AMPA receptor/TARP stoichiometry visualized by single-molecule subunit counting

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Edited* by David Julius, University of California, San Francisco, CA, and approved February 15, 2013 (received for review November 1, 2012)

Members of the transmembrane AMPA receptor-regulatory protein (TARP) family modulate AMPA receptor (AMPA-R) trafficking and function. AMPA-Rs consist of four pore-forming subunits. Previous studies show that TARPs are an integral part of the AMPA-R complex, acting as accessory subunits for mature receptors in vivo. The TARP/AMPA-R stoichiometry was previously measured indirectly and found to be variable and dependent on TARP expression level, with at most four TARPs associated with each AMPA-R complex. Here, we use a single-molecule technique in live cells that selectively images proteins located in the plasma membrane to directly count the number of TARPs associated with each AMPA-R complex. Although individual GFP-tagged TARP subunits are observed as freely diffusing fluorescent spots on the surface of Xenopus laevis oocytes when expressed alone, coexpression with AMPA-R–mCherry immobilizes the stargazin-GFP spots at sites of AMPA-R–mCherry, consistent with complex formation. We determined the number of TARP molecules associated with each AMPA-R by counting bleaching steps for three different TARP family members: γ-2, γ-3, and γ-4. We confirm that the TARP/AMPA-R stoichiometry depends on TARP expression level and discover that the maximum number of TARPs per AMPA-R complex falls into two categories: up to four γ-2 or γ-3 subunits, but rarely above two for γ-4 subunit. This unexpected AMPA-R/TARP stoichiometry difference has important implications for the assembly and function of TARP/AMPA-R complexes.

Results

Counting GFP-Labeled AMPA-R Subunits with TIRF Microscopy. In neurons, most GluA1 subunits of AMPA-Rs assemble with GluA2 into GluA1/GluA2 heteromers. When expressed alone in heterologous systems, however, functional homomorphic receptors can be formed by either subunit, which provides a useful system for studying AMPA-R structure and function in vitro. Taking advantage of this property of the AMPA-Rs, we first validated our single-molecule approach in Xenopus laevis oocytes, our

Glutamate is the main excitatory neurotransmitter in the mammalian CNS. Most fast excitatory synaptic transmission in the brain is mediated by AMPA receptors (AMPA-Rs). Four AMPA-R subunits, GluA1–4, contribute to the heterotetrameric assemblies of the AMPA-R (1–4). The localization of AMPA-Rs to the postsynaptic membrane is regulated by a large number of proteins through multiple mechanisms and plays important roles in synaptic plasticity (5–9).

Transmembrane AMPA-R regulatory proteins (TARPs) represent a family of AMPA-R regulatory proteins that are tightly associated with AMPA-Rs, and can be considered auxiliary AMPA-R subunits (10–12). TARPs regulate AMPA-R function by several mechanisms. They mediate the efficient cell surface expression of AMPA-Rs, modulate gating, affect agonist efficacy, and even attenuate intracellular polyamine block of calcium-permeable AMPA-Rs (13–22). The effect of stargazin (γ-2) and other TARPs on GluA1 is not neuron specific and can be accurately mimicked in nonneuronal mammalian cells (18, 23–25), as well as Xenopus oocytes (13, 14, 17, 26, 27), suggesting that the basic mechanisms for TARP/AMPA-R interaction and TARP-mediated regulation of AMPA-R trafficking are preserved in nonneuronal cells.

