Reconstitution of Homomeric GluA2<sup>flop</sup> Receptors in Supported Lipid Membranes

**FUNCTIONAL AND STRUCTURAL PROPERTIES**

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**Background:** Ionotropic glutamate receptors mediate fast excitatory synaptic transmission in the vertebrate CNS.

**Results:** Conformational flexibility and dimensions of functional membrane-embedded full-length GluA2<sup>flop</sup> receptors are characterized by atomic force microscopy.

**Conclusion:** Conformational flexibility and dimensions are both strongly affected by receptor density.

**Significance:** The reconstitution protocol lays the foundation for correlated structure-function analysis of membrane-embedded glutamate receptors.

AMPARs (AMPARs) are glutamate-gated ion channels ubiquitous in the vertebrate central nervous system, where they mediate fast excitatory neurotransmission and act as molecular determinants of memory formation and learning. Together with detailed analyses of individual AMPAR domains, structural studies of full-length AMPARs by electron microscopy and x-ray crystallography have provided important insights into channel assembly and function. However, the correlation between the structure and functional states of the channel remains ambiguous particularly because these functional states can be assessed only with the receptor bound within an intact lipid bilayer. To provide a basis for investigating AMPAR structure in a membrane environment, we developed an optimized reconstitution protocol using a receptor whose structure has previously been characterized by electron microscopy. Single-channel recordings of reconstituted homomeric GluA2<sup>flop</sup> receptors recapitulate key electrophysiological parameters of the channels expressed in native cellular membranes. Atomic force microscopy studies of the reconstituted samples provide high-resolution images of membrane-embedded full-length AMPARs at densities comparable to those in postsynaptic membranes. The data demonstrate the effect of protein density on conformational flexibility and dimensions of the receptors and provide the first structural characterization of functional membrane-embedded AMPARs, thus laying the foundation for correlated structure-function analyses of the predominant mediators of excitatory synaptic signals in the brain.

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<sup>‡</sup>The abbreviations used are: AMPAR, AMPA receptor; ATD, amino-terminal domain; LBD, ligand-binding domain; AFM, atomic force microscopy; DDM, n-dodecyl β-D-maltoside; DM, decyl β-D-maltoside; CTZ, cyclothiazide; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo|f|quinoxaline-7-sulfonamide; pS, picosiemens.
(AFM) provided the first images of the membrane-embedded AMPARs (15). Although there are some significant differences in the observed structures, both EM and x-ray crystallography studies report proteins that are ~18 nm in length and ~15 nm across; in addition, EM of native AMPARs indicates large conformational flexibility (12). On the other hand, AFM images of GluA3 receptors show membrane-bound receptors with a substantially greater lateral extent and a much reduced height. It is not clear whether the AFM data arise from the intrinsic flexibility of the bulky extracellular domains, artifacts introduced by tip-protein interactions, or protein misfolding introduced by the reconstitution.

In this work, we report a novel reconstitution protocol for unedited GluA2flop homomers that were studied previously by EM (13): AFM images confirm the receptors are indeed full-length, and single-channel measurements reveal channel conductances consistent with those of native membranes. By spatially confining the receptors to achieve surface densities similar to those found in the postsynaptic membrane, we observed receptor dimensions similar to those obtained by EM and x-ray techniques. High receptor density also resulted in less structural heterogeneity, i.e. lower conformational flexibility of the receptors. More broadly, this is the first case in which an AMPAR has been successfully reconstituted, yielding single-channel recordings with physiologically plausible conductance levels and AFM images corresponding to the full-height extracellular structure seen by other techniques. The reconstitution protocol provides the possibility of two-dimensional crystallization (16) and imaging the receptor domains (ATDs and LBDs) in different conformational states via domain deletion and drug application. Overall, for the first time, biochemical and EM data (13, 17) are available in concert with AFM and electrophysiology for a purified AMPAR of known composition.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification*—Protein expression and purification were performed as described (18). Briefly, a tagged GluA2(Q)flop baculovirus construct was engineered with an insect cell leader sequence fused to a FLAG epitope, followed by the mature coding sequence of the unedited GluA2flop splice variant (accession number NP_001077280) (13). In the GFP-GluA2 construct, GFP was fused upstream of the GluA2 ATD. The identity and homogeneity of the purified protein (~20 μg/ml) were assessed by silver staining/SDS-PAGE, blue native PAGE, and Western blotting (17).

