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Molecular constituents of neuronal AMPA receptors

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Dynamic regulation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) underlies aspects of synaptic plasticity. Although numerous AMPAR-interacting proteins have been identified, their quantitative and relative contributions to native AMPAR complexes remain unclear. Here, we quantitated protein interactions with neuronal AMPARs by immunoprecipitation from brain extracts. We found that stargazin-like transmembrane AMPAR regulatory proteins (TARPs) copurified with neuronal AMPARs, but we found negligible

binding to GRIP, PICK1, NSF, or SAP-97. To facilitate purification of neuronal AMPAR complexes, we generated a transgenic mouse expressing an epitope-tagged GluR2 subunit of AMPARs. Taking advantage of this powerful new tool, we isolated two populations of GluR2 containing AMPARs: an immature complex with the endoplasmic reticulum chaperone immunoglobulin-binding protein and a mature complex containing GluR1, TARPs, and PSD-95. These studies establish TARPs as the auxiliary components of neuronal AMPARs.

Introduction

Most excitatory synapses in brain use glutamate as a neurotransmitter. Fast signaling at these synapses occurs primarily by activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type and *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, and these two receptors sub-serve different functions at the synapse. AMPA receptors (AMPA) are glutamate-gated monovalent cation channels whose activation provides most of the postsynaptic depolarization that drives neuronal firing. NMDA receptors are permeable both to monovalents and to calcium, which induces synaptic plasticity. This NMDA receptor-dependent synaptic plasticity involves rapid changes in the density of synaptic AMPARs.

AMPA channels comprise heterotetramers of subunits GluR1–4 (Hollmann and Heinemann, 1994). Furthermore, a variety of proteins can interact with AMPARs *in vitro*. Most of these associated proteins were identified by yeast two-hybrid screening using the cytoplasmic tails of AMPAR subunits. Many AMPAR-interacting proteins contain PDZ motifs that bind to the extreme COOH-terminal tails of specific AMPAR subunits. The final four amino acids of GluR2/3 bind to the

PDZ proteins GRIP1/ABP and PICK1 (Dong et al., 1997; Srivastava and Ziff, 1999; Xia et al., 1999). The COOH terminus of GluR1 binds to the PDZ protein SAP-97 (Leonard et al., 1998). More proximal regions of the AMPAR subunits can also bind to non-PDZ proteins. The adaptor protein AP-2 and the *N*-ethyl maleimide sensitive factor NSF, which are proteins involved in vesicle budding and fusion, bind to overlapping regions in GluR2/3 (Song et al., 1998; Luthi et al., 1999; Noel et al., 1999; Lee et al., 2002). Finally, the cytoskeletal protein band 4.1N associates with GluR1 and 4 (Shen et al., 2000). Genetic studies have not yet established that any of these interactions with the cytoplasmic tails of AMPARs are essential for receptor trafficking.

The first transmembrane protein found to interact with AMPARs is stargazin (Chen et al., 2000), which is mutated in stargazer mice (Letts et al., 1998). These mice show absence epilepsy and lack functional AMPARs at cerebellar granule cells (Chen et al., 1999, 2000; Hashimoto et al., 1999). Stargazin plays two roles in trafficking AMPARs to synapses. First, stargazin can associate with all four AMPAR subunits and traffic them to the plasma membrane. Second, the extreme COOH terminus of stargazin can bind to PSD-95 and other PDZ proteins to mediate synaptic clustering of AMPARs (Chen et al., 2000). Although AMPARs function normally in forebrain of stargazer mice, a family of related transmembrane AMPAR regulatory proteins (TARPs) is expressed in brain and can also traffic AMPARs (Tomita et al., 2003). Therefore, the TARP mechanism may be universal in trafficking AMPARs in all neurons. Despite

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Abbreviations used in this paper: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; CBP, calmodulin-binding peptide; IAP, immunoprecipitation; LTD, long-term depression; NMDA, *N*-methyl-D-aspartate; TARP, transmembrane AMPAR regulatory protein.

The online version of this article includes supplemental material.