Although much is known about the interaction domains on the classical TARPs (γ-2, γ-3, γ-4, and γ-8) and AMPA-Rs that mediate assembly and modulation (13, 23, 24, 27, 28), far less is known about the stoichiometry of the TARP/AMPA-R complexes. The magnitude of modulation of AMPA-Rs has been shown to be proportional to the level of TARP expression, suggesting that complex stoichiometry is not fixed (20). One recent study examined the stoichiometry between AMPA-Rs and γ-2 (also called stargazin), and found that AMPA-R/γ-2 complex under overexpression conditions has a variable stoichiometry (one to four γ-2 per complex) but that one γ-2 unit was sufficient to modulate AMPA-R activity. It was also observed that in neurons γ-2 has a fixed stoichiometry on AMPA-Rs (29). Another study showed that increasing TARP expression level increases kainate efficacy (the amplitude of kainate-evoked currents as a fraction of glutamate-evoked current amplitude), with low kainate efficacy for AMPA-R alone, intermediate efficacy when two of the four AMPA-R subunits are fused to a TARP, and maximal kainate efficacy when all four AMPA-R subunits are fused to a TARP or when the free AMPA-R is coexpressed with very high levels of free TARP (30).

We examined the stoichiometry of TARP/AMPA-R complexes directly using a single-molecule method in total internal reflection fluorescence (TIRF) microscopy (31). We find that green fluorescent protein (GFP)-tagged TARP subunits diffuse freely in the membrane of Xenopus oocytes when expressed alone, whereas fluorescently tagged AMPA-R subunits expressed alone are immobile. When they are coexpressed, the TARP subunits colocalize with the AMPA-Rs and become immobile. We examined four TARP family members, γ-2, γ-3, γ-4, and γ-8 (γ-8 function was affected by tagging and therefore not included in the analysis). We find that the TARP/AMPA-R stoichiometry depends on TARP expression level, as shown earlier. Strikingly, the maximum number of TARPs bound to each AMPA-R complex falls into two categories: up to four γ-2 or γ-3 subunits, but only up to two γ-4 subunits.
chosen expression system for the study, by applying it to the stoichiometry of homotetrameric AMPA-Rs.

Our single-molecule technique is based on the photobleaching of GFP tags fused to the protein of interest (31). This technique involves counting irreversible steps of photobleaching of GFP in areas of membrane in intact oocytes where the protein of interest is expressed at a low enough density that individual complexes can be resolved as single fluorescent spots. Under such conditions, the number of bleaching steps reflects the number of GFP tags and thus the number of protein subunits. The high sensitivity and low background necessary for the observation of single fluorescent proteins were achieved by using TIRF microscopy, where a laser beam is reflected at the coverslip/sample interface and excitation is restricted to the plasma membrane of the cell.

We fused GFP to the C terminus of GluA1 (GluA1-GFP) and confirmed the functionality of the GluA1-GFP construct by two-electrode voltage clamping. At 12–24 h after injection of 25 ng of RNA encoding GluA1-GFP per cell, we mechanically removed the vitelline membrane of several cells and placed them on a coverslip. Upon illumination with 488-nm laser light in TIRF, single molecules of GluA1-GFP appeared as bright immobile fluorescent spots on a dark background (Fig. 1A). The immobility of the AMPA-Rs is reminiscent of what is seen in cyclic nucleotide-gated channels and NMDA receptors and may reflect tethering to the cytoskeleton through C-terminal protein-binding motifs (31, 32). Typically, the fluorescence intensity decreased in several discrete steps, indicating photobleaching of the individual GFP tags (Fig. 1B).

We counted the bleaching steps from a total of 568 spots in 14 movies from different cells injected with GluA1-GFP. Most of the spots had either two or three bleaching steps, with a minority bleaching in one or four steps (Fig. 1C, red bars). Rarely, we observed five bleaching steps, which could be accounted for by the rare instance of two receptors within a distance below the diffraction limit. As we had already shown in earlier work, the occurrence of spots with fewer than four bleaching steps can be accounted for by 20% of the GFP tags being nonfluorescent (31). The observed distribution of bleaching steps for GluA1-GFP closely resembles the predicted binomial distribution for homotetramers with an 80% probability of the GFP being fluorescent (Fig. 1C, blue bars).