*Reconstitution*—All reconstitutions reported here were performed with the porcine brain lipid extract unless stated otherwise. The lipids were purchased from Avanti Polar Lipids, Inc., as chloroform solutions. As specified by the manufacturer, the brain lipid extract is a mixture (w/w) of phosphatidylethanolamine (16.7%), phosphatidylserine (10.6%), phosphatidylcholine (9.6%), phosphatidic acid (2.8%), phosphatidylinositol (1.6%), and unknown (58.7%). For liposome preparation, chloroform was evaporated under argon, and the lipid film was exposed to vacuum overnight to remove residual solvent. The film was hydrated by vortexing in buffer A (5 mM EDTA, 1 mM EGTA, and 30 mM HEPES, pH 7.4) to a final lipid concentration of 4 mg/ml. The lipid suspension was alternately placed in liquid N2 and warm water (35 °C) for six cycles. The lipids were then extruded through a series of filters, starting with a 1-μm pore size (Avanti Polar Lipids Extruder). Prepared unilamellar liposomes were solubilized in CHAPS detergent (10 mM); C12E8 (9.8 mM), n-dodecyl β-D-maltoside (DDM; 2.8 and 8.4 mM), and decyl β-D-maltoside (DM; 0.3 and 30 mM) detergents were also tested (see Fig. 2A). The liposome/detergent mixture was first sonicated in a water bath at room temperature for 15 min, followed by 1 h of incubation at room temperature. The purified protein was added to the lipid/detergent mixture at a lipid/protein mass...
ratio of 320:1. The protein/lipid/detergent mixture was incubated for 1 h at room temperature with gentle shaking. For detergent removal, pretreated Bio-Beads SM-2 (Bio-Rad) were added to the mixture at a bead/detergent mass ratio of 30:1, and the suspension was shaken for 1 h at room temperature. The proteoliposomes were stored at +4 °C until used.

**Fluorescence**—Receptor reconstitution was monitored using two-color fluorescence microscopy. Liposomes composed of brain lipids were prepared with 0.01% (w/w) 18:1 phosphatidylcholine-N-[(lissamine rhodamine B sulfonyl) (referred to as 18:1 Liss Rhod PE; Avanti Polar Lipids, Inc.; λex = 557 nm and λem = 571 nm) at 2 mg/ml in buffer A. The liposomes were extruded through a 1-μm filter before reconstitution with the GFP-tagged GluA2(Q)lop construct. Imaging was performed with an Olympus IX81 inverted microscope equipped with a Hamamatsu ORCA-ER CCD camera (C4742-80). Fluorescence was excited sequentially from the lipid and protein, and images were merged. The background signal arising from out-of-focus liposomes was subtracted from the images without any further processing.

**Electrical Recordings of Lipid Bilayers**—The activity of reconstituted proteins was assessed by electrical recordings of lipid bilayers formed using the tip-dip method adapted from Refs. 19 and 20: a quartz patch pipette was pulled (P-2000 puller, Sutter Instrument Co.) to 2–4 μm in diameter, and a lipid bilayer was formed at the tip of the pipette without fire polishing. For bilayer formation, 1,2-diphytanoyl-sn-glycero-3-phosphocholine was dissolved in decane at 2 mg/ml. A lid from a 2-ml microcentrifuge tube was used as a bath (total volume of 200 μl). Both bath and pipette were filled with recording buffer (125 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, and 5 mM Tris-HCl, pH 7.4). Before bilayer formation, the bath was precoated with 5 μl of 1,2-diphytanoyl-sn-glycero-3-phosphocholine in decane. Once the bilayer of gigohm resistance was formed, proteoliposomes were added to the bath. Stirring of the bath (SPIN-2 bilayer stir plate, Warner Instruments) for at least 20 min was necessary to achieve protein incorporation into the bilayer. The receptor density was determined from AFM images and AFM imaging parameters as described (21). The images were flattened using SPIP (scanning probe image processor, Image Metrology, Hørsholm, Denmark). No other image processing was performed.

