Reconstitution of homomeric GluA2\textsubscript{flop} receptors in supported lipid membranes: functional and structural properties*

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*Running title: Structure and function of reconstituted AMPA receptors

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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\textsubscript{flop} 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.

**SUMMARY**

Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (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 structure and functional states of the channel remains ambiguous, particularly since these functional states can only be assessed with the receptor bound within an intact lipid bilayer. To provide a basis for investigating AMPAR structure in a membrane environment, we have developed an optimized reconstitution protocol using receptors whose structure has previously been characterized by electron microscopy. Single-channel recordings of reconstituted homomeric GluA2\textsubscript{flop} receptors recapitulate key electrophysiological parameters of the channels expressed in native cellular membranes. Atomic-force microscopy (AFM) studies of the reconstituted samples provide high-resolution images of full-length membrane-embedded 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.

AMPARs are ionotropic glutamate receptors (iGluRs) that mediate fast excitatory synaptic transmission. Their number and composition change with synaptic activity, and their dynamics are closely associated with the processes of
Structure and function of reconstituted AMPA receptors

Due to their essential physiological roles, AMPARs have been implicated in numerous disorders of the CNS such as Alzheimer’s disease, epilepsy, schizophrenia, fragile X syndrome and ischemia (2). Functional AMPARs are homo or hetero-tetrameric complexes made up of various combinations of the subunits GluA1-4; although their subunit composition varies, almost all of them contain a GluA2 dimer (3). Unlike the other subunits, GluA2 can be RNA edited to contain an arginine (R) instead of a glutamine (Q) at a core residue in the pore loop. Channels containing edited GluA2 subunits are nearly Ca\(^{2+}\)-impermeable (4) and exhibit near-linear current–voltage (I/V) relationships (5).

Each AMPAR subunit has the modular structure shown in Fig. 1A, consisting of extracellular amino-terminal (ATD) and ligand-binding domains (LBD) connected via flexible linkers, a transmembrane region (TM) and a cytoplasmic carboxyl terminal domain (CTD) (6). The ATDs are implicated in iGluR trafficking and subtype-specific assembly of AMPARs (7); the LBDs bind agonists and competitive antagonists, leading to changes in channel activity (8); the transmembrane region contains the (cation-selective) ion channel (9); and the CTD is a point of contact between AMPARs and cytoplasmic regulatory proteins (10). X-ray crystallography has revealed the structure of the un-edited homomeric GluA2 receptor in complex with a competitive antagonist (Fig. 1B) (11); in order to assist crystallization, C-terminus and part of the ATD-LBD linker were deleted, and several point mutations removed predicted glycosylation sites. The structure confirmed a dimer-of-dimers subunit arrangement within a tetramer. Electron microscopy (EM) has also been used to study full-length receptors (12-14), and atomic force microscopy (AFM) provided first images of the membrane-embedded AMPARs (15). Although there are some significant differences in the observed structures, both EM and X-ray crystallography report proteins that are approximately 18 nm in length and ~15 nm across; in addition, EM of native AMPARs indicated large conformational flexibility (12). AFM images of GluA3 receptors, on the other hand, showed 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 paper we report a novel reconstitution protocol for unedited GluA2\(_{\text{flop}}\) homomers which 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 post-synaptic membrane, we observe 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 opens the possibility of 2D crystallization (16) and of 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 in (18). Briefly, a tagged GluA2(Q)\(_{\text{flop}}\) baculovirus construct was engineered with an insect-cell leader sequence fused to a FLAG epitope, followed by the mature coding sequence of the unedited GluA2\(_{\text{flop}}\) splice variant (sequence ID: NP_001077280 (13)). In the GFP::GluA2 construct, GFP was fused upstream of the GluA2 N-terminal domain. The identity and homogeneity of purified protein (~20 μg/mL) were assessed with silver-stained 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 (PE, 16.7%), phosphatidylserine (PS, 10.6%), phosphatidylcholine (PC, 9.6%), phosphatidic acid (PA, 2.8%), phosphatidylinositol (PI, 1.6%) and unknown (58.7%). For liposome preparation, chloroform was evaporated under Ar 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, 30 mM HEPES, pH 7.4) to a final lipid concentration of 4 mg/mL. The lipid suspension was alternately placed in liquid N\textsubscript{2} 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 and finishing with a 0.2 µm pore size (Avanti Polar Lipids Inc. extruder). Prepared unilamellar liposomes were solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent (10 mM); octaethylene glycol monododecylether (C\textsubscript{12}E\textsubscript{8}; 9.8 mM), n-dodecyl \textbeta-D-maltoside (DDM; 2.8 and 8.4 mM) and decyl-\textbeta-D-maltoside (DM; 0.3 and 30 mM) detergents were also tested (Fig. 2A). The lipid-detergent mixture was first sonicated in a water bath at room temperature for 15 min., followed by 1h incubation at room temperature. The purified protein was added to the lipid-detergent mixture at lipid-to-protein mass ratio of 320:1. The protein-lipid-detergent mixture was incubated for 1h at room temperature with gentle shaking. For detergent removal, pre-treated Bio-Beads SM2 (Bio-Rad) were added to the mixture at a beads-to-detergent mass ratio of 30:1 and the suspension was shaken for 1h at room temperature. The proteoliposomes were stored at +4°C until use.