**TARP Mobility in the Absence and Presence of AMPA-Rs.** We began with an examination of the first TARP identified, Stargazin (γ-2). To visualize γ-2 behavior in the membrane, we expressed the GFP-tagged γ-2 (γ-2-GFP) alone and imaged at high speed using TIRF microscopy. In contrast to the immobile AMPA-Rs, a large fraction of the γ-2-GFP moved laterally in the membrane (Fig. 2A). We predicted that TARPs may become immobile once they bind to the AMPA-Rs, because these are immobile on their own. To test this prediction, we coexpressed γ-2-GFP and GluA1 tagged with the red fluorescent protein mCherry (GluA1-mCherry) and sequentially imaged first the red fluorescence from the immobile GluA1-mCherry to identify the location of AMPA-Rs, and then the green fluorescence from γ-2-GFP. We obtained the trajectories of the γ-2-GFP spots (Fig. 2B) using an automated tracking program and determined for each individual spot the maximum displacement from its starting position, which we used as a criterion for mobility, and the distance to the closest GluA1-mCherry spot, which defined whether or not the γ-2-GFP was colocalized with the GluA1-mCherry. When the two proteins were coexpressed, a large fraction of γ-2-GFP spots colocalized with the

**Fig. 1.** Single-molecule subunit counting of GFP-tagged GluA1. (A) Single molecules of GluA1-GFP in a Xenopus oocyte membrane patch appear as bright spots under 488-nm illumination. The circles mark spots used in bleaching steps statistics. (Scale bar, 2 μm.) (B) Intensity from example spots with four, three, two, and one bleaching steps. The green arrows mark fluorescence intensity levels. (C) Histogram of bleaching steps for GluA1-GFP (red) and fit with 64% probability of GFP to be fluorescent (blue) (a total of 568 spots from 14 experiments was analyzed).

**Fig. 2.** Movement of TARPs with and without coexpression of GluA1 and GluK2. (A) GluA1-GFP was immobile, and γ-2-GFP (γ-2 = stargazin [Stg]) was mobile. Binding to GluA1 immobilized Stg. GluK2 does not bind Stg and did not immobilize Stg. The red crosses in the lower panels mark the AMPA-R positions. (Scale bar, 250 nm.) (B) Histogram of distances from initial positions that Stg-GFP molecules travel until they photobleach, alone (n = 1,192 spots), or coexpressed with GluA1 (n = 584) or GluK2 (n = 457). Mobile fraction shaded light, fraction colocalizing with AMPA-Rs shaded dark. (C) Fractions of mobile spots and spots colocalizing with AMPA-Rs for different amounts of RNA injected (all values in nanograms) for Stg alone or with GluA1 or GluK2 (n = 6–11 movies per condition). (D) Mobile fractions of four TARPs (γ-2, γ-3, γ-4, and γ-8) and γ-1, which does not bind to GluA1, alone or coexpressed with GluA1 (n = 3–8 movies per condition). All error bars indicate SEM.
GluA1-mCherry spots, and the fraction of mobile γ-2–GFP spots decreased (Fig. 2A and B). In contrast, a kainate receptor subunit GluK2 (tagged with mCherry), which is structurally similar to AMPA-Rs, but does not interact with γ-2 (26), did not reduce the movement of γ-2–GFP or colocalize with γ-2–GFP molecules (Fig. 2A and B).

To maximize counting of γ-2–GFP that are associated with AMPA-Rs, we first determined the expression conditions at which most of the γ-2–GFP becomes immobilized by varying the ratio of GluA1-mCherry to γ-2–GFP expression. We changed the amount of injected γ-2–GFP RNA between 0.1 and 2.5 ng per cell while keeping the amount of GluA1-mCherry RNA constant at 25 ng. At injection levels of 0.25 ng or less of γ-2–GFP RNA, the fraction of immobile γ-2–GFP molecules was above 90%. With increasing amounts of γ-2–GFP RNA in the injection mixture, the fraction of mobile γ-2–GFP spots increased and the fraction of γ-2–GFP spots that colocalized with GluA1-mCherry decreased (Fig. 2C).