**RESULTS**

**Reconstitution Protocol**—In a previous AFM study, homomeric GluA3 receptors were reconstituted in a mixed-lipid system (phosphatidylcholine from egg yolk and phosphatidyserine from porcine brain) using n-octyl β-D-glucopyranoside detergent, which was removed subsequently by dialysis (15). The protocol resulted in sparsely reconstituted GluA3 receptors protruding only a few nanometers above the lipid bilayer, much less than ~13 nm expected on the basis of x-ray data (11). In this study of homomeric GluA2 receptors, we aimed to increase the reconstitution efficiency and to investigate any impact of the reconstitution process on the receptor conformation. We investigated a variety of gentler detergents (C12E8, CHAPS, DDM, and DM), which were subsequently removed by Bio-Beads SM-2 (Fig. 2A). Different parameters of the protocol were adjusted to increase the protein density for the purposes of AFM imaging (Fig. 2, A–F) (22).

First, we used different optical techniques to monitor the various stages of the reconstitution process. Differential interference microscopy (Olympus BX61 microscope, 60× immersion objective, numerical aperture of 1.42) allowed us to exclude the possibility of vesicle aggregation, which could result in the proteoliposomes being unusable for structural or functional studies. No vesicle aggregation was observed at any stage of the reconstitution process. The mean radius of the proteoliposomes was determined by dynamic light scattering to be 71.4 ± 1.1 nm (n = 4) (data not shown). Fluorescence microscopy showed that receptors with GFP fused upstream from the ATD were reconstituted into 18:1 Liss Rhod PE-labeled liposomes (Fig. 3). First, negative controls containing only liposomes or GFP-tagged proteins were imaged (Fig. 3, insets). In the reconstituted samples, the co-localization of red liposomes with GFPs gave rise to yellow spots, confirming the association of the receptors with the liposomes (Fig. 3, C and D, insets).

The receptor density was determined from AFM images (once the proteoliposomes were adsorbed to mica): the surface area of the bilayer in each image was determined using ImageJ software (23), and the protein particles were counted manually for each image. A particle was counted as a protein if it protruded <20 nm above the surrounding bilayer and had 20–40 nm in lateral extent; the mean density is expressed as the number of protein particles/μm² of the supported lipid bilayer. Under these criteria, some artifacts and contaminants were
inevitably counted as proteins, but the negative control data presented in Figs. 2 and 6A show that the background control is not significant: the reconstituted samples have much higher particle counts than the negative controls. The surface density measurements show that the use of CHAPS for liposome solubilization had the biggest effect on the reconstituted receptor density. As shown in Fig. 2A, although DM, DDM, and C12E8 all yielded similar densities, the density was an order of magnitude higher with CHAPS. Decreasing the lipid/protein mass ratio resulted in higher protein density but also in higher aggregation of proteins.

**Effect of different reconstitution parameters on density of reconstituted GluA2 receptors.** Black bars are reconstituted samples, and white bars are negative controls (reconstitutions without protein). The reconstitutions were performed with brain lipid extract and CHAPS detergent unless stated otherwise. All data are shown as means ± S.E. A, effect of different detergents: CHAPS (10 mM), C12E8 (9.8 mM), DDM (2.8 and 8.4 mM), and DM (0.3 and 20 mM). The highest protein density of 78.4 ± 10.8 proteins μm⁻² (n = 33) was achieved with CHAPS. B, two different concentrations of DDM were tested. At 2.8 mM DDM, liposomes were saturated with the detergent when the protein was added, and at 8.4 mM DDM, the liposomes were solubilized as described (22). Solubilizing and saturating concentrations were also tested for DM (data not shown). C, the receptors were reconstituted in different lipid mixtures: brain total lipid extract, fusion lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine/cholesterol at a 10:5:5:4 molar ratio), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPG). For CHAPS-mediated reconstitutions, the average density of AMPARs was 78.4 ± 10.8 μm⁻² (n = 33). However, it should be noted that the reconstitution was spatially heterogeneous: some areas of the supported lipid bilayers appear quite empty, whereas other areas reveal values considerably higher than the mean.