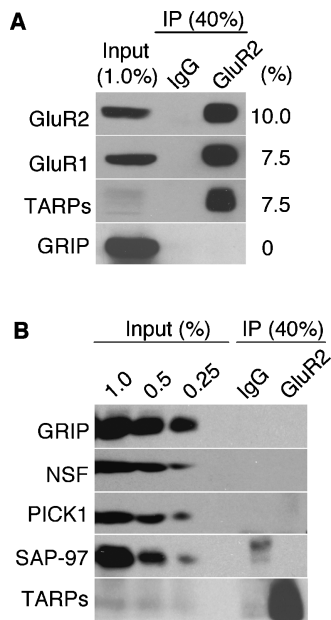


Figure 1. **Immunoprecipitation of AMPARs from wild-type mice.** (A and B) Brain extracts solubilized with 1% Triton X-100 were used for immunoprecipitation with anti-GluR2 antibody or nonimmune mouse IgG. AMPAR subunits and previously reported AMPAR binding proteins (see text) were examined by immunoblotting. Input lanes contain the indicated percentage of proteins used for immunoprecipitation (IP). (A) The percentages of binding (%) are indicated.

this wealth of AMPAR binding proteins, their relative and quantitative contribution to neuronal AMPARs remains uncertain. Also unclear is whether the cytoplasmic AMPAR-interacting proteins and TARPs bind to distinct or overlapping populations of AMPAR proteins. Here, we used immunoprecipitation studies to quantify protein interactions with neuronal AMPARs.

Results and discussion

We solubilized whole brain extracts with 1% Triton X-100 and purified AMPAR complexes using a well-characterized antibody to GluR2. Under these conditions, ~10% of GluR2 was isolated and along with it came ~7.5% of total GluR1 (Fig. 1 A). Recovery of TARPs showed similar efficiency. In contrast, we were unable to detect any of the other reported AMPAR binding proteins including GRIP, NSF, PICK1, or SAP-97 (Fig. 1, A and B).

To permit more efficient isolation of GluR2 complexes, we designed an epitope-tagged GluR2 construct that has both a FLAG-recognition site and a calmodulin-binding peptide (CBP), which permit efficient and selective protein isolation from crude homogenates (Yang et al., 2002; Fig. 2 A). We first used this construct in heterologous expression systems and found that it expressed an appropriate and functional GluR2. As expected, the expressed protein migrated at a slightly larger monomeric molecular mass than did GluR2 without the engineered tags (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200501121/DC1>). We found that glutamate-evoked currents from this GluR2 expressed in *Xenopus laevis* oocytes were vastly increased by co-expression of stargazin cRNA (Fig. 2 B). We also determined