**Fluorescence** - Receptor reconstitution was monitored using two-colour fluorescence microscopy. Liposomes composed of brain lipids were prepared with 0.01% (w/w) 18:1 phosphoethanolamine-N-lissamine rhodamine B sulfonyl (18:1 Liss Rhod PE, \( \lambda_{ex} = 557 \text{ nm}, \lambda_{em} = 571 \text{ nm}; \) Avanti Polar Lipids Inc.) at 2 mg/mL in buffer A. The liposomes were extruded through a 1 µm filter before reconstitution with the GFP-tagged GluA2(Q\textsubscript{flop}) construct. Imaging was performed with an Olympus IX81 inverted microscope equipped with a CCD camera (Hamamatsu Orca ER, C47 42-80). 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 (19,20): a quartz patch pipette was pulled (P-2000 puller, Sutter Instrument, California, U.S.A.) 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 (DPhPC) was dissolved in decane at 2 mg/mL. A lid from a 2 mL microfuge tube was used as a bath (200 µL total volume). Both bath and pipette were filled with the recording buffer: 125 mM NaCl, 5 mM KCl, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4} and 5 mM Tris HCl, pH 7.4. Before bilayer formation, the bath was pre-coated with 5 µL of DPhPC in decane. Once the bilayer of gigaohm resistance was formed, proteoliposomes were added to the bath. The signal was elicited by the addition of 5 mM L-Glu and 250 µM cyclothiazide (CTZ; signal potentiator and desensitization modulator of GluA2\textsubscript{flop} variant); the signal was absent in the presence of the saturating amounts of antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[\textit{f}]quinoxaline-7-sulfonamide (NBQX) and in the negative control. All recordings were obtained using an Axopatch 200B amplifier and Digidata 1440A digitizer (Molecular Devices LLC, Sunnyvale, U.S.A.). Pipette offset was adjusted before every recording. The signal was sampled at 50 kHz and low-pass filtered at 10 kHz for acquisition and 2 kHz for presentation. Single-channel conductances were determined by Gaussian fit to amplitude histograms. The number and the time constants of the open and closed states were determined through the exponential log probability fit of the dwell time histograms. The search method was variable metric and the minimization method maximum likelihood. Data analysis was performed with Clampfit 10.2.0.14 (Molecular Devices LLC, Sunnyvale, U.S.A.).
Sunnyvale, U.S.A.) and Origin 8.0 (OriginLab Corporation, Northampton, U.S.A.).