**Ion Channel Activity of Reconstituted AMPARs—**Proteoliposomes in tip-dip bilayers generated a clear signal in response to 5 mM L-Glu and 250 μM CTZ (Fig. 4A, upper trace). This response was blocked by the addition of the NBQX antagonist, as shown in Fig. 4A (middle trace), and was absent in the negative control (lower trace). Analysis of >1000 open events yielded an exponential log probability fit to a dwell time histogram revealing two open states with time constants τ₁ = 0.9 ± 0.1 ms and τ₂ = 5.6 ± 0.1 ms (Fig. 4B). A similar fit to the dwell time of closed states resulted in three time constants: τ₁ = 0.6 ± 0.1 ms, τ₂ = 5.3 ± 0.1 ms, and τ₃ = 44.7 ± 0.3 ms (Fig. 4C). The open state probability for a recording of duration of 9 s was found to be 0.45. We found clear evidence for conductance levels of 5, 9, 12, and 20 pico siemens (pS) in three separate recordings (Fig. 4, D–F), with subconductance levels of 9 and 12 pS resolved within the same recording (A and E). Current fluctuations did not allow a more detailed investigation of subconductance levels for each recording. Similar single-channel conductance levels have been elicited by glutamate in both native membranes and membranes containing recombinantly expressed AMPARs (24, 25) and have been associated with independent subunit gating (26). It is not clear what caused the dominant conductance level of reconstituted AMPARs to vary between recordings, and the observed heterogeneity might be specific to reconstituted systems with complex lipid mixtures.

In the majority of our measurements, proteoliposome fusion with the bilayer resulted in only one channel being active at a time (based on the absence of multistep openings). On one occasion, however, at least two channels were simultaneously gating in the bilayer, as indicated by the presence of current plateaus at multiple values of the fundamental. Double con-
ductance events are clearly visible in Fig. 5A. In these recordings, an additional conductance level of ~47.5 pS was present, as well as its multiple at ~97.5 pS (Fig. 5B). A high conductance state of 54 pS has previously been reported for reconstituted native AMPARs (27). The highest reported conductance for AMPARs in native membranes is 44 pS (24).

FIGURE 3. Fluorescence microscopy illustrating protein reconstitution in liposomes. Receptors with GFP fused upstream from the ATD were reconstituted into 18:1 Liss Rhod PE-labeled liposomes, which was confirmed by co-localization of both emission wavelengths. A, negative control showing only liposomes with 0.01% (w/w) 18:1 Liss Rhod PE. B, non-reconstituted protein only. C and D, co-localization of fluorescently labeled membrane (red) and protein (green) in reconstituted samples resulted in yellow spots (enlarged in the insets). All images were acquired at magnification ×100; insets are ×10 zooms of the corresponding image. Red circles mark areas used for background subtraction.

