



LTP requires postsynaptic PDZ-domain interactions with glutamate receptor/auxiliary protein complexes

Nengyin Sheng^{a,b,c,1}, Michael A. Bemben^c, Javier Díaz-Alonso^c, Wucheng Tao^c, Yun Stone Shi^{c,d}, and Roger A. Nicoll^{c,e,1}

^aState Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, 650223 Yunnan, China; ^bCenter for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming, 650223 Yunnan, China; ^cDepartment of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143; ^dThe Model Animal Research Center, Key Laboratory of Model Animal for Disease Study of Ministry of Education, Nanjing University, 210061 Nanjing, China; and ^eDepartment of Physiology, University of California, San Francisco, CA 94143

Contributed by Roger A. Nicoll, February 23, 2018 (sent for review January 16, 2018; reviewed by Rodrigo Andrade and Christian Luscher)

Long-term potentiation (LTP) is a persistent strengthening of synaptic transmission in the brain and is arguably the most compelling cellular and molecular model for learning and memory. Previous work found that both AMPA receptors and exogenously expressed kainate receptors are equally capable of expressing LTP, despite their limited homology and their association with distinct auxiliary subunits, indicating that LTP is far more promiscuous than previously thought. What might these two subtypes of glutamate receptor have in common? Using a single-cell molecular replacement strategy, we demonstrate that the AMPA receptor auxiliary subunit TARP γ -8, via its PDZ-binding motif, is indispensable for both basal synaptic transmission and LTP. Remarkably, kainate receptors and their auxiliary subunits Neto proteins share the same requirement of PDZ-binding domains for synaptic trafficking and LTP. Together, these results suggest that a minimal postsynaptic requirement for LTP is the PDZ binding of glutamate receptors/auxiliary subunits to PSD scaffolding proteins.

long-term potentiation | glutamate receptor | auxiliary protein | PDZ-binding domain

One of the most remarkable properties of excitatory synapses in the brain is their ability to undergo long-lasting functional changes, referred to as long-term potentiation (LTP). Most glutamatergic excitatory synaptic transmission is mediated by AMPA receptors (AMPA) and NMDA receptors (NMDARs). It is generally accepted that LTP requires the activation of NMDARs, which, via CaMKII and a series of poorly understood steps, results in the rapid accumulation of AMPARs at the activated synapses (1–5). Contrary to existing dogma, recent work has demonstrated that LTP is surprisingly promiscuous and any AMPAR subunit expresses normal LTP (6). Even more remarkable, kainate receptors (KARs), a separate class of glutamate receptor with very limited homology to AMPARs that are normally not expressed at these synapses, express normal LTP (6). Furthermore, in addition to their limited homology, AMPARs and KARs assemble with distinct auxiliary subunits; transmembrane AMPAR regulatory proteins (TARPs) assemble with AMPARs (7–9) and Neto proteins assemble with KARs (9, 10). What is the basis for the promiscuity? What might these two classes of receptor have in common? Providing answers to these questions might shed light on fundamental underlying steps in LTP.

TARPs comprise a family of proteins, which share considerable sequence homology, including the presence of a C-terminal PDZ-binding motif (11), and therefore would be expected to have similar properties. Although it is well established that TARPs selectively bind to AMPAR subunits and that their PDZ-binding motifs are essential and specific for the synaptic trafficking of AMPARs in cerebellar granule cells, they are widely expressed throughout the brain and this model has been difficult to apply to other synapses (12–15). In the hippocampus, γ -8 is the highest expressed TARP; however, the presence of other TARPs has obscured its role in basal synaptic transmission and synaptic plasticity in pyramidal neurons (15, 16). Here we have used a

single-cell molecular replacement strategy to demonstrate that both basal synaptic transmission and LTP require the PDZ-binding motif of the AMPAR auxiliary subunit TARP γ -8. Remarkably, kainate receptors and their auxiliary subunits Netos share the same requirement of PDZ-binding domains for synaptic trafficking and LTP. These results suggest that the PDZ binding of glutamate receptors/auxiliary subunits to PSD scaffolding proteins is the minimal postsynaptic requirement for LTP.