We next extended our analysis of colocalization and immobilization to other TARPs that are known to interact with and modulate the functions of AMPA-Rs (17, 19, 20, 33). We chose γ-3, γ-4, and γ-8, which are functionally similar to γ-2. We also included γ-1 as a negative control because it does not interact with GluA1 and bears only low homology to γ-2 (16, 26, 34). Based on our observation that a large excess (>100:1) of GluA1-mCherry RNA over γ-2–GFP RNA resulted in an almost complete immobilization of γ-2–GFP, we injected GFP-tagged γ-1, γ-3, γ-4, and γ-8, either alone or together with at least 100-fold excess of GluA1-mCherry. Similar to what we observed with γ-2–GFP, the fraction of mobile γ-3–GFP, γ-4–GFP, or γ-8–GFP spots strongly decreased when GluA1-mCherry was coexpressed. In contrast, γ-1–GFP was not immobilized by GluR1-mCherry (Fig. 2D).

Taken together, these results support previous findings of specific interactions between the classical TARPs (γ-2, γ-3, γ-4, and γ-8) and the GluA1 subunit of AMPA-Rs (13, 17, 26, 27, 33) and establish that TARPs are strong enough to permit biochemical copurification (11), are stable enough in the membranes of live cells to persist for at least tens of seconds.

**Counting Bleaching Steps of TARPs Bound to AMPA-R.** Previous studies have shown that TARPs directly interact with AMPA-Rs (11, 13, 27) and regulate both their localization and gating (14–20, 22). The stoichiometry of TARP/AMPA-R complexes was recently deduced indirectly from functional population assays to be a maximum of four TARPs per AMPA-R complex (30). Having established that TARPs remain bound to immobile AMPA-Rs for the duration of imaging, we decided to directly count the number of TARP subunits present at individual AMPA-R complex using our single-molecule photobleaching assay.

The AMPA-R/TARP stoichiometry deduced from our single-molecule photobleaching assay hinges on one important assumption—the GFP tag on the TARPs does not interfere with its function. In addition, we also prefer to have GluA1 tagged with mCherry to count the photobleaching steps of only the GFP-TARPs associated with AMPA-Rs. Therefore, we next evaluated whether tagging alters the properties of the interaction between TARPs and GluA1.

We first evaluated whether tagging alters the modulatory effect of TARPs on the function of GluA1. Untagged GluA1 cRNAs were injected into oocytes either alone or together with cRNAs encoding either the native untagged TARPs or the GFP-tagged TARPs, and glutamate-evoked currents were measured 1 d after injection using two-electrode voltage-clamp recording. We tested the untagged and tagged versions of all four TARPs. As shown earlier (26, 33), in all of these cases, the expression of untagged versus GFP-tagged TARPs significantly increased the glutamate-evoked currents (Fig. S1). However, whereas the GFP-tagged γ-2, γ-3, and γ-4 enhanced the glutamate-evoked currents to a similar degree as did the untagged γ-2, γ-3, and γ-4, the GFP-tagged γ-8 failed to significantly increase glutamate-evoked current, indicating compromised function (Fig. S1). Thus, we are confident that GFP-γ-2, γ-3, and γ-4 are suitable for the stoichiometry analysis, but that, although we observed clear colocalization of γ-8–GFP and GluA1-mCherry on the surface membrane of oocytes, we could not rule out the possibility that the interference with function by the GFP tag was accompanied by (or even due to) an alteration in stoichiometry. For this reason, although we could determine the stoichiometry of γ-8 (Fig. S2), we left it out of our main analysis and interpretation and focused on γ-2, γ-3, and γ-4.