FIGURE 4. Activity of reconstituted GluA2 receptors. A, single-channel current measured at +80 mV in 5 mM L-Glu and 250 μM CTZ, indicating conductance levels of 9 and 12 pS. Closed and open levels are marked as c, o1, and o2, respectively. The signal was absent in the presence of NBQX (+80 mV; middle trace) and in the negative control (liposomes without receptors, +100 mV; lower trace). B, dwell time histogram of open events with a two-term exponential fit yielding time constants $\tau_{o1} = 0.9 \pm 0.1$ ms and $\tau_{o2} = 5.6 \pm 0.1$ ms. N, number of bin counts. C, dwell time histogram of closed events with a three-term exponential fit yielding time constants $\tau_{c1} = 0.6 \pm 0.1$ ms, $\tau_{c2} = 5.3 \pm 0.1$ ms, and $\tau_{c3} = 44.7 \pm 0.3$ ms. D–F, single-channel conductance was determined by Gaussian fit to amplitude histograms. Conductances of 5 pS (D), 9 and 12 pS (E), and 20 pS (F), characteristic of AMPARs, were measured at +100, +80, and +40 mV, respectively.
AFM Imaging of Reconstituted GluA2 flop—Incorporation of purified protein into membrane was visualized by AFM in the absence of ligands. Fig. 6 shows scans of reconstituted samples: the flat light brown area is the lipid bilayer containing reconstituted proteins; the dark brown areas correspond to membrane defects, i.e. regions where there is no membrane, so the AFM tip can approach the mica surface. GluA2 homomers reconstituted using the above protocol appeared most frequently as isolated particles surrounded by membrane, but small clusters were also observed because of the relatively high protein density achieved by this protocol. Fig. 6B presents a large-area AFM image that shows protein incorporated predominantly as isolated particles distributed quite uniformly across the membrane. Alternatively, the reconstitutions presented in Fig. 6 (C and D) show a greater tendency for proteins to cluster, i.e. they appear to be in contact with each other.

More detailed AFM image analysis of isolated receptors (Fig. 7) shows the thickness of the brain lipid bilayer to be 4.0 ± 0.8 nm (n = 177), with isolated GluA2 receptors protruding 2–5 nm above the membrane. The spread of heights here arises largely from the fact that the AFM tip does not scan over the peak position but instead samples the height at different lateral positions. The result of a more detailed analysis that involved locating and measuring the height of each receptor individually is presented in Fig. 7C. The full height of the protein was 11.4 ± 3.1 nm (mean ± S.D., n = 179). These measurements were repeated using a softer cantilever (0.08 newtons/m) and resulted in a full receptor height of 11.1 ± 3.9 nm (mean ± S.D., n = 122) (data not shown). The height of the receptor was also unaffected by different lipids (brain lipid extract, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol), or fusion lipids

FIGURE 5. Multichannel recording of reconstituted GluA2 receptors. A, multiple opening levels indicate concurrent gating of more than one channel in the bilayer. The holding voltage was +40 mV. Closed and open levels are marked as c, o1, and o2, respectively. B, when multiple openings were recorded, a higher conductance of 47.5 pS was observed, together with its multiple at 97.5 pS.

FIGURE 6. AFM images of reconstituted GluA2 homomers showing isolated and clustered proteins. The lipid bilayer is the flat light brown area. Defects in the lipid bilayer expose imaging surface (mica; dark brown area). Bright specks are proteins reconstituted in the lipid bilayer. Big white clumps in C and D are unresolved aggregates of proteins and/or lipids. A, negative (protein-less) control of reconstituted samples. Note the absence of the proteins (i.e. bright specks) in the flat lipid bilayer. Scale bar = 500 nm. B, isolated proteins surrounded by only the membrane (average density of 27 proteins µm⁻²). Three isolated proteins are indicated by black arrowheads. Scale bar = 500 nm. C and D, small clusters (circled) containing up to eight receptors (average density of 223 particles µm⁻²). Examples of isolated proteins are indicated by black arrowheads. Scale bars = 500 nm (C) and 200 nm (D). Samples in C and D were incubated overnight at +4 °C.
(1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine/cholesterol at a 10:5:5:4 molar ratio), its location in the bilayer (i.e. proximity to the edge), or the pH of the imaging buffer (pH 6, 7.4, or 8) (data not shown). It is clear from these data that the height is significantly smaller than the values of 18 nm determined by x-ray measurements (11) and 17 nm obtained by EM (13). EM of synaptic AMPARs resulted in two classes of structures with heights of 20 nm (type 1) and 13 nm (type 2) (12).