AFM - Proteoliposomes (5 µL) were deposited onto a freshly cleaved mica disc and incubated at least 20 min. in a humid chamber at room temperature to allow supported lipid bilayer (SLB) formation. Before AFM imaging, the sample was rinsed three times (50 µL/rinse) with AFM buffer (40 mM NaCl, 260 mM KCl, 30 mM HEPES, pH 7.4) to remove residual material not adsorbed onto the substrate. Imaging was performed in AFM buffer using a Dimension 3100™ (Digital Instruments Veeco Metrology, Woodbury, U.S.A). Silicon nitride cantilevers with pyramidal tips (spring constant of 0.57 Nm⁻¹, TR800PSA, Olympus, UK) were used unless stated otherwise. All images were acquired in tapping mode in liquid at 512 x 512 pixel resolution using imaging parameters as described in (21). The images were flattened in SPIP (Scanning Probe Image Processor, Image Metrology, Horsholm, 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 phosphatidylserine from porcine brain) using n-octyl-β-D-glucopyranoside (OG) detergent, the latter being removed subsequently by dialysis (15). The protocol resulted in sparsely reconstituted GluA3 receptors protruding only a few nm above the lipid bilayer, much less than ~13 nm expected on the basis of X-ray data (11). In the present 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 SM2 (Fig. 2A). Different parameters of the protocol were adjusted in order to increase the protein density for the purposes of AFM imaging (Fig. 2A-F) (22).

To begin with we used different optical techniques to monitor the various stages of the reconstitution process. Differential interference microscopy (Olympus, BX61, 60x objective, immersion, N.A. 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 (DLS) 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-labelled liposomes (Fig. 3). Firstly, negative controls containing only liposomes or GFP tagged proteins were imaged (Fig. 3A and B respectively). In the reconstituted samples, the co-localization of red liposomes with green-fluorescent proteins gave rise to yellow spots, confirming the association of the receptors with the liposomes (insets in Fig. 3C and D).

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 per µm² of the SLB. 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 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 by far the biggest effect on the reconstituted receptor density. As shown in Fig. 2A, while DM, DDM and C₁₂E₈, all yielded similar densities, the density is an order of magnitude higher with CHAPS. Decreasing the lipid-to-protein mass ratio resulted in the higher protein density, but also increased the presence of aggregates (Fig. 2F). For CHAPS-mediated reconstitutions the average density of AMPARs was 78.4 ± 10.8 µm² (n=33). However, it should be noted that the reconstitution is spatially heterogeneous: some areas of the SLBs 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 top trace). This response was blocked by the addition of NBQX antagonist, as shown in Fig. 4A (middle trace), and was absent in the negative control (Fig. 4A bottom trace). Analysis of >1000 open events yielded an exponential log probability fit to a dwell time histogram revealing two open states with time constants τc1 = 0.9 ± 0.1 ms and τc2 = 5.6 ± 0.1 ms (Fig. 4B). A similar fit to the dwell time of closed states resulted in three time constants: τo1 = 0.6 ±0.1 ms, τo2 = 5.3 ± 0.1 ms and τo3 = 44.7 ± 0.3 ms (Fig. 4C). The open state probability for a recording of duration of 9s is found to be 0.45. We found clear evidence for conductance levels of 5, 9, 12 and 20 pS in three separate recordings (Fig. 4D-F), with subconductance levels of 9 and 12 pS resolved within the same recording (Fig. 4A 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 multi-step 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 conductance events are clearly visible in Fig. 5A. In the absence of ligands, 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, and so the AFM tip can approach the mica surface. GluA2 homomers reconstituted using the above protocol appear most frequently as isolated particles surrounded by membrane, but small clusters are also observed because of the relatively high protein density achieved by this protocol. Fig. 6B presents a large-area AFM image which shows protein incorporated predominantly as isolated particles distributed quite uniformly across the membrane. Alternatively, the reconstitutions presented in Fig. 6C 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 which involved locating and measuring the height of each receptor individually is presented in Fig. 7C. The full height of the protein is 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 which involved locating and measuring the height of each receptor individually is presented in Fig. 7C. The full height of the protein is 11.4 ± 3.1 nm (mean ± s.d.; n=179). These measurements were repeated using a softer cantilever (0.08 Nm⁻¹) and resulted in the full receptor height of 11.1 ± 3.9 nm (mean ± s.d.; n=122; data not shown). The receptor height was also unaffected by different lipids (brain lipid extract, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG) or fusion lipids (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 10:5:5:4 molar ratio), its location in the bilayer (i.e. proximity to the edge) or pH of the imaging buffer (6, 7.4 or 8) (data not shown). It is clear from these data that the height is significantly smaller than the value of 11.1 nm determined by X-ray measurements (11), and 17 nm obtained by EM (13). EM of synaptosomal 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 isolated, reconstituted GluA2 receptors presented in Fig. 7D reveal considerable heterogeneity in size, shape and coordination. The lateral dimensions of approximately 30 nm x 30 nm are also significantly larger than the X-ray measurements, 9 nm x 15 nm (11), or the EM measurements, 11 nm x 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).

