Published in final edited form as:

Science. 2019 April 26; 364(6438): . doi:10.1126/science.aav9011.

Architecture of the heteromeric GluA1/2 AMPA receptor in complex with the auxiliary subunit TARP $\gamma 8$

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Abstract

AMPA-type glutamate receptors (AMPA receptors) mediate excitatory neurotransmission, and are central regulators of synaptic plasticity, a molecular mechanism underlying learning and memory. Although AMPARs act predominantly as heteromers, structural studies have focused on homomeric assemblies. Here we present a cryo-EM structure of the heteromeric GluA1/2 receptor associated with two TARP $\gamma 8$ auxiliary subunits, the principal AMPAR complex at hippocampal synapses. Within the receptor, the core subunits arrange to give the GluA2 subunit dominant control of gating. This structure reveals the geometry of the Q/R-site controlling calcium flux, suggests association of TARP-stabilized lipids, and demonstrates that the extracellular loop of $\gamma 8$ modulates gating by selectively interacting with the GluA2 ligand-binding domain. Collectively, this structure provides a blueprint for deciphering the signal transduction mechanisms of synaptic AMPARs.

Introduction

Ionotropic glutamate receptors (iGluRs), tetrameric cation channels gated by L-glutamate, are the primary mediators of signal transmission at excitatory synapses in the brain (1, 2). Two principal iGluR subtypes, NMDA (N-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type receptors (NMDARs and AMPARs respectively), coexist at synapses, with complementary roles in synaptic communication. AMPARs drive baseline transmission, while NMDARs trigger synaptic plasticity, inducing long-term changes in synaptic efficacy. A prominent mechanism of synaptic plasticity is the recruitment or removal of synaptic AMPARs through processes such as long-term potentiation (LTP) or depression (LTD), which are considered to be cellular mechanisms underlying learning and memory (3).

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Author contributions: I.H.G. conceptualised and supervised the study. B.H. designed and performed protein purification. B.H. and J.G.-N. carried out cryo-EM data collection. B.H. performed cryo-EM data processing. J.G.-N. performed model building. J.F.W., H.H. and O.C. designed and performed electrophysiology experiments. All authors performed data analysis and contributed to the preparation of the manuscript.

Competing interests: The authors declare no competing interests.

Data and materials availability: Cryo-EM density and PDB models are available from the PDB (6QKZ and 6QKC) and EMDB (EMD-4575 and EMD-4572).

Synaptic signaling and trafficking of iGluRs are determined by their subunit composition. The four AMPAR subunits, GluA1-4, assemble into hetero-tetramers of various compositions, preferentially containing the GluA2 subunit (1). RNA editing of GluA2 changes a glutamine to an arginine at a site in the conduction pore (the Q/R site) (4). Through this mechanism, the GluA2 subunit controls Ca^{2+} permeability (5–7), channel block by polyamines (8–10), channel conductance (11) and subunit assembly (1, 12). Editing at this site is essential for survival of the organism (13). iGluRs are comprised of two conformationally distinct, diagonally opposed subunit pairs termed AC (or ‘pore-proximal’) and BD (or ‘pore-distal’) (14) (Fig. 1A). The BD chains dominate channel gating, and in NMDARs are occupied by the GluN2 subunits (15, 16), however the subunit placement rules of heteromeric AMPARs have not been firmly established.

AMPA tetramers associate with various auxiliary subunits (1, 17). Principal amongst these are proteins from the TARP (transmembrane AMPAR regulatory protein) family, with subtypes associated with AMPARs across the brain, controlling receptor trafficking, gating, ion permeation and pharmacology (1, 18, 19). The six TARPs belong to three subgroups (Ia, Ib and II), with the cerebellar TARP, $\gamma 2$ or stargazin (group Ia), being the most extensively studied. Structural studies of GluA2 homomers in complex with $\gamma 2$ show that up to four $\gamma 2$ s can associate around the AMPAR transmembrane sector, with their two extracellular segments (Ex1 and Ex2; Fig. 1B) poised to interact with the ligand-binding domain (LBD) (20, 21).

While $\gamma 2$ controls AMPAR function in the cerebellum, GluA1/2 heteromers associated with TARP $\gamma 8$ are the most abundant AMPAR complex in the hippocampus (19, 22), the seat for episodic memories, driving the majority of synaptic transmission (23) and mediating synaptic plasticity at the Schaffer collateral-CA1 synapse (3, 24). Thus far, structural studies have focused on homomeric GluA2 complexes, which appear to play no appreciable role in synaptic transmission (23), while structural insights into the architecture of both GluA1 and $\gamma 8$ are so far absent, and only a single heteromeric AMPAR structure has been reported (GluA2/3) (25).