Results

Our understanding of AMPAR trafficking in the hippocampus has been hampered by the fact that pyramidal cells express multiple TARPs with seemingly redundant roles (15–20). To circumvent this redundancy, we developed a strategy to replace the endogenous AMPARs with GluA1-TARP tethered receptors (Fig. S1). We used in utero electroporation in triple-floxed mice (*Gria1^{fl/fl}Gria2^{fl/fl}Gria3^{fl/fl}*) followed by recording from acute slices at postnatal days 17–21 (P17–P21). This allowed us to examine, in isolation, the properties of the tethered receptor, using simultaneous dual whole-cell recordings from a transfected neuron and a neighboring control neuron (6, 21, 22). Consistent with our previous studies (21), we verified that expressing Cre alone in CA1 neurons deleted all AMPAR function (Fig. 1A). We next coexpressed GluA1 that was tethered to the full-length γ -8 (GluA1- γ -8) to replace the endogenous AMPARs. In this case we recorded a full rescue of the synaptic responses (Fig.

Significance

Long-term potentiation (LTP) is the most compelling cellular and molecular model for learning and memory. Both AMPARs and KARs, two separate classes of glutamate receptor with very limited homology, express normal LTP in pyramidal neurons. However, the general underlying molecular mechanism remains a mystery. Here, with the strategy of single-cell molecular replacement we show that the PDZ-binding domains of AMPAR/TARP and KAR/Neto receptor complexes are essential for basal synaptic transmission and LTP. Our work suggests that the glutamate receptors share the same postsynaptic requirement for LTP.

Author contributions: N.S. and R.A.N. designed research; N.S., M.A.B., J.D.-A., W.T., and Y.S.S. performed research; M.A.B., J.D.-A., W.T., and Y.S.S. contributed new reagents/analytic tools; N.S. and R.A.N. analyzed data; and N.S. and R.A.N. wrote the paper.

Reviewers: R.A., Wayne State University School of Medicine; and C.L., University of Geneva.

The authors declare no conflict of interest.

Published under the PNAS license.

¹To whom correspondence may be addressed. Email: shengnengyin@mail.kiz.ac.cn or roger.nicoll@ucsf.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1800719115/-DCSupplemental.

Published online March 26, 2018.

of stargazin (19) or TARP γ -8 (18) impair LTP. When we expressed the tethered TARP γ -8 containing the phosphor-null mutations (18), basal synaptic transmission was not fully rescued (Fig. S3A), but LTP in these neurons was normal (Fig. S3B). These results suggest that these sites are not essential for the expression of LTP. Perhaps the differences in results stem from different experimental strategies. Another possibility is that, while the phosphorylation of the TARP C-tail might contribute to LTP of heteromeric AMPARs, it may play a minimal role during LTP of GluA1 homomeric receptors, as we report in this study.

One of the most surprising recent results in the LTP field is the finding that the CA1 neurons, in which the endogenous AMPARs were replaced with KAR/Netos (GluK1/Neto2) that have limited homology with AMPARs and do not bind to TARPs, exhibit normal LTP expression (6). What might these two subclasses of glutamate receptors have in common? Might the critical role of the PDZ-binding domain of γ -8 provide insight into the seemingly promiscuous nature of LTP? It has been found that GluK2 binds to the postsynaptic scaffolding protein PSD-95 through the last four amino acids EMTA (25). Our previous studies have shown that cotransfection of Neto1 or Neto2 proteins is essential for GluK1 synaptic targeting (26), suggesting that pyramidal cells do not express appreciable amounts of either Neto1 or Neto2. Therefore, Neto1 or Neto2 was coexpressed with GluK1 to replace the endogenous AMPARs for later electroporation and electrophysiological experiments. It should be noted that the last four amino acids of GluK1 (ETVA) and Neto1 (TTRV) share homology with the type 1 PDZ-binding motif. We therefore first examined whether the GluK1/Neto1 receptor complex can bind to PSD-95. When PSD-95 was expressed together with wild-type HA-tagged GluK1 and Neto1 in HEK cells, we were able to immunoprecipitate PSD-95 with the HA antibody (Fig. 2). In contrast, when the mutants without the last four amino acids (HA-GluK1 Δ 4 and Neto1 Δ 4) were used for coexpression, the level of coimmunoprecipitated PSD-95 was greatly reduced (Fig. 2), indicating that the last four amino acids in GluK1 and Neto1 are functional PDZ-binding motifs mediating their interaction with PSD-95. These results suggest that the PDZ-binding motifs in the GluK1/Neto1 receptor complex might also be necessary for the receptors' interaction with postsynaptic scaffolding proteins and