To determine whether the fluorescent tags on GluA1 disturb the TARP–GluA1 interaction, we repeated a subset of the experiments with untagged GluA1. In the first experiment, we performed single-molecule subunit counting on oocytes in which we coexpressed GFP-labeled γ-2 with untagged GluA1 (Fig. S3A). Similar to the previous results from oocytes coexpressing GluA1-mCherry and γ-2–GFP, we observed up to four GFP bleaching steps from the immobile fluorescent spots, suggesting that up to four γ-2–GFPs can assemble with an untagged GluA1 receptor. When we coexpressed untagged GluA1 with γ-8–GFP, most of the immobile fluorescent spots had one bleaching step and up to 25% of spots had two bleaching steps (Fig. S3B). Very few spots had three bleaching steps, and none had more than three. The near absence of spots with more than two bleaching steps suggests that a maximum of two γ-8-GFPs can assemble with an untagged GluA1 receptor, as observed in the experiments with GluA1-mCherry. The pull-down efficiency for the untagged versus the untagged GluA1 was a higher occurrence of spots with only one bleaching step in the untagged GluA1. This can be explained by γ-8–GFP monomers that are not associated with GluA1 but are immobile. In this experiment with untagged GluA1, these free γ-8–GFPs cannot be distinguished from the GluA1-associated γ-8–GFPs because of the lack of a fluorescence tag on GluA1. In contrast, in the experiments with GluA1-mCherry, free γ-8–GFPs that were not associated with GluA1 were excluded from the statistics because they did not colocalize with the red fluorescence from GluA1-mCherry.

For γ-2 and γ-8, we next examined the pull-down efficiency of coimmunoprecipitation between GFP-tagged TARPs (γ-2 or γ-8) and GluA1 and asked whether the C-terminal mCherry tag on GluA1 could affect the interaction (Fig. S4). The pull-down efficiency for mCherry-GluA1 was 0.62 ± 0.12 for γ-2 (n = 4 independent experiments) and 0.72 ± 0.37 for γ-8 (n = 4 independent experiments) when normalized to untagged GluRA1. This modestly lower efficiency of coimmunoprecipitation fell within the range of variability for pull-down efficiency of GluA1 by the GluA1 antibody (1.00 ± 0.33, n = 4 for the γ-2 experiment; 1.00 ± 0.37, n = 4 for the γ-8 experiments), as well as the range of variability of pull-down efficiency of mCherry-GluA1 by the GluA1 antibody (1.00 ± 0.17, n = 4 for the γ-2 experiments; 1.00 ± 0.23, n = 4 for the γ-8 experiments), all calculated from the data from the same experiments (P > 0.5).

Having established that mCherry-tagging on GluA1 and GFP-tagging on TARPs (with the exception of γ-8) do not seem to interfere with their function, we proceeded to the single-molecule photobleaching experiments. GFP-tagged TARPs were coexpressed with GluA1-mCherry, and green TARP-GFP spots that were colocalized with red GluA1-mCherry spots were analyzed (Fig. 3A and B). For γ-2 and γ-3, we mainly observed spots with one or two bleaching steps at low TARP expression, and as the amount of γ-2 or γ-3 RNA increased (with the amount of GluA1-mCherry RNA held constant), the distribution of bleaching steps shifted toward three and four bleaching steps (Fig. 3C). The behavior of γ-4 was very different from that of γ-2 and γ-3. At low TARP expression levels, most γ-4 spots had one bleeding step, a few with two steps, and even fewer with three bleeding steps (Fig. 3C). At higher γ-4 expression, the fraction of spots with two steps increased more than 30%, but the occurrence of spots with three or four bleaching steps stayed at a very low level (<2%). This observation of low bleeding step numbers was in stark contrast to γ-2 and γ-3, where the shift toward higher occurrence of two bleaching steps was always accompanied by an increase of events with three or four bleaching steps.
Correction for Undercounting Due to Nonfluorescent GFP. To determine the actual numbers of TARP subunits bound to the AMPA-R from the distribution of bleaching steps, we needed to correct for the underestimation of the numbers of GFPs present in each complex due to the 20% of GFP tags that are typically nonfluorescent (31, 32, 35). We corrected for this undercounting to obtain the distribution of TARP subunits for each of the expression levels (Fig. S5). An examination of these distributions showed that γ-2 and γ-3 reached four TARP subunits per AMPA-R, consistent with the receptor having four identical GluA1 subunits that provide four TARP binding sites. However, although four γ-2 and γ-3 subunits were often found, the majority of observations were of three or fewer per receptor. The tendency to have a submaximal number of TARPs per complex was much more pronounced for γ-4, which rarely had three TARP subunits per receptor and almost never had four.