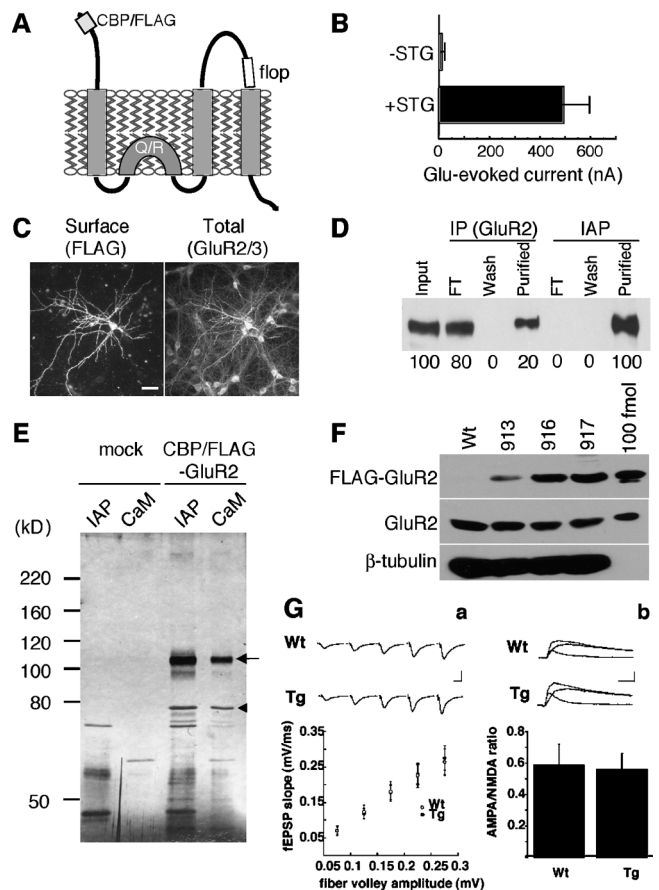


Figure 2. **Functional expression of CBP/FLAG-GluR2 in heterologous cells and in transgenic mouse brain.** (A) Schematic presentation of CBP/FLAG-GluR2. Calmodulin-binding peptide (CBP) and FLAG peptide sequences were inserted after the signal sequence of mouse GluR2 (flop form edited at position 586 (Q/R)). (B) In *X. laevis* oocytes expressing CBP/FLAG-GluR2, glutamate-evoked currents were vastly increased by coexpression of stargazin cRNA. (C) Surface receptors expressed in hippocampal neurons were live labeled with anti-FLAG M2 and total GluR2 was stained with anti-GluR2/3. Bar, 20 μ m. (D) More efficient isolation of CBP/FLAG-GluR2 by immunoaffinity purification (IAP; anti-FLAG M2 agarose) than by conventional immunoprecipitation (anti-GluR2 antibody). Almost 100% of solubilized CBP/FLAG-GluR2 was isolated by IAP. FT, flow-through. (E) Two-step purification of CBP/FLAG-GluR2. The extracts of HEK cells transfected with mock vector or CBP/FLAG-GluR2 were subjected to sequential affinity chromatography: IAP and calmodulin-affinity chromatography (CaM). The arrowhead indicates the copurified 78-kD protein (identified as BiP/Grp78 by mass spectrometry). (F) Whole brain extracts (50 μ g) from the indicated transgenic mouse lines and 100 fmol of CBP/FLAG-GluR2 purified from HEK cells were probed with the indicated antibodies. CBP/FLAG-GluR2 represents ~50% of the endogenous protein in line 917. (G) Basal synaptic transmission is normal in transgenic GluR2 mice. (a) Input-output curve for basal synaptic transmission in hippocampal slices from wild type (Wt; $n = 16$) and transgenic (Tg; $n = 18$) mice. Each point represents the mean \pm SEM for each bin. Sample fEPSPs at different stimulus intensities are shown on top. Bars: (y-axis) 0.5 mV; (x-axis) 20 ms. (b) AMPA/NMDA ratios were calculated by evoking dual-component EPSCs (Wt, $n = 5$; and Tg, $n = 9$). Histogram represents the AMPA/NMDA ratio mean \pm SEM. Sample traces of the mixed and isolated AMPA- and NMDA-mediated EPSCs from wild-type and transgenic mice are shown on top. Bars: (y-axis) 100 pA; (x-axis) 20 ms.

that this construct was expressed appropriately on the surface of transfected hippocampal neurons (Fig. 2 C).

Immunoaffinity purification (IAP) from transfected HEK293T cells using FLAG antibody-conjugated agarose al-