Here we present structural and functional studies elucidating the organization of the GluA1/2 receptor in complex with TARP $\gamma 8$. We demonstrate preferential placement of the GluA2 subunit in the gating-dominant BD position, reveal the AMPAR- $\gamma 8$ contacts at side chain resolution and describe a $\gamma 8$ Ex1 interaction with the upper lobe of the GluA2 LBD, which modulates gating. This structure also offers a view of the predominant Q/R site configuration in the selectivity filter, and reveals strong density indicative of annular lipids. These line the channel fenestrations above the Q/R site, specifically stabilized between GluA1 and $\gamma 8$ where they have the potential to modulate ion flux.

Functional characterization of $\gamma 8$ -associated GluA1/2 receptors

AMPA receptors contain two non-equivalent positions for TARP association, the A’C’ and B’D’ sites (Fig. 1A) (21), resulting from a switch between the four-fold symmetric ion channel and a two-fold symmetric LBD layer (14). Structural studies with $\gamma 2$ suggest that TARPs preferentially occupy the B’D’ position (20). Although not firmly established (26), type Ib-

TARPs ($\gamma 4$ and $\gamma 8$) appear to preferentially associate with the AMPAR in a 2-TARP stoichiometry (two TARP subunits per receptor tetramer) (22, 27, 28), especially in the hippocampus where $\gamma 8$ is known to co-assemble with other AMPAR auxiliary subunits, such as cornichon homologue 2 (CNIH-2) (22).

We studied a GluA1/2 receptor (both flip splice variants, and GluA2 edited at the Q/R and R/G sites (29, 30)) associated with two $\gamma 8$ subunits, by co-expressing GluA1 with GluA2 in the ‘TARP-tandem’ configuration (26), where the C-terminus of GluA2 is fused to the N-terminus of $\gamma 8$ (GluA2- $\gamma 8$), in HEK293 cells. The resulting 2-TARP complex, termed ‘A1/2- $\gamma 8$ ’, elicited glutamate-evoked currents characteristic of TARP-associated AMPARs, with slowed deactivation and desensitization kinetics compared to TARP-free GluA1/2 (Fig. 1C and Fig. S1A). Equivalent results were obtained by expressing a GluA1- $\gamma 8$ fusion with GluA2 (Fig. 1C and Fig. S1A-D). A functional comparison with a 4-TARP stoichiometry was achieved by co-expressing GluA1/2 with an excess of free $\gamma 8$, allowing the association of four $\gamma 8$ subunits. The increased $\gamma 8$ occupancy showed a resensitizing current, a hallmark of AMPARs associated with four $\gamma 8$ s (Fig. S1B) (28), larger steady-state currents (Fig. S1C) and a subtly slower entry into desensitization (Fig. S1A), while two TARPs were sufficient for maximal effects on receptor deactivation and recovery from desensitization (Fig. 1C and Fig. S1D). Thus, this A1/2- $\gamma 8$ configuration presents a functional AMPAR-TARP complex (28), allowing mechanistic studies of how these auxiliary subunits act to modulate gating of the receptor heteromer.

Subunit arrangement in GluA1/2 AMPARs

We probed the arrangement of the GluA1 and GluA2 subunits in the tetramer using two functional assays. The M3 linkers, which connect the channel gate to the LBD, show conformational differences between AC and BD chain pairs, implying that the BD linkers exhibit a greater pulling force on the gate (see below) (31, 32). If GluA2 predominantly occupies the BD positions in GluA1/2 heteromers, as has been suggested (33), then mutation of the GluA2 M3 linker would have a dominant effect on gating in the heteromer. To test this, we mutated an isoleucine residue, which controls linker tension by associating with the lower lobe of the LBD (I629 in GluA1 and I633 in GluA2; denoted ‘IA’ mutation). This mutation attenuates gating in GluA2 homomers, with no influence on receptor surface trafficking (31, see also Fig. S7E). As anticipated, this mutation severely limited channel activation in both GluA1 and GluA2 homomers (Fig. 1D). In heteromeric receptors, mutation of GluA2 reduced receptor gating to a greater extent than mutating GluA1 (Fig. 1E), supporting an arrangement with GluA2 preferentially in the pore-distal ‘BD’ positions.