Occupancy of TARP Binding Sites at the AMPA-R. Having seen that the number of TARPs per complex was often less than four, we set out to calculate TARP occupancy, i.e., the fraction of the receptor’s binding sites that was occupied. For illustration of the different behavior of the four TARPs, we use the highest TARP expression levels from the corrected count distributions in Fig. S1. By calculating the least-squares fit of the TARP subunit distributions to a binomial distribution assuming four possible binding sites, we determined the occupancy p (Fig. 4; fits for all expression conditions in Fig. S6).

For γ-2 and γ-3, the fits closely matched the observed distributions (Fig. 4A and B) and gave estimates of high occupancy (p_γ-2 = 0.77 and p_γ-3 = 0.74) for the highest RNA injections. We also obtained a good fit for γ-4 (Fig. 4C), but the occupancy was much lower (p_γ-4 = 0.33). We also performed a least-square fit of each TARP distribution by assuming two binding sites per receptor. The sum of the residuals representing the discrepancy between the data and the estimates from the fit for γ-4 was about equal for the two- and four-binding-site models, but for γ-2 and γ-3 the sum of the residuals was approximately sevenfold and approximately fourfold larger for the model with two binding sites, respectively. Thus, for γ-2 and γ-3, the four-binding-site model gives a better fit; for γ-4, one cannot distinguish between the models.

Dependence of Occupancy on TARP:AMPA-R Ratio. To assess the dependence of occupancy on TARP expression, we calculated p across the series of experiments that used different levels of TARP RNA. Occupancy was plotted semilogarithmically against the ratio of the number of TARP subunits to the number of AMPA-Rs in the field of view (Fig. 4D and Fig. S7) (Materials and Methods). Under the assumption that there are four binding sites for each of the TARPs, γ-2 and γ-3 were seen to increase monotonically toward an individual binding-site occupancy of 1.0, whereas γ-4 only slowly increased and did not rise above an individual binding-site occupancy of 0.4. In contrast, under the assumption of a two-binding-site model, the binding curve of γ-4 increased at a similar steepness to what was seen for γ-2 and γ-3, and reached a maximum occupancy of 0.64.

Discussion

We used a direct subunit-counting approach to explore TARP/AMPA-R stoichiometry under conditions in which TARPs were free to associate with GluA1. By imaging coexpressed GFP-TARP subunits and mCherry-GluA1 subunits via TIRF microscopy, we were able to monitor the TARP-GluA1 interaction via the immobilization of otherwise highly mobile TARPs at sites of GluA1. Our observation of TARP mobility suggests that TARPs do not interact with scaffolding or cytoskeletal proteins in oocytes, but does not say how mobile TARPs would be in neurons where TARPs can interact with postsynaptic density-95 (PSD95) (36). The stability of these interactions and TARP mobility has not been determined. AMPA-R mobility has been studied within synapses using single-molecule tracking (over much longer time periods than our 20-s bouts of observation) and found to be complex, with highly mobile and less mobile pools of AMPA-Rs that are subject to interchange and whose presence could explain at least some aspects of recovery from desensitization experiments (37–39). The immobilization of AMPA-Rs appears likely to reflect several parameters, including anchoring of the AMPA-Rs and TARPs by PSD-95/Discs large/zona occludens-1 (PDZ) domain-containing proteins [e.g., to glutamate receptor-interacting protein (GRIP)
and PSD95, respectively), and protein crowding. In this sense, the oocyte may recapitulate only one aspect of the complex immobilization of AMPA-Rs produced by their anchoring, possibly to the cytoskeleton.