While 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 hrs on mica at +4°C and in 50 μL of AFM buffer: the combination of larger reconstitution volume and longer incubation time on mica appear to be the important experimental factors. To compare densities of different clusters, clustered particles were counted within a defined square area of 200 nm x 200 nm (area of a smaller synapse (28)). Up to 8 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 density of AMPARs in the adult rat cerebellum (437±277 μm⁻²) (28).

When clustered, the receptors exhibit 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 is 18 nm. The increase in the protein height observed upon clustering confirms that imaged AMPARs are indeed, full-length and that their ATDs have 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 ~2 nm deep and ~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 Cα of the following residues: the distance between proximal subunits was measured between Arg30 of each subunit and is 5.7 nm; the distance between distal subunits was measured between Glu324 of each subunit and is 8.8 nm; the depth of the pore was measured between Ile221 and Glu269 of subunit D and is 3.5 nm (the numbering is according to PDB entry 3kg2 and distal and proximal subunits are defined as in (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 C- or N-terminus lying outside the vesicles. The bulky extracellular (N-terminus) domains extend ~13 nm above the membrane whereas intracellular (C-terminus) 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 ≤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 C-terminus. In the case of inverted orientation, with the C-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 AMPAR, OG detergent was used at the liposome destabilization stage (15,20,29). However, because of the negative impact of OG on the structural integrity (30) and ligand-binding activity (18) of GluA2 receptors, we developed a novel OG-free reconstitution protocol in order 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 it 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-denaturing, zwitterionic detergent CHAPS resulted in the highest protein density. CHAPS was also used in the purification of native AMPARs for observation by electron microscopy (12). Due to its widespread use in the reconstitution of membrane proteins (33,34), the success of CHAPS in GluA2 flop 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.

While single-channel recordings of over-expressed GluA2Q flop channels have been observed on numerous occasions (25,35,36), the electrical measurements presented here are the first single-channel recordings of homomeric GluA2Q flop 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 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 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 presented data 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 flop 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 (though bilayer formation was confirmed using gramicidin), low rate of fusion of proteoliposomes with the bilayer, low protein activity, in addition to 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 full 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-labelled proteoliposomes. Furthermore, the height of 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, a 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 mGluR1 (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). In order to restrict their movements, reconstituted proteins can be prepared as 2D 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 receptors are unlikely to undergo large conformational changes (≥5 nm) when in postsynaptic membrane.

The only sub-molecular feature that could be resolved on the clustered receptors was a central pore, also present in the crystal (11) and EM structures (12,13). 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 \text{ 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 full-length reconstituted AMPARs. Future work to further increase the receptor density will potentially allow 2D crystals to be produced, permitting sub-nm 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 into the working mechanism of these receptors.

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Structure and function of reconstituted AMPA receptors


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FIGURE LEGENDS

**FIGURE 1.** Modular architecture of AMPARs. A. Schematic representation of an AMPAR monomer comprising an amino terminal domain (ATD), a ligand binding domain (LBD), a transmembrane region (TM) consisting of three membrane helices (M1, M3, M4) and a re-entrant M2 and a carboxyl terminal domain (CTD); the N-terminus is positioned extracellularly and C-terminus intracellularly; the pink star represents an agonist molecule bound in the LBD cleft. B. The crystal structure of the near full-length GluA2 homotetramer; individual monomers are color coded (blue, green, red and yellow) and domain layers are enclosed in boxes to emphasize the modular structure (PDB entry: 3kg2 (11)).