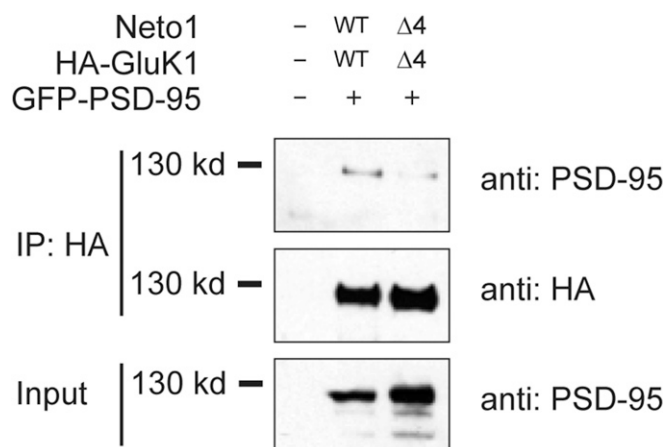


Fig. 2. GluK1/Neto1 receptor complex interacts with PSD-95 through PDZ-binding motifs. HEK293T cells were transfected with expression plasmids of GFP-tagged PSD-95 together with wild-type HA-tagged GluK1 and Neto1 or respective mutants deleting the last four amino acids (HA-GluK1 Δ 4 and Neto1 Δ 4) as indicated. Western blot was performed to analyze the levels of PSD-95 and GluK1 in the HA immunoprecipitates (IP) and whole-cell lysate (Input) with indicated antibodies.

synaptic trafficking, similar to the requirement of PDZ domain interaction for AMPAR/TARPs.

As an initial test of this hypothesis, we overexpressed these constructs in slice cultures on a wild-type background. As previously reported (26), both Neto1 (Fig. S4A and E) and Neto2 (Fig. S4B and E) promoted GluK1 receptor targeting to synapses, leading to a large enhancement of synaptic currents mediated by GluK1. As predicted, exogenous coexpression of mutated GluK1 Δ 4 and Neto1 Δ 4 failed to enhance synaptic transmission (Fig. S4C and E). Similarly, expression of GluK1 Δ 4 together with Neto2 Δ 4 substantially reduced the enhancement (Fig. S4D and E). It is important to note that deleting the last four amino acids in either Neto1 or Neto 2 alone, while keeping the last four amino acids in GluK1 intact, had no effect (26), suggesting functional redundancy.

We next returned to in utero electroporation to replace endogenous AMPARs with these same constructs (Fig. 3). Coexpression of GluK1 and Neto1 under these conditions resulted in a substantial rescue of synaptic currents (Fig. 3A1). Cells coexpressing GluK1 and Neto1 also exhibited normal LTP (Fig. 3A2). In striking contrast, the coexpression of GluK1 Δ 4 and Neto1 Δ 4 failed to rescue synaptic currents (Fig. 3B1 and Fig. S4F), and LTP was absent (Fig. 3B2). We repeated these experiments with Neto2. In agreement with previous results (6), coexpression of GluK1 and Neto2 caused a partial rescue of synaptic currents (Fig. 3C1), and LTP was normal (Fig. 3C2). In a final series of experiments, we coexpressed GluK1 Δ 4 together with Neto2 Δ 4. This resulted in minimal rescue of synaptic currents (Fig. 3D1 and Fig. S4F), and LTP was severely reduced (Fig. 3D2). Importantly, in all of the experiments, the NMDAR synaptic currents were unaltered (Fig. S5). Thus, any impairment in LTP cannot be attributed to the loss of NMDARs. Taken together, these results indicate that the PDZ domains of the GluK1/Neto receptor complex are necessary for both basal synaptic transmission and LTP.