By counting steps of irreversible photobleaching of GFP-labeled TARPs in single GluA1 receptor complexes, we determined the distribution of TARP subunits at hundreds of individual GluA1 receptors on the cell surface. Counting of bleaching steps was done across a wide range of ratios of TARP-to-GluA1 expression, allowing us to observe the saturating level of binding sites in the GluA1 receptor complex by TARPs. We examined four different TARPs: γ-2, γ-3, γ-4, and γ-8 using single-molecule imaging. Three of these TARPs—γ-2, γ-3, and γ-4—provided interpretable results because we could show in control experiments that the GFP-tagged versions of these TARPs were fully capable of normally regulating AMPA-Rs. Consistent with earlier work (33), each of these TARPs associated with GluA1. The TARP-GluA1 associations were stable for the duration of our experiments (up to 40 s), consistent with high affinity binding. Strikingly, we found different maximal occupancies for the different TARPs. For γ-2 and γ-3, we counted up to four TARPs bound to each GluA1 receptor, which agrees with previous studies using neuronal tissues (29, 30) and, importantly, validates our approach. Our analysis of the relationship of occupancy to the TARP-to-GluA1 ratio was consistent with the expectation that a receptor made of four identical subunits provides four γ-2 or γ-3 TARP association sites.

By contrast, we found that the number of TARPs per receptor rarely exceeded two for the γ-4 isoform, and that the slope of the dependence of occupancy on the ratio of TARP-to-GluA1 expression was considerably lower for γ-4 than it was for γ-2 and γ-3. The frequency distributions followed a binomial function with four possible binding sites, but could be equally well fitted by a more parsimonious two–binding-site model. The behavior of γ-4 thus significantly deviated from that of γ-2 and γ-3, suggesting a fundamentally different interaction mode.

One concern about the unexpected behavior of γ-4 is that GFP tagging may affect its function. However, we think this is highly unlikely. Previous studies (29, 30, 40) had shown for γ-2 and γ-8 that there is a dose-dependent effect of TARPs on AMPA-R properties, including the amplitude of glutamate-evoked responses—i.e., the size of glutamate-evoked responses is significantly bigger in four-TARP complex than in two-TARP complexes. We have shown that the tagged γ-4 enhances the glutamate-evoked response ~10-fold, similar to the effect of wild-type, untagged γ-4, indicating that tagging does not interfere with either the normal association of γ-4 with AMPA-Rs or the modulation of AMPA-Rs by γ-4. It is worth pointing out, moreover, that the ~10-fold boost we see with tagged γ-4 is similar to values reported in neurons (33). Thus, our electrophysiological assay provides strong evidence for a normal function of GFP-tagged γ-4, permitting us to conclude that the 2:1 stoichiometry is likely to be correct.

In hippocampal pyramidal neurons, the pharmacological profile of AMPA-Rs (measured as relative efficacy of kainite and glutamate as agonists) most closely resembles that of a receptor complex formed by the heterologous expression of a fusion protein in which the γ-8 subunit is attached to the GluA1 subunit—a situation that is expected to force a stoichiometry of four γ-8 subunits per tetrmeric receptor (30). This seemingly straightforward conclusion may be complicated by several issues. First, AMPA-Rs interact with other proteins, including cytosine-knot AMPA-R–modulating protein 44 (CKAMP44) and cornichons (41, 42); and both AMPA-Rs and their auxiliary subunits, including TARPs, are subject to posttranslational modification—factors that could affect the relative efficacy of kainate vs. glutamate in neurons. Second, it was unclear whether the actions of TARPs are equivalent for tethered and nontethered conditions at each of the stoichiometries. Third, the macroscopic analysis of glutamate-evoked responses could not determine whether the summed current reflected pure populations of AMPA-Rs with a single stoichiometry, either in HEK cells where the fusion may not be 100% preserved because of protease activities, or in neurons where receptors may be loaded with different numbers of TARPs. This does not diminish the importance of those studies, which were the first to address the question of TARP/AMPA-R stoichiometry, but it prevented them from being definitive. Our study addresses the same question with a completely different approach that avoids these pitfalls and has the benefit of determining association by three distinct assays (immobilization, colocalization, and single-molecule counting), each of which reveals the properties of dozens of individual complexes. Our results both confirm earlier interpretation and provide an unexpected observation, namely that TARP/AMPA-R associations may differ among TARPs. Although γ-2 and γ-3 follow the established 4:1 stoichiometry, γ-4 does not, preferring 2:1, suggesting that not all TARPs are created equal.