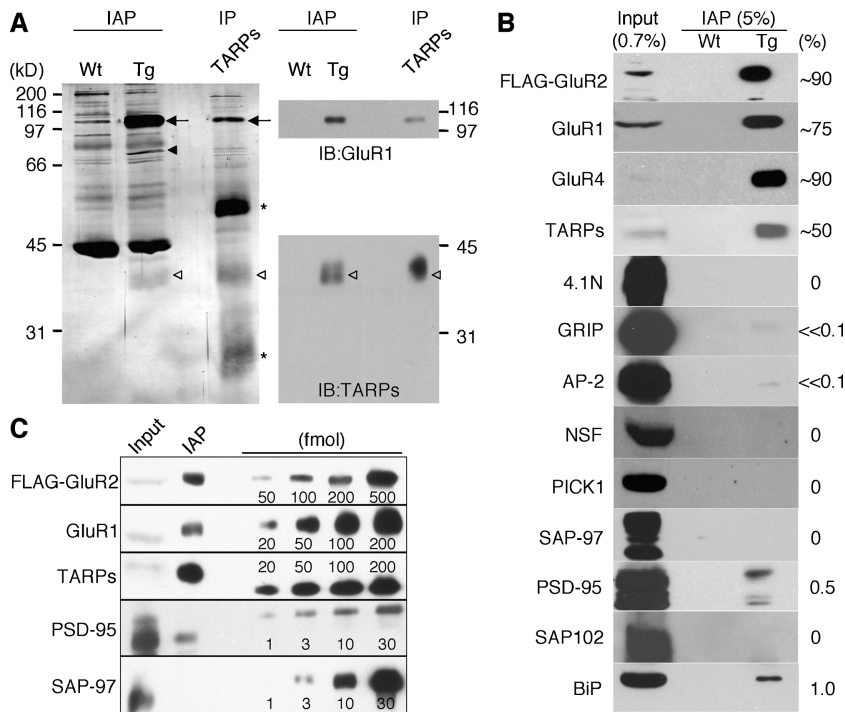


Figure 3. Quantitative association of TARPs with immunopurified AMPARs. (A) Gold colloidal total protein staining of immunoaffinity-purified CBP/FLAG-GluR2 (IAP) and immunoprecipitated TARPs (IP). The same preparations were also analyzed by Western blotting with anti-GluR1 or TARP antibody. The proteins with molecular masses of 110 (arrow), 78 (closed arrowhead), and 35 kD (open arrowhead) were detected in the transgenic mouse (Tg) but not in wild-type (Wt). 110- and 35-kD proteins were also detected in TARPs-IP, which correspond to AMPARs including GluR1 and TARPs, respectively. Asterisks denote the bands of IgG heavy and light chains. (B) IAP elution in A was immunoblotted with the indicated antibodies. The recovery (%) of each protein from input is indicated. (C) Quantitative binding of TARPs with AMPARs. For CBP/FLAG-GluR2, GluR1, and TARPs, input (1%) and purified AMPARs (IAP; 1%) from transgenic mouse brain were analyzed with the indicated amounts of purified CBP/FLAG-GluR2, HA-GluR1, and His₆-stargazin COOH terminus. For PSD-95 and SAP-97, input (0.1%) and IAP elution (4%) were analyzed with purified PSD-95-GFP and SAP-97-GFP. These data are representative of five independent experiments.

lowed us to quantitatively isolate the expressed GluR2. Immunoblot analysis revealed that GluR2 was more efficiently isolated with the IAP method than with conventional anti-GluR2 immunoprecipitation (Fig. 2 D). Silver staining of eluates showed specific bands of 110 and 78 kD (Fig. 2 E). These interactions were specific because both protein bands copurified after the application to calmodulin resin. The 110 kD corresponds to GluR2. Mass spectrometry analysis revealed that the 78-kD protein is BiP/Grp78 (immunoglobulin binding protein/glucose regulated protein 78), a ubiquitous protein chaperone that occurs in the ER. Previous studies showed that BiP also binds to AMPARs in brain extracts (Rubio and Wenthold, 1999).

To use our epitope-tagged construct for isolation of AMPAR complexes from brain, we developed a transgenic mouse model. We put the CBP/FLAG-GluR2 construct downstream of the Thy 1 promoter. Highest protein expression in brain was found in line 917, and this line was used for subsequent studies (Fig. 2 F and Fig. S1 B). As expected, the Thy 1 promoter drove protein expression exclusively in brain and not in peripheral tissues (Fig. S1 C; Luthi et al., 1997). We found GluR2 transgene expression in the cerebral cortex, hippocampus, and striatum and much less expression in the cerebellum (Fig. S1 D). Basal synaptic transmission in transgenic mice was not different from wild-type mice (Fig. 2 G).

We used brains from these mice to isolate neuronal AMPAR complexes. Forebrain extracts were solubilized with Triton X-100. Purification from transgenic mouse brain extracts yielded protein bands of 110, 78, and 35 kD (Fig. 3 A). Quantitative Western blotting showed that the 110-, 78-, and 35-kD proteins corresponded to AMPAR subunits, BiP, and TARPs, respectively (Fig. 3, A–C; and see Fig. 4). We saw no other protein bands copurified specifically with AMPARs. As previously described (Tomita et al., 2004), when TARPs were im-

muno-precipitated from brain, a protein complex comprising 110- and 35-kD proteins, corresponding to AMPARs and TARPs respectively, was isolated (Fig. 3 A).