Discussion

Despite many decades of investigation, the rules governing AMPAR trafficking and LTP remain confusing. In the present study, we took a single-cell genetic approach that allowed us to study the behavior of a defined AMPAR/TARP complex in isolation. With this approach we established that the PDZ ligand of TARP γ -8 is essential for the translocation of surface AMPARs to the synapse. Furthermore, this PDZ ligand is also essential for LTP. Using γ -8 Δ 4 knock-in mice, it was previously found that the PDZ-binding motif of TARP γ -8 is involved in constitutive synaptic transmission, but not required for synaptic plasticity (17). This seemingly inconsistent result may be caused by the redundancy of other TARPs expressed in pyramidal cells. Most importantly, our findings provided a clue as to why exogenously expressed KARs, with minimal homolog to AMPARs, exhibit normal LTP. These receptors, together with their auxiliary Neto subunits, also contain PDZ ligands, which we demonstrate are essential both for basal synaptic targeting and for LTP.

Previous studies directly compared the properties of TARP γ -8 to that of TARP γ -2 in stargazin cerebellar granule cells (27). Both TARP γ -8 Δ 4 and TARP γ -2 Δ 4 trafficked to the surface the same as their respective wild type but both profoundly reduced the frequency of miniature EPSCs (mEPSCs), confirming the importance of the PDZ-binding motif for both TARPs in synaptic targeting. Interestingly, although the amplitude of the few remaining mEPSCs in TARP γ -2 Δ 4 expressing cells was reduced, a significant reduction was not observed with TARP γ -8 Δ 4, perhaps suggesting a minor PDZ-binding independent component to trafficking. However, our present results in hippocampal pyramidal cells indicate that the PDZ-binding domain of TARP γ -8 can fully account for its synaptic targeting. Furthermore, our findings suggest that the PDZ-domain interactions between glutamate receptors/auxiliary subunits and PSD scaffolding proteins are a general and minimal postsynaptic

passive role. The present results support a fundamentally different model that focuses on the PSD as the target for modification during LTP, with the glutamate receptor/auxiliary subunit complexes playing passive roles.

Materials and Methods

Experimental Constructs. The GluA1-TARP tethered constructs were made as previously described (22) and were subcloned into the pCAGGS vector for biolistic transfection and in utero electroporation.

Mouse Genetics. Animals were housed according to the Institutional Animal Care and Use Committee Guidelines at the University of California, San Francisco and the Institutional Review Board of Kunming Institute of Zoology, Chinese Academy of Sciences. *Gria1^{fl/fl}Gria2^{fl/fl}Gria3^{fl/fl}* mice were generated and genotyped as previously described (21).

Electrophysiology. Voltage-clamp recordings were taken from CA1 pyramidal neurons in acute hippocampal slices or organotypic slice cultures. For acute slices, 300- μ m transverse slices were cut using a Microslicer DTK-Zero1 (Ted Pella) in chilled high-sucrose cutting solution containing the following (in mM): 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 7 D-glucose, 210 sucrose, and 1.3 ascorbic acid. The slices were then incubated for 30 min at 34 °C in artificial CSF (ACSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 26.2 NaHCO₃, 1 NaH₂PO₄, and 11 D-glucose. For acute slices, 2.5 mM CaCl₂ and 1.3 mM MgSO₄ were added to the ACSF, which was bubbled with 95% O₂ and 5% CO₂ to maintain pH, and the slices were allowed to recover at room temperature for 30 min to 1 h before recording at room temperature. For organotypic slice cultures, 4 mM CaCl₂ and 4 mM MgSO₄ were added. For recording, slices were transferred to a perfusion stage on an Olympus BX51WI upright microscope and perfused at 2.5 mL/min with ACSF containing 0.1 mM picrotoxin. Synaptic responses were evoked by stimulating with a bipolar metal electrode in stratum radiatum of CA1. To ensure stable recording, membrane holding current, input resistance, and pipette series resistance were monitored throughout the recording. Data were gathered through a MultiClamp 700B amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 10 kHz.