As AFM probes the extracellular surface structure of the receptor, we can expect the images to reveal details of the subunit coordination at the level of ATDs. However, the AFM images of the isolated reconstituted GluA2 receptors presented in Fig. 7D reveal considerable heterogeneity in size, shape, and coordination. The lateral dimensions of ~30 × 30 nm are also significantly larger than the x-ray measurements, 9 × 15 nm (11), or the EM measurements, 11 × 14 nm (12, 13). This is perhaps unsurprising given the inherent flexibility of the structure and the absence of any spatial confinement in the membrane environment. Nevertheless, the AFM images of isolated reconstituted GluA2 receptors do reflect a tetrameric stoichiometry with substantial internal structure (Fig. 7D). Although there are several possibilities leading to the observed high conformational heterogeneity of the isolated receptors in AFM, allowing the receptors to cluster yielded less structural heterogeneity and receptor heights more consistent with x-ray and EM structures.

**AFM Imaging of Receptor Clusters**—Receptor clusters were observed to form in supported membranes (reconstituted in CHAPS) and kept for >12 h on mica at +4 °C and in 50 μl of AFM buffer: the combination of a larger reconstitution volume and a longer incubation time on mica appear to be the important experimental factors. To compare the densities of different clusters, clustered particles were counted within a defined square area of 200 × 200 nm (area of a smaller synapse) (28). Up to eight individual receptors could be resolved within these clusters, which corresponds to a protein density of ≥200 proteins μm⁻², a value that is comparable to the density of AMPARs in the adult rat cerebellum (437 ± 277 μm⁻²) (28).

When clustered, the receptors exhibited dimensions closer to the values found in the EM and x-ray data. Fig. 8A shows a small linear cluster of three proteins in close contact. The height scan data (Fig. 8, B and C) show that the full height of these receptors was 18 nm. The increase in the protein height observed upon clustering confirms that imaged AMPARs were indeed full-length and that their ATDs had remained intact during the reconstitution and imaging. This result strongly suggests that protein-protein interactions stabilize the structural conformation of the extracellular domains and that neither thermal fluctuations nor tip-sample interactions are sufficiently strong to disassociate the ATDs.
The lateral extent of the receptors is also more compact, although the tip geometry is too large to be able to completely resolve the proteins along the line of contact. Nevertheless, the cross-section is now clearly elongated. Unlike the images of the isolated receptors in Fig. 7, the internal structure arising from individual subunits is not resolved, but there is evidence of a central pore on the symmetry axis: a dip \( \pm 2 \) nm deep and \( \pm 6.5 \) nm across (limited by the image pixel size). The pixel intensity measurements show that the pore signal was significantly stronger than the background (mica and bilayer; \( p < 0.05 \)); however, the exact pore size might have been affected by the AFM tip. The dimensions of the central pore located at the symmetry axis at the top of the ATDs in the full-length GluA2 crystal structure (11) were measured between C\( \alpha \) atoms of the following residues: the distance between proximal subunits was measured between the Arg-30 residues of each subunit and is 5.7 nm; the distance between distal subunits was measured between the Glu-324 residues of each subunit and is 8.8 nm; and the depth of the pore was measured between Ile-221 and Glu-269 of subunit D and is 3.5 nm (the numbering is according to Protein Data Bank code 3kg2, and distal and proximal subunits are defined as described (11)). The depth of the pore is somewhat smaller than measured in the crystal structure of the GluA2 tetramer, due, most likely, to the combined effects of tip geometry and thermal fluctuations of the protein in the liquid environment (Fig. 8D).

Receptor Orientation—In principle, the receptors can be incorporated into membranes with either the carboxyl or amino terminus lying outside the vesicles. The bulky extracellular (amino-terminal) domains extend \( \sim 13 \) nm above the membrane, whereas intracellular (carboxyl-terminal) domains are only 50 amino acids long (for the rat GluA2 subunit (P19491)) and are expected to be significantly shorter (no structural data are available for AMPAR intracellular domains). This large difference in domain height is potentially beneficial for the identification of protein orientation using AFM. Fig. 8 clearly shows GluA2 receptors oriented with their extracellular domains protruding above the bilayer. This orientation of the proteins agrees well with that observed for reconstituted GluA3 homomers (15). However, the orientation of the isolated proteins, which protrude \( \pm 7 \) nm above the surrounding membrane, is less conclusive. This protrusion, although significantly shorter than the 13–14 nm expected for the extracellular domain of GluA2 tetramers, is unlikely to be associated with the carboxyl terminus. In the case of inverted orientation, with the carboxyl terminus lying above the lipid bilayer, AFM images would show protrusions superimposed on top of a bulge caused by the presence of the bulky extracellular domains lying between the mica surface and the bilayer proximal leaflet; no such structures were observed.