To extend this finding to TARP-containing receptors, we took advantage of the C528L mutation in the GluA2 M1 transmembrane helix that limits TARP modulation without preventing association with the receptor (C524L in GluA1, denoted ‘CL’ mutation) (34). As TARPs preferentially dock to the B’D’ site (20, and confirmed for $\gamma 8$ below), mutation of the M1 helix in only the AC subunits should affect the action of TARPs (Fig. 1F). Combining CL mutation of both GluA1 and GluA2 subunits in the A1/2- $\gamma 8$ complex sped receptor desensitization and reduced steady-state currents (Fig. 1G-I), consistent with prevention of TARP-modulation (Fig. S1A and C). However, when mutating either the

GluA1 or GluA2 subunit, only GluA1_{CL} but not GluA2_{CL} exhibited ‘TARP-free’, quickly desensitizing responses (Fig. 1H) and trend-wise reduced steady-state currents (Fig. 1I). Therefore, placement rules in GluA1/2 heteromers dictate that GluA1 preferentially occupies the pore-proximal and GluA2 the pore-distal positions, both in the absence and presence of $\gamma 8$, providing GluA2 with dominant control of receptor gating.

Cryo-EM structure of a GluA1/2- $\gamma 8$ receptor

Overall architecture of the full-length receptor

The A1/2- $\gamma 8$ receptor (wild-type sequence except for a 23-residue truncation of the GluA2 C-terminus for TARP fusion) was extracted from HEK Expi293F^{TM} cells in digitonin (Fig. S2A-C), trapped in a resting state with the competitive antagonist NBQX (6-nitro-2,3-dioxo-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide) and imaged with a Titan Krios microscope equipped with a K2 Summit detector and a Volta phase plate (Fig. S2D). Three-dimensional (3D) classification and refinement resulted in a model with a homogeneous TMD-LBD sector and a heterogeneous NTD layer (Fig. S2E and S3), a feature commonly observed with AMPARs (25, 35–37). Further 3D classification resulted in models with incompletely resolved NTDs rather than discrete conformations, suggesting a continuum of conformers (Fig. S3). A subset of particles exhibiting a clear three-layered, ‘Y-shaped’ structure produced a map with an overall resolution of 6.3 Å, with highest resolution in the LBD/TMD/TARP sector (Fig. S3B and S4, A, B and E). Rigid body fitting of crystal structures from individual AMPAR domains provided a first model of a GluA1/2 heteromer comprising the N-terminal domain (NTD) layer, the LBDs and the TMD surrounded by two $\gamma 8$ subunits in the B’D’ positions (Fig. 2A-D). Subunit identification was aided by a subunit-specific glycosylation signature of the GluA1 NTD. Clearly resolved glycosylation at Asn45 was visible on AC chains, but not BD chains (Fig. 2E and F), providing structural confirmation of the localization of GluA2 to the functionally dominant pore-distal position, in accordance with the functional data (Fig. 1G-I).

Features of the NTD-layer

The ‘Y’-shape of A1/2- $\gamma 8$ is established by an interface between the GluA2 NTDs (Fig. 2A-C), a hallmark of homomeric GluA2 receptors that is also observed in crystal structures of isolated GluA2 and GluA2/3 NTDs (14, 25, 38). The center of mass (COM) distance between NTD and LBD layers is increased by ~4.5 Å compared to GluA2 structures with shortened NTD-LBD linkers (14, 20). However, relative to wild-type GluA2 (PDB code 4UQJ), the inter-layer distance is reduced by ~3 Å (COM ~63 Å versus 65.8 Å), resulting in a closer apposition of the GluA1 NTD and LBD (Fig. S5A and B). A charged loop (GluA1 residues L170-E179), emanating from the most flexible NTD segment (helix αF) (39), together with an N-glycan projecting from the NTD at N231, are poised to mediate interlayer contacts with the LBD (Fig S5B and C). Interestingly, in another iGluR family member, GluD, a helical extension of the equivalent NTD αF engages the LBD for interlayer signaling (40). This raises the possibility that this region could play a more general role in iGluR function.

Relative to GluA2 homomers in the resting state (PDB codes 3KG2, 5KBU), we observe rearrangements of the NTD layer, including a clockwise rotation of the entire layer around the overall 2-fold axis of symmetry and a wider opening angle between the NTD dimers (Fig. S5D). Also of note is a visible loosening of the GluA1/2 NTD dimer compared to homomeric crystal structures, which is unexpected given the high stability of this interface (41, 42). Whether this is caused by the presence of the receptor core is currently unclear and requires higher resolution structures.