**FIGURE 2.** Effect of different reconstitution parameters on the density of reconstituted GluA2 receptors. Black columns are reconstituted samples and white columns negative controls (reconstitutions without protein). The reconstitutions were performed with brain lipid extract and CHAPS detergent unless stated otherwise. All data are shown as mean ± SEM. A. Effect of different detergents: 3-[(3-
cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; 10 mM), octaethylene glycol monododecyl ether (C_{12}E_{8}, 9.8 mM), n-dodecyl β-D-maltoside (DDM; 2.8 and 8.4 mM) and decyl-β-D-maltoside (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 are saturated with the detergent when the protein is added and at 8.4 mM DDM, the liposomes are solubilized as described in (50); 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 (POPE: POPC: POPS: cholesterol at 10:5:5:4 molar ratio), POPA and POPC (see text for abbreviations). D. The reconstitutions were performed at room temperature and +4°C. E. The mixture of lipids, proteins and detergent was mixed with Bio-Beads in order to remove detergent; the length of the Bio-Beads treatment was varied between 1h and overnight. F. Different lipid-to-protein mass ratios were tested: 13 and 667; lower lipid-to-protein mass ratio resulted in higher protein density but also in higher aggregation of proteins.

**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 labelled 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 labelled membrane (red) and protein (green) in reconstituted samples results in yellow spots (enlarged in insets). All images were acquired at 100x magnification (insets are 10x 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, bottom trace). B. Dwell time histogram of open events with a two-term exponential fit yielding time constants $\tau_{o1} = 0.9 \pm 0.1\text{ ms}$ and $\tau_{o2} = 5.6 \pm 0.1\text{ ms}$. C. Dwell time histogram of closed events with a three-term exponential fit yielding time constants $\tau_{c1} = 0.6 \pm 0.1\text{ ms}$, $\tau_{c2} = 5.3 \pm 0.1\text{ ms}$ and $\tau_{c3} = 44.7 \pm 0.3\text{ ms}$. D-F. Single-channel conductance was determined by Gaussian fit to amplitude histograms; conductance of D. 5 pS, E. 9 and 12 pS and F. 20 pS characteristic of AMPARs were measured at +100, +80 and +40 mV respectively.

**FIGURE 5.** Multi-channel recording of the reconstituted GluA2 receptors. A. Multiple opening levels indicate concurrent gating of more than one channel in the bilayer; 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. Lipid bilayer is the light brown, flat 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 only by the membrane (average density: 27 proteins μm^{-2}), three isolated proteins are indicated by black arrowheads; scale bar: 500 nm. C.-D. Small clusters (encircled) containing up to 8 receptors (average density: 223 particles μm^{-2}); examples of isolated proteins are indicated by black arrowheads; scale bars: C. 500 nm, D. 200 nm; samples in C. and D. underwent overnight incubation at +4°C.

**FIGURE 7.** A. AFM image of isolated receptors reconstituted in the lipid bilayer (scale bar: 500 nm). The heights obtained from the linescan are given in B.; yellow and green arrows indicate protein features
covered by the scan line. C. Distribution of extracellular protrusions corresponding to a height of 11.4 ± 3.1 nm (n=179). D. High-resolution AFM images of isolated receptors revealing internal structure which is mostly tetrameric; subunit contours for each receptor are shown below the corresponding scan (scale bars: 20 nm).

FIGURE 8. AFM scan of clustered GluA2 receptors. A. Small linear cluster containing three receptors (encircled) in contact (scale bar: 20 nm). B. 3D reconstruction of the cluster revealing the presence of central pore in each receptor. C. The height profile of the black dotted line marked in A. D. Inset shows the uppermost receptor from the cluster in A and the pore dimensions are visible from the height profile of the white line in the inset (6.5 nm across and 2 nm deep).
Figure 2

A. Normalized protein density by detergent type.

B. Normalized protein density by DDM concentration.

C. Normalized protein density by lipid composition.

D. Normalized protein density by temperature.


F. Normalized protein density by lipid-to-protein mass ratio.
Figure 3
Figure 4

A

B

C

D

E

F

Structure and function of reconstituted AMPA receptors
Figure 5

A

2 pA

500 ms

B

Frequency

100000

80000

60000

40000

20000

0

Amplitude (pA)

Closed

47.5 pS

97.5 pS
Figure 6

A

B

C

D

Structure and function of reconstituted AMPA receptors
Figure 8
Reconstitution of homomorphic GluA2^flop receptors in supported lipid membranes: functional and structural properties
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