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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	SerialEM 3.7, plcamp 10
Data analysis	Relion-3.0, GraphPad Prism-7, CryoSPARC -v2.14, COOT-0.9, Phenix-1.17-, Chimera-1.11.2, Pymol-2.3.4, ChimeraX-1.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The cryo-EM maps and coordinates for overall, ATD layer and LBD-TMD layer of the A1A2A1A2-symmetric (S) and A1A2A1A2-asymmetric (AS) complexes have been deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers EMD-23283 and EMD-23284 and in the Protein Data Bank (PDB) under accession codes 7LDD and 7LDE, respectively. The cryo-EM maps for overall, ATD layer and LBD-TMD layer of A1A2A3A2-AS1, A1A2A3A2-AS2, A3A2A3A2-S and A3A2A3A2-AS complexes have been deposited in the EMDB under accession numbers EMD-23285, EMD-23286, EMD-23287 and EMD-23288, respectively. The cryo-EM maps of A1A2A3A2-AS3 and A1A2AXA2 have been deposited in the EMDB under accession numbers EMD-23289 and EMD-23290, respectively. The cryo-EM map and coordinates for the LBD-TMDmix complex has been deposited in the EMDB and PDB under accession codes EMD-23292 and 7LEP, respectively.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were not predetermined for this study. Sample sizes of cryo-EM data were determined by the availability of microscope time. For the functional experiments, electrophysiology recordings were repeated at least five times, on different cells. Single molecule pull down experiments were carried out by collecting at least 25 images from two independent samples on different days. The sample sizes of these both experiments were determined based on the consistency and variability.
Data exclusions	No data were excluded from the analyses.
Replication	Cryo-EM related experiments including purification, SDS-PAGE gel and Western Blot were reproduced three times independently. electrophysiology experiments were reproduced with at least five different cells. Single molecule pull down experiments were reproduced on a two independent samples on different days.
Randomization	Our experiment is not randomized. Cryo-EM related experiments including purification, SDS-PAGE gel and Western Blot were repeated several times with different batches of mice. For electrophysiology experiments, cells with GFP and mCherry fluorescence were randomly selected. For the single molecule pull down experiments, images were randomly acquired in different regions of the sample chamber.
Blinding	The investigators were not blinded; it was not technically or practically feasible to do so for cryo-EM analysis, electrophysiology experiments, or single molecule pull down experiments.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	In house antibodies: 11B8 scFv anti-GluA1 (produced by our lab); 15F1 Fab anti-GluA2 (produced by our lab); 5B2 Fab anti-GluA3 (produced by our lab); E3 mAb anti-GluA4 (produced by our lab); 13A8 mAb/Fab anti-TARP-γ8 (produced by our lab); Commercial antibodies: anti-GluA1 (Millipore, N453, 04-823,), anti-GluA2 (Thermo Fisher, N/A, PA5-19496), anti-GluA3 (Invitrogen, 3B3,32-0400), anti-GluA4 (Millipore, N/A, ab1508), anti-PSD95 (Abcam, N/A, ab18258) and anti-synDIG4 mAb (NeuroMab, L102/45, 75-409). We have stated in the method that the antibodies used for structural determination were not diluted, antibodies used for single molecule pull down experiments were diluted to at a concentration of 1 to 3 μg/mL and antibodies used for Western Blot were diluted in1: 1000.
Validation	Validation of 11B8 scFv anti-GluA1, 5F1 Fab anti-GluA2 and 5B2 Fab anti-GluA3 used for cryo-EM structure determination and single molecule pull down experiments can be found in the previous published literature (Zhao, Y. et al. Architecture and subunit arrangement of native AMPA receptors elucidated by cryo-EM. Science 364, 355-362 (2019)). Validation of E3 mAb anti-GluA4 and 13A8 mAb/Fab anti-TARP-γ8 for single molecule pull down experiments and Western Blot were performed in this studies through biochemistry analysis in the Extended Data Figure. 2. The validation of anti-synDIG4 mAb for single molecule pull down experiments could be found in the website: https://www.antibodiesinc.com/products/syndig4-l102-45. The validation of commercial antibodies nti-GluA1, anti-GluA2, anti-GluA3, anti-GluA4 and anti-PSD95 for western blot could be found in the websites: https://www.emdmillipore.com/US/en/product/Anti-phospho-GluR1-Ser831-Antibody-clone-N453-rabbit-monoclonal,MM_NF-04-823?ReferrerURL=https%3A%2F%2Fwww.google.com%2F; https://www.thermofisher.com/antibody/product/GluR2-Antibody-Polyclonal/

PA5-19496; https://www.thermofisher.com/antibody/product/GluR3-Antibody-clone-3B3-Monoclonal/32-0400; https://www.emdmillipore.com/US/en/product/Anti-Glutamate-Receptor-4-Antibody,MM_NF-AB1508 and https://www.abcam.com/psd95-antibody-synaptic-marker-ab18258.html, respectively. The sources for all the in house antibodies are from mouse. The sources for commercial antibodies anti-GluA1, anti-GluA2, anti-GluA3, anti-GluA4 and anti-PSD95 are from rabbit, rabbit, mouse, rabbit and mouse, respectively.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	Sf9 cells for expression of Baculovirus are from Thermofisher (12659017, lot 421973). HEK293S GnTI- cells (Ric15) for protein expression and electrophysiology studies are from: Reeves P, et al. Structure and function in rhodopsin: High-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. Proc. Natl. Acad. Sci. USA 99 (21): 13419-13424, 2002. PubMed: 12370423.
Authentication	The cells were routinely maintained in our lab. They were not authenticated experimentally for these studies.
Mycoplasma contamination	Sf9 cells and HEK293S GnTI- cells (Ric15) were tested negative
Commonly misidentified lines (See ICLAC register)	No commonly misidentified lines were applied.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Adult (6-8 weeks) C57BL/6 mice (both male and female) were ordered from Charles River Laboratories for hippocampal dissection. The housing conditions were set as: temperature 68-72F; humidity 40-60%; Dark/light cycle 12:12. No experimental manipulations were performed on these animals.
Wild animals	The study did not involve wild animals.
Field-collected samples	No filed-collected samples were used in this study.
Ethics oversight	All mice were euthanized under proper Institutional Animal Care and Use Committee (IACUC) protocols, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) and carried out only by members of Dr. Gouaux's lab approved on the IACUC protocol - TR01_IP00000905.

Note that full information on the approval of the study protocol must also be provided in the manuscript.