DISCUSSION

In all previous studies of reconstituted AMPARs, \( n \)-octyl \( \beta \)-\( \delta \)-glucopyranoside detergent was used at the liposome destabilization stage (15, 20, 29). However, because of the negative impact of \( n \)-octyl \( \beta \)-\( \delta \)-glucopyranoside on the structural integrity (30) and ligand-binding activity (18) of GluA2 receptors, we developed a novel \( n \)-octyl \( \beta \)-\( \delta \)-glucopyranoside-free reconsti-
tution protocol to obtain high-resolution AFM images of functional receptors. The protocol was optimized to produce densely reconstituted proteins, comparable to the densities observed in synaptic membranes; this not only creates a more biologically relevant environment but also provides a system that is more amenable to high-resolution AFM imaging (31, 32). Although many parameters were varied during the protocol development, including detergents, membrane lipids, and pH, none influenced the density of the reconstituted protein as much as the detergent used for liposome solubilization. We found that the non-denaturating zwitterionic detergent CHAPS resulted in the highest protein density. CHAPS was also used in the purification of native AMPARs for observation by EM (12). Due to its widespread use in the reconstitution of membrane proteins (33, 34), the success of CHAPS in GluA2(Q)flip reconstitution is unlikely to be explained by specific detergent-protein interactions. As the protein used in our studies was purified using DM, and CHAPS was only used to solubilize the liposomes, the efficacy of CHAPS probably lies in its interaction with brain lipids and/or its effective removal by Bio-Beads.

Although single-channel recordings of overexpressed GluA2(Q)flip channels have been observed on numerous occasions (25, 35, 36), the electrical measurements presented here are the first single-channel recordings of homomeric GluA2(Q)flip channels and the first of reconstituted recombinant GluA2 receptors. The signal obtained in our experiments using tip-dip bilayers was elicited in saturating concentrations of L-Glu and CTZ and was sensitive to the AMPAR antagonist NBQX. The signal was not observed with the negative control (protein-free vesicles). The tip-dip bilayers containing only one channel gating at a time exhibited conductance levels (5, 9, 12, and 20 pS) and open states characteristic of AMPARs (24, 25, 27, 37). However, the dominant conductance level varied between recordings, a property so far observed only for AMPARs reconstituted in a complex lipid mixture of brain lipid extract. Multiple openings, which indicate the presence of more than one active channel in the bilayer, were observed in only one recording; the conductance level of ~50 pS recorded on that occasion resembles the previously reported conductance of native AMPARs, both reconstituted (20, 27, 38) and non-reconstituted (24). Vaithianathan et al. (27) reported a 54-pS conductance state for synaptosomal AMPARs, which they assigned to the simultaneous opening of two channels resulting from cooperativity among the receptors. Indeed, we have recorded ~50-pS conductance states only when multiple channels were present in the bilayer, indicating the possibility of cooperation between the channels. However, recordings of lower noise are needed before more detailed analysis can be performed. In addition, the data presented were obtained in the presence of saturating concentration of CTZ, which could have affected the conductance properties.

The success rate of our tip-dip bilayer experiments with the reconstituted GluA2(Q)flip receptors was generally not high (~30%). Various factors might be responsible, such as the presence of organic solvents in the bilayer, low bilayer formation rate (although bilayer formation was confirmed using gramicidin), low rate of fusion of proteoliposomes with the bilayer, and low protein activity, in addition to a low number of proteins per proteoliposome.