Structure of the A1/2_γ8 LBD-TMD sector

To improve resolution of the LBD/TMD/TARP sector, we masked the NTD and carried out consecutive steps of focused refinement, followed by 3D classifications without alignment (Fig. S3). This procedure gave rise to a model with an overall resolution of 4.4 Å, with the TMD layer and the ion conduction pore reaching 3.4 Å (Fig. 3A, and Figs S3 and S4C-E). Model building was aided by side chain density throughout the TMD/TARP region, most of the M1 and M3 linkers, which connect the LBD to the TMD, and parts of the LBD (Fig. 3A, C and Fig. S6A-D).

Features of the GluA1/2 LBD layer

The LBDs, bi-lobate ‘clamshell’ structures that coordinate L-glutamate and competitive antagonists in their inter-lobe cleft (43), are arranged as two heterodimeric pairs, and adopt a conformation characteristic of resting-state AMPARs (35, 44) (Fig. 3A-B). Density for the competitive antagonist NBQX is apparent in the inter-lobe cleft of the GluA1 and GluA2 LBDs, forcing the clamshells into a cleft-open, antagonized conformation (Fig. S7A-B) (43). A closer inspection suggests that GluA2 LBD cleft is subtly more closed relative to GluA1 (by ~2°), which is possibly related to the greater pulling force exerted by subunits in the BD positions (32, 44).

The stability of the LBD dimer interface, formed between the upper LBD lobes, is a central determinant of gating kinetics (45), and is tuned by alternative splicing (at the flip/flop exons of both GluA1 and GluA2) (30) and by RNA editing at the GluA2 R/G site (29). Residual side chain density is observed for Arg739 in GluA1 (which is not subject to editing, 29), but not for the edited Gly743 of GluA2 (Fig. S7C).

The LBD dimers associate to form a ‘gating ring’ (Fig. 3B), that opens in an iris-like fashion upon receptor activation between helices G of the AC chains (Fig. S7D) (35). The central opening of the ring is within the range of GluA2 resting-state structures associated with TARP γ2 (PDB 5KBU and 5KK2), and the height of LBD layer, relative to the plane of the membrane, is also comparable to these structures (Fig. S7E). The base of the LBD connects via linkers to the TMD to transduce binding of L-glutamate to opening of the ion channel (Fig. 3A and C and Fig. S7E).

The TMD sector

The outer gate, formed by a helical bundle of the four M3 transmembrane helices adopts a closed conformation as expected for a resting state channel (31, 35), and is ‘capped’ by the GluA1 M625 side chains, projecting towards the pore axis (Fig. S8A). The level of

constriction at the narrowest point (A617 in GluA1, A621 in GluA2) is similar to resting-state GluA2 determined at a comparable resolution (PDB code 3KG2). The importance of this region is highlighted by a rare mutation of this conserved Ala to Thr in GluA3, which stabilizes the closed-gate conformation and is linked to severe intellectual disability (46). The structural deviation of the M3 gating linkers between the AC and BD chains (14, 31, 44), exemplify how the horizontally oriented GluA2 linkers exert gating dominance (Fig. 3C). We also see side chain density of the Ile residues that determine linker tension (GluA1 I629/GluA2 I633) (31), and of the arginines that shape desensitization (GluA1 R624/GluA2 R628) (47).

The inner pore constriction is formed by the M2 pore loop. At its apex, where GluA1 contains a glutamine (Q582), RNA editing at the Q/R site introduces Arg586 into GluA2 (4) (Fig. 3D). This arginine provides key signatures of heteromeric AMPARs: preventing both Ca^{2+} ion permeation and polyamine block (1). Our structure shows that the GluA2 R586 guanidinium groups project into the water-filled cavity above, ideally positioned to restrict cation influx (5–7, 11), while the GluA1 Q582 side chains bend toward the adjacent GluA2 subunit, in H-bonding distance of the R586 main chain and the Q587 side chain (the +1Q), effectively lining the ‘entrance’ of the selectivity filter (Fig. 3D-E). Deeper into the filter, the +1Q side chains swing anti-clockwise (when viewed from the top) in all four subunits to interact with a tryptophan projecting from the M3 helix (GluA1 W602 and GluA2 W606) of the neighboring subunit (Fig. 3E).