Whole-Cell Synaptic Recordings and LTP. Simultaneous dual whole-cell recordings were made between GFP- and mCherry-positive experimental cells as identified by epifluorescence and neighboring nontransfected control cells. The internal recording solution contained (in mM): 135 CsMeSO₄, 8 NaCl, 10 Hepes, 0.3 EGTA, 5 QX314-Cl, 4 Mg-ATP, 0.3 Na-GTP, and 0.1 spermine. Osmolarity was adjusted to 290–295 mOsm, and pH was buffered at 7.3–7.4. AMPAR-mediated responses were isolated by voltage-clamping the cell at –70 mV, whereas NMDAR-mediated responses were recorded at +40 mV, with amplitudes measured 100 ms after stimulation to avoid contamination by AMPAR current. LTP was induced by stimulating at 2 Hz for 90 s while clamping the cell at 0 mV, after recording a stable 3- to 5-min baseline, but not more than 6 min after breaking into the cell. To minimize run-up of baseline responses during LTP, slices were stimulated for ~10 min before breaking in, and both cells were held as cell-attached for 2–5 min before breaking into the whole-cell mode. Before breaking in, stimulation intensity was calibrated just below the threshold required to elicit an action potential from the wild-type control neuron.

Immunoblotting. HEK293T cells were maintained and transfected as previously described (37). For coimmunoprecipitation, cells were washed in PBS and lysed in a TBS buffer containing 1 mM EDTA (Sigma), protease inhibitors (Roche), and 1% Triton X-100 (Fisher Scientific). Of the total whole-cell lysates, 2.5% was saved as input, and the rest was then incubated with ~10 μ L

of monoclonal anti-HA agarose antibody-conjugated beads (Sigma) at 4 °C overnight. The following day, the immunoprecipitations were washed in TBS buffer containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, protease inhibitors, and 0.1% Triton X-100. The antibody-conjugated beads were resuspended in SDS/PAGE sample buffer and subjected to Western blotting. Antibodies used in the study were PSD-95 (MS; Neuromab) and HA (Rat; Roche).

Neuronal Transfection. Organotypic hippocampal slice cultures were made from P6–P8 mice as previously described (38). Biolistic transfections were carried out on day 2 after culturing using a Helios Gene Gun (Bio-Rad) with 1- μ m DNA-coated gold particles. When expressing two plasmids, gold particles were coated with equal amounts of each plasmid expressing different fluorescent markers. Observed frequency of coexpression was nearly 100%. Slices were maintained at 34 °C with media changes every other day.

For in utero electroporations, ~E15.5 pregnant *Gria1-3^{fl/fl}* mice were anesthetized with 2.5% isoflurane in O₂ and injected with buprenorphine for analgesic. Embryos within the uterus were temporarily removed from the abdomen and injected with 2 μ L of mixed plasmid DNA into the left ventricle via a beveled micropipette. FUGW-Cre:mCherry and pCAGGS-GluA1-TARP:EGFP plasmids were typically diluted to final concentration of approximate 0.5 μ g/ μ L each. Each embryo was electroporated with 5 \times 50 ms, 35 V pulses. The positive electrode was placed in the lower right hemisphere, and the negative electrode was placed in the upper left hemisphere (39). After electroporation, the embryos were sutured into the abdomen and killed on P17–P21 for dual whole-basal synaptic transmission or LTP recording (6).