To evaluate the composition of the endogenous AMPAR complex more carefully, we used additional antibodies for Western blotting (Fig. 3 B). Again, we found that GluR1, GluR4, and TARPs copurified with FLAG-tagged GluR2. High recovery of GluR1 and GluR4 indicates that physiologically relevant AMPARs were isolated. In contrast, we found no significant association of protein 4.1N, GRIP, AP-2, NSF, PICK1, SAP-97, or SAP-102. We did find some association of PSD-95 with the purified AMPAR complex (Fig. 3, B and C). However, the recovery of PSD-95 was ~0.5%, which confirms that PSD-95 does not directly bind to AMPARs (see Fig. 4) and indicates that a large proportion of PSD-95 occurs in other neuronal protein complexes. Quantitative Western blots revealed that CBP/FLAG-GluR2, GluR1, and TARPs are at the molar ratio of ~5:1:1 in the purified complex (Fig. 3 C; representative from five independent experiments). Our failure to isolate previously reported binding proteins in our AMPAR complex cannot be explained by poor recovery, as we isolated ~90% of the solubilized GluR2 (Fig. 3 B and Fig. S1 E). We considered that our conditions may not have solubilized synaptic AMPAR complexes. Therefore, we repeated our procedure using deoxycholate, which provides a more efficient extraction of cytoskeletal and synaptic proteins (Luo et al., 1997; Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200501121/DC1>). Deoxycholate severely reduced the recovery of purified AMPARs and TARPs, and increased nonspecific binding (Fig. S2, Wt). However, we again found that TARPs interacted with AMPARs more efficiently than other proteins.

We wondered whether our purification isolated a single AMPAR complex or multiple ones with distinct compositions.

To address this question, we reprecipitated the protein complexes with antibodies to either BiP or TARPs (Fig. 4). Silver staining revealed that BiP reprecipitation quantitatively isolated 110- and 78-kD proteins, whereas TARP reprecipitation quantitatively isolated 110- and 35-kD proteins, confirming that 78- and 35-kD protein bands that copurified with AMPARs (Fig. 3 A) are BiP and TARPs, respectively. The BiP reprecipitation showed that this ER chaperone associates with GluR2 alone, as we found no association with GluR1, PSD-95, or TARPs. In contrast, we found that the TARP reprecipitates contained both GluR1 and PSD-95. This finding suggests that BiP binds to an immature GluR2 complex whereas TARPs bind to a more mature one containing heteromeric GluR subunits and the synaptic tether PSD-95.

This study identified stargazin as the only major detectable auxiliary component of mammalian AMPARs. Our failure to identify cytosolic AMPAR-binding proteins in immunoprecipitates from forebrain extracts conflicts with data and conclusions from numerous previously published papers (Osten et al., 1998, 2000; Nishimune et al., 1998; Song et al., 1998; Srivastava et al., 1998; Luthi et al., 1999; Noel et al., 1999; Xia et al., 1999; Daw et al., 2000; Perez et al., 2001; Hanley et al., 2002; Lee et al., 2002; Wyszynski et al., 2002; Hirbec et al., 2003; Lee et al., 2004). Reasons for this discrepancy are not clear, but likely do not reflect differences in methodology; we carefully followed previously published protocols and used at least two kinds of solubilization conditions for all our experiments. One possibility is that the previously published studies were not done quantitatively and were not done with comparison to TARPs. These studies may have identified minimal amounts of cytosolic-interacting proteins that were dismissed as negligible in our experiments. We cannot discount the possibility that these previously identified AMPAR-interacting proteins bind under specialized circumstances or bind to receptors that are not solubilized in nondenaturing detergents. However, our protocol did solubilize ~90% of total AMPARs, so the previously reported non-TARPs seem not to be general or core-interacting proteins with the AMPAR complex.

Genetic analyses support our contention that stargazin, but not other known proteins, plays a fundamental role in trafficking of AMPARs. Mice lacking stargazin lack surface AMPARs in specific neuronal populations (Hashimoto et al., 1999; Chen et al., 2000). In addition to mediating synaptic AMPAR trafficking, identified cytosolic proteins are proposed to participate in synaptic plasticity. A widely published "subunit rule" model posits that protein interactions with the tail of GluR1 mediate hippocampal long-term potentiation by trafficking GluR1/2 heteromers, whereas proteins interacting with GluR2/3 heteromers mediate hippocampal long-term depression (LTD; Shi et al., 2001; Lee et al., 2004). However, genetic studies are generally not in accord with the currently defined models. Mice lacking GluR1 retain significant postsynaptic long-term potentiation during development and in certain regions of mature hippocampus (Zamanillo et al., 1999; Jensen et al., 2003). Mice lacking GluR3 or GluR2 can only express heteromers containing GluR1. That these mice show normal LTD (Jia et al., 1996; Meng et al., 2003) conflicts with the model that trafficking of GluR2/3 heteromers