Despite these difficulties, the protocol succeeded in achieving reconstituted receptor densities that were sufficiently high that clustering could be observed in the AFM images. However, when imaged at low density, GluA2 receptors exhibited structural heterogeneity, with extracellular domains adopting a range of conformations as reported for reconstituted GluA3 receptors (15). The dimensions of the receptors were substantially different from those reported by EM (12, 13) and x-ray crystallography (11) studies: instead of the expected ~18 nm in length, the receptors were ~11 nm high, which could be interpreted as indicating that the ATD got detached during the reconstitution or imaging. However, fluorescence studies performed with GFP fused upstream of the ATD of GluA2 confirmed co-localization of the receptors with 18:1 Liss Rhod PE-labeled proteoliposomes. Furthermore, the height of the clustered protein was measured to be ~18 nm, which confirms that full-length receptors were indeed reconstituted. Some degree of structural heterogeneity is expected for isolated receptors imaged in near-physiological conditions. For example, cross-linking studies of full-length AMPARs in the apo state indicate high mobility of LBDs (39), which would give rise to structural heterogeneity. Also, subunit-specific dynamics have been shown for isolated ATDs, with GluA3 ATDs exhibiting higher structural heterogeneity than GluA2 ATDs (40). Different conformations of apo states (“active” and closed) have also been noted for the soluble LBDs of metabotropic glutamate receptor 1 (41), which are homologous to the ATDs of AMPARs. It is also plausible, however, that such tall and flexible structures are perturbed as a result of the AFM tip-sample interactions. A similar “flattening” of isolated spatially unconfined proteins when imaged by AFM has been reported for nicotinic acetylcholine receptors (42), inositol 1,4,5-triphosphate receptors (43), and P2X receptors (44). The larger than expected lateral dimension and reduced height of the isolated receptors could indicate that the ATDs are displaced from their mean equilibrium positions but remain attached to the LBDs through the linker. The bending of the receptor due to the imaging force would in that case also explain why tetrameric structure and dimer-of-dimers association are not resolved for every isolated receptor.

Clustered GluA2 receptors exhibited more tightly packed ATDs than isolated receptors and displayed less structural heterogeneity. Protein-protein interactions may in fact stabilize the full-length structure against external perturbations and reduce intrinsic dynamical effects, but it does seem surprising that the effects can be so marked, even for a cluster with as few as three receptors. Nevertheless, it is well established that high-resolution imaging of tall flexible samples such as AMPARs requires some degree of immobilization (45). To restrict their movements, reconstituted proteins can be prepared as two-dimensional crystals (45, 46) or as dense protein arrays (32, 47, 48). The density achieved in our reconstitutions is lower than the crystal density, but it is closer to the native synaptic membranes (28, 49). Lower conformational flexibility of clustered receptors in near-physiological conditions indicates the recep-
 tors are unlikely to undergo large conformational changes (≥5 nm) when in postsynaptic membranes. The only submolecular feature that could be resolved on the clustered receptors was a central pore, also present in the crystal (11) and EM (12, 13) structures. Although the extent of the ATD separation in published EM and x-ray structures differs, with antagonist-treated structures exhibiting a more pronounced ATD separation (11, 12) than the apo structure (17), the current AFM data are not as yet sufficient to distinguish clearly between the models. AFM is, however, well suited to address the conformation of AMPAR ATDs, as more high-resolution images are obtained.

The reconstitution protocol described here is a straightforward method of producing functional GluA2 flop receptors in a membrane environment, amenable to biophysical studies. It should also be applicable to other glutamate receptors. High protein densities have been achieved using CHAPS as the liposome-solubilizing detergent; subsequent receptor clustering has for the first time permitted the observation of reconstituted full-length AMPARs. Future work to further increase the receptor density will potentially allow two-dimensional crystals to be produced, permitting subnanometer studies of different functional states to be investigated. As the spatial resolution is increased, structural details revealed by AFM will give insights into the behavior of these neuroreceptors in near-physiological conditions: in liquid at physiological pH and at densities comparable to those in native membranes. At sufficiently high expression levels, AFM could image association points between AMPARs and auxiliary subunits. Due to the liquid environment, the protein dynamics may be visualized in real time using high-speed AFM. For AMPARs, this means the potential to directly image open and desensitized states and shed new light on the working mechanism of these receptors.

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