Outside-Out Patches. Outside-out patches were taken from CA1 pyramidal neurons of organotypic hippocampal slices. Initially, cells were recorded in whole-cell mode at –70 mV with a 4- to 5-M Ω patch pipette. The pipette was then slowly pulled away from the soma until a high-resistance seal reformed. The external Hepes-ACSF contained (in mM): 140 NaCl, 5 KCl, 1.4 MgCl₂, 1 CaCl₂, 5 EGTA, 10 Hepes, 1 NaH₂PO₄, 10 D-glucose, with pH adjusted to 7.4. Glutamate currents were evoked by perfusion over the tip of the pipette of the same solution with the addition of 1 mM L-glutamic acid, 0.1 mM D-APV, 0.1 mM picrotoxin, 0.1 mM cyclothiazide, and 0.5 mM tetrodotoxin. A ValveLink 8 (AutoMate Scientific) was used for fast perfusion of control and glutamate-containing Hepes-ACSF. During outside-out patch experiments, experimental cells were interleaved with nontransfected control cells.

Statistical Analysis. Significance of evoked dual whole-cell recordings compared with controls was determined using the two-tailed Wilcoxon signed-rank sum test. For all experiments involving unpaired data, including all outside-out patch data, a Mann–Whitney *U* test with Bonferroni correction for multiple comparisons was used. Data analysis was carried out in Igor Pro (Wavemetrics), Excel (Microsoft), and GraphPad Prism (GraphPad Software).

ACKNOWLEDGMENTS. We thank M. Cerpas and D. Qin for technical assistance and all members of the R.A.N. laboratory for discussion of and comments on the manuscript. This work was funded by grants from the National Institute of Mental Health (to R.A.N.), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB13000000), the National Natural Science Foundation of China (31741055), and the Chinese Academy of Sciences Pioneer Hundred Talents Program (to N.S.). Y.S.S. is supported by National Natural Science Foundation of China Grants (31371061 and 31571060), Ministry of Science and Technology of China Grants (2014CB942804 and 2015BAI08B02), and Natural Science Foundation of Jiangsu Province Grant (BK20140018).

- Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* 40:361–379.
- Murakoshi H, Yasuda R (2012) Postsynaptic signaling during plasticity of dendritic spines. *Trends Neurosci* 35:135–143.
- Huganir RL, Nicoll RA (2013) AMPARs and synaptic plasticity: The last 25 years. *Neuron* 80:704–717.
- Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* 25:103–126.
- Collingridge GL, Isaac JT, Wang YT (2004) Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci* 5:952–962.
- Granger AJ, Shi Y, Lu W, Cerpas M, Nicoll RA (2013) LTP requires a reserve pool of glutamate receptors independent of subunit type. *Nature* 493:495–500.
- Jackson AC, Nicoll RA (2011) The expanding social network of ionotropic glutamate receptors: TARPs and other transmembrane auxiliary subunits. *Neuron* 70:178–199.
- Greger IH, Watson JF, Cull-Candy SG (2017) Structural and functional architecture of AMPA-type glutamate receptors and their auxiliary proteins. *Neuron* 94:713–730.
- Straub C, Tomita S (2012) The regulation of glutamate receptor trafficking and function by TARPs and other transmembrane auxiliary subunits. *Curr Opin Neurobiol* 22:488–495.
- Copits BA, Swanson GT (2012) Dancing partners at the synapse: Auxiliary subunits that shape kainate receptor function. *Nat Rev Neurosci* 13:675–686.
- Tomita S, et al. (2003) Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. *J Cell Biol* 161:805–816.
- Chen L, et al. (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408:936–943.
- Tomita S, et al. (2005) Stargazin modulates AMPA receptor gating and trafficking by distinct domains. *Nature* 435:1052–1058.
- Menuz K, O'Brien JL, Karmizadegan S, Bredt DS, Nicoll RA (2008) TARP redundancy is critical for maintaining AMPA receptor function. *J Neurosci* 28:8740–8746.