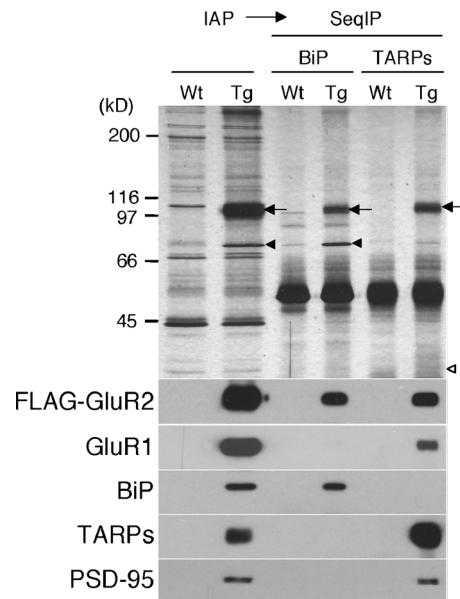


Figure 4. Two distinct AMPAR complexes. Immunoaffinity-purified brain AMPAR complexes (IAP) were reprecipitated (seqIP) with antibodies to either BiP or TARPs and analyzed by silver staining (top) and immunoblotting with the indicated antibodies (bottom). The BiP reprecipitation isolates 110- (arrow) and 78-kD (closed arrowhead; BiP) proteins; the TARP reprecipitation isolates 110- and 35-kD (open arrowhead; TARPs) proteins. The BiP reprecipitates include only GluR2, but not GluR1, TARPs, or PSD-95, whereas TARP reprecipitates include GluR1 and PSD-95 in addition to GluR2.

mediates LTD. Even more problematic for such a model is that mice lacking both GluR2 and GluR3, which form GluR1 homomers, also show LTD (Meng et al., 2003). Although we cannot fully discount the possibility that the currently defined subunit rules play roles in specialized circumstances or in isolated neuronal populations, they are not necessary for established plasticity models in hippocampus, where they were initially reported.

Agreeing with previous studies (Rubio and Wenthold, 1999), we find a small population of AMPARs that associates with the chaperone BiP. This population of subunits likely represents immature receptors, as BiP resides in the ER. Indeed, previous studies suggested that a subpopulation of GluR2 subunits resides in a monomeric or dimeric state in the ER (Greger et al., 2003). Accordingly, we find that the BiP-associated GluR2 subunits are homomeric and are not associated with other proteins. Association of AMPARs with TARPs is entirely independent from their interaction with BiP. This may suggest that TARPs displace BiP from GluR2 subunits to facilitate surface trafficking. This would fit with our previous results showing that TARPs facilitate AMPAR progression through the secretory pathway (Tomita et al., 2003).

Data here also indicate that TARPs play roles beyond that of chaperone, as TARPs remain associated with AMPARs at the synapse. This work, together with the aforementioned genetic studies by others, questions the general importance of previously described non-TARP mechanisms for receptor trafficking. These data establish a unique role for TARPs, as integral components essential for trafficking and synaptic function of neuronal AMPARs.

Materials and methods

Antibodies

The following antibodies were used: rabbit polyclonal antibodies to GluR1, GluR2/3, and GluR4 (Chemicon), TARPs (Tomita et al., 2003), GRIP (a gift from R. Huganir, Johns Hopkins University, Baltimore, MD), and NSF and PICK1 (Santa Cruz Biotechnology, Inc.); mouse monoclonal antibodies to GluR2 (Chemicon), SAP97 and BiP (StressGen Biotechnologies), PSD-95 (MA1-046; Affinity BioReagents, Inc.), FLAG (M2), β -tubulin and AP-2 (Sigma-Aldrich), and 4.1N (BD Biosciences).

