or amyotrophic lateral sclerosis, in which relatively high expression of Ca^{2+}-permeable AMPA receptors has been proposed or reported (Pellegrini-Giampietro et al., 1992; Carriedo et al., 2000) despite substantial GluR2 expression in the affected neurons.

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