The pore diameter at the filter entrance, measured at the R586 Ca atoms, is comparable to a GluA2 homomer trapped in an active state at a comparable resolution (PDB code 6DLZ; distance 11.7 Å in A1/2_γ8 versus 10.7 Å in 6DLZ) (Fig. S8B-C). This comparison indicates that the inner constriction does not dilate markedly upon gating in AMPARs, despite a number of contacts between M2 with both M1 and the M3 gating helix (Fig. S8D-E), including a cation- π interaction between a tryptophan in M2 (GluA1 W574, GluA2 W578) and an arginine in M3 (GluA1 R595, GluA2 R599) (Fig. S8E). This behavior is reminiscent of the related K^+ channels (48) but deviates from transient receptor potential (TRP) channels, where both the outer gate and selectivity filter widen on activation (49). Also noteworthy is strong density within the filter, close to the +3 cysteine (Fig. 3D, and Fig. S8F), a residue modified by palmitoylation (50). While unlikely to be palmitoyl groups, due to the side-chain geometry, the nature of this density is currently uncertain. The cytoplasmic entrance to the filter, which contributes to polyamine binding and channel rectification, is lined by a ring of aspartate residues (GluA1 D586, GluA2 D590) (Fig. 3D and Fig. S8G).

Lipid-like densities decorate the TMD

We observe strong density associated with the receptor, perpendicular to the presumed plane of the membrane. These signals are characteristic of lipid molecules, although further work and higher-resolution structures are required to ascertain this (Fig. 3A and 4A). Marking the boundary of the outer membrane leaflet, these densities specifically fill the cavity between γ8 and the GluA1 subunit, where they line the GluA1 pre-M1 ‘cuff helix’ (Fig. 4B) but are absent from the equivalent region of GluA2 (Fig. 4C-D). This suggests they may be stabilized by the presence of γ8. We modelled five lipid molecules (L1-5) in this cavity,

while an additional molecule (L6) locates to the perimeter of $\gamma 8$, next to W86 (Fig. 4A, C and D). Of note, 'L6' mirrors lipid density located at an analogous Trp residue, W49 (Fig. S9A), in a crystal structure of claudin-15 (PDB 4P79), a protein structurally related to $\gamma 8$ (see below) (51). The presumed head groups of L1 and L2 engage the extracellular β -sheet of $\gamma 8$, at Y45 ($\beta 1$) and H197 ($\beta 5$), respectively, with their acyl chains packing against the TARP and receptor ($\gamma 8$ -TM4 and GluA1-M1), respectively (Fig. 4A). The third lipid, L3 fills the periphery of the cavity, connecting L1 and L2 to L4 and L5 (Fig. 4C-D).

The density of L4 projects diagonally towards the lateral portals of the channel above the Q/R site, between the GluA1 M1 and M4 helices, engaging the M2 helix through F580 and M581 (Fig. 4B). L4 and L5 are positioned by interactions with the densities of L2 and L3 as well as a number of GluA1 residues along M1 (Y529), M4 (I794, L801), the M1 cuff (F511), and M3 of GluA2 (F607). The location of L4, at the heart of the channel, gives it the potential to control ion flux, perhaps in a $\gamma 8$ -dependent manner. Of note, a comparably positioned lipid has recently been observed in molecular dynamics simulations of the NMDAR channel (52). Lipids are known to modulate other pore-loop channels such as the closely related kainate receptors (KARs), dependent on the Q/R-editing state (53), and can penetrate into the fenestrations of voltage-gated sodium channels, where they may occupy docking sites for hydrophobic drugs (54).

The TARP $\gamma 8$ interaction region

TARP $\gamma 8$ docks to the receptor TMD through its TM3 and TM4 helices, forming extensive contacts with M1 of GluA1 and M4 of GluA2 (Fig. 5A-B). While $\gamma 8$ -TM4 almost exclusively engages GluA1 (M1), $\gamma 8$ -TM3 wedges between both subunits, effectively crosslinking M1 and M4 of the receptor (Fig. 5B). As a result of this, GluA1-M1 and GluA2-M4 pack more tightly upon $\gamma 8$ interaction compared to the TARP-free A'C' TARP-docking site (Fig. 5C). On closer inspection, comparison of the A'C' and B'D' sites reveals rearrangements that accompany the binding of $\gamma 8$, with the GluA1 Y519 and M523 side chains swinging towards the TARP to facilitate formation of an H-bond between E520 of the receptor and Y