Generation of transgenic mice

CBP and FLAG peptide sequences were inserted after the signal sequence of mouse GluR2 cDNA (CBP/FLAG-GluR2). For generation of transgenic mice, cDNA of CBP/FLAG-GluR2 was subcloned into the Thy 1-expression cassette (a gift from D. Monard, Friedrich Miescher Institut, Basel, Switzerland; Luthi et al., 1997). Transgenic mice were generated by Y. Chen-Tsai (Stanford Transgenic Research Facility, Stanford, CA). Eight transgenic founders were crossed with C57BL/6J mice to produce the CBP/FLAG-GluR2 transgenic lines.

Immunoprecipitation from mouse brain

Mouse brains were homogenized with buffer A (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA, 0.32 M sucrose, and 100 μ g/ml PMSF). P2 membrane fractions were solubilized with buffer B (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 50 μ g/ml PMSF) for 1 h. After centrifugation at 37,000 g for 1 h, the extracts were precleared by the addition of protein A-Sepharose (Amersham Biosciences). The supernatant (~5–8 mg of protein) was incubated with 5 μ g of anti-GluR2 mAb (Chemicon) or anti-TARP antibody for 2 h, and then with 50 μ l of protein A-Sepharose for 1 h.

IAP of CBP/FLAG-GluR2

CBP/FLAG-GluR2 expressed in HEK cells (one 10-cm plate) was tandemly purified using anti-FLAG M2 agarose and CaM Sepharose as described previously (Yang et al., 2002), except for the use of Triton X-100 instead of CHAPS. For purification of CBP/FLAG-GluR2 from transgenic mice, forebrain extracts from wild-type or transgenic mice were prepared as described in the previous section. Deoxycholate extraction followed the method of Luo et al. (1997). Solubilized and precleared proteins (~5–8 mg protein) from P2 membrane fractions were incubated with 75 μ l of FLAG-M2 agarose (Sigma-Aldrich) for 2 h, washed with buffer B six times, and eluted with 375 μ l of 0.25 mg/ml FLAG peptide (Sigma-Aldrich) in buffer B. Purified proteins were separated by SDS-PAGE and transferred to PVDF membranes. Proteins on the membrane were visualized by colloidal gold total protein stain (Bio-Rad Laboratories) or Western blotting analysis. For quantitative Western blot analysis, CBP/FLAG-GluR2, HA-GluR1, PSD-95-GFP, or SAP-97-GFP was expressed in HEK cells and purified by immunoprecipitation with anti-FLAG, HA, or GFP antibody. Purified proteins were quantitated by Coomassie brilliant blue or gold colloidal staining using BSA for calibration. Scanned signals were analyzed by NIH Image software. For reprecipitation of BiP and TARP from immunoprecipitated AMPAR complexes, the IAP elution was incubated with anti-BiP or anti-TARP antibody, and then with protein A-Sepharose.

Mass spectrometry analysis

The protein band migrating at 78 kD was excised from a silver-stained gel, reduced with 10 mM dithiothreitol, and alkylated with iodoacetamide. This extract was digested with trypsin (12 ng/ μ l) overnight at 37°C. The extracted peptides were then separated via HPLC using a reverse phase C-18 column (LC Packings) on a 1100 series HPLC (Agilent Technologies). The LC eluent was coupled to a micro-ion-spray source attached to a QSTAR Pulsar mass spectrometer (MDS Sciex). 44 peptides obtained from the 78-kD band were identified for BiP and these peptides accounted for 57% of the entire BiP sequence (NP_005338).

Image acquisition

Microscopic image of hippocampal neurons was taken at RT using a fluorescence microscope (Axiovert S100TV; Carl Zeiss MicroImaging, Inc.) with a 20 \times /0.75 NA objective and a charge-coupled device camera (model CA742-95; Hamamatsu) controlled by Metamorph software (Ver. 4.1.3; Universal Imaging Corp.). Adobe Photoshop was used for image cropping and adjustment.

Electrophysiology

Electrophysiology using *X. laevis* oocytes was performed as described previously (Tomita et al., 2004). Electrophysiology in hippocampal slices was performed as described previously (Schnell et al., 2002).

Online supplemental material

Fig. S1 shows the expression pattern of CBP/FLAG-GluR2 in generated transgenic mice and efficient purification of CBP/FLAG-GluR2 from transgenic mouse brain. Fig. S2 shows protein composition of AMPAR complexes purified from the deoxycholate-extracted brain. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200501121/DC1>.

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