Although our results suggest that γ-8 may be compromised by GFP tagging, the stoichiometry of the γ-8/AMPA-R complex we observed is similar to that of the γ-4/AMPA-R complex, suggesting that a 2:1 TARP/AMPA-R stoichiometry may be shared by both TARPs. Directly tethering auxiliary subunits, including TARPs, are subject to posttranslational modification—factors that could affect the relative efficacy of kainate vs. glutamate in neurons. It is worth noting, moreover, that the ~10-fold boost we see with tagged γ-4 is similar to values reported in neurons (33). Thus, our electrophysiological assay provides strong evidence for a normal function of GFP-tagged γ-4, permitting us to conclude that the 2:1 stoichiometry is likely to be correct.

Fig. 4. Fit of binding-site occupancy. The probability of each TARP binding site of the AMPA-R to be occupied by a TARP had been fitted (blue) to the distribution of bound TARP subunits (red) as calculated from the experiments with the highest amounts of injected RNA (n = 4–7 per condition). Lower concentrations are in Fig. S1 (A) γ-2 and (B) γ-3 have high occupancy around 0.8, whereas (C) γ-4 has a lower occupancy around 0.3. Four equivalent binding sites were assumed. (D) Occupancy p as a function of TARP-to-AMPA-R ratio for all three TARPs using the model with four binding sites (solid lines) and for γ-4 with the two–binding-site model (dashed lines).

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experiments, where individual protein domains need to be spatially resolved.

Although the molecular basis of the differences in binding for the different TARPs remains to be elucidated, two possible explanations come to mind, which can be addressed in future studies. First, the receptor may have four equal TARP binding sites, but for some TARPs, docking of one TARP may sterically hinder binding of another TARP molecule at a neighboring site, so that for example only two TARPs may bind with high affinity at diagonally situated positions on the receptor. Allosteric effects of TARP binding on receptor conformation could have the same result without there being a direct steric interference. Alternatively, AMPA-Rs may not have four equal docking sites. Indeed, the recent cryo-EM structure of GluA2 provided clues to such a scenario. Whereas the membrane spanning portion of the receptor exhibits a fourfold symmetry, the extracellular domain breaks this symmetry by pairing ligand binding domains into a dimer of dimers and by complex domain swapping between subunits (43). Interestingly, the differences in kainate efficacy between the four TARPs depend on the first extracellular domain between transmembrane segments 1 and 2, where γ-4 differs from γ-2 and γ-3 in possessing an additional proline-rich motif (19). Because the extracellular domains of tetrameric AMPA-Rs exhibit a two-by-two structure of dimers and by complex domain swapping between subunits (43), it is possible that there are only two binding sites for γ-4 or two kinds of binding sites with differing affinities.

The single-molecule approach presented here provides a more detailed view of the interactions between TARPs and AMPA-Rs than is possible to obtain from ensemble measurements of the average readout of many AMPA-Rs. With the future development of more photostable red and blue fluorescent proteins that can be used for single-molecule experiments, it should become possible to determine the subunit composition for two interacting partners at the same time. This will open the way for elucidating complexes formed by mixtures of GluA1 and GluA2 receptor subunits with TARPs, and the interaction between TARPs and other newly discovered AMPA-R-interacting proteins such as cornichons (41) and CKAP44 (42).

Materials and Methods

Microscopy was performed as described in ref. 31. All microscopy data were processed using custom MATLAB (Mathworks), Labview, or Mathematica software. Other related experimental and analysis procedures are described in SI Methods and Materials.

ACKNOWLEDGMENTS. We thank Sarah Bell for help with two-electrode voltage clamp. The work was supported by National Institutes of Health Grants 1R01MH091193 and 1PS05MH084603 (to L.C.), and R01NS35549 and 2P22Y018241 (to E.Y.C.), by the Excellence Initiative of the German Federal and State Governments (EXC 294) (M.H.U.), and by National Basic Research Program of China (973 Program) Grant 2012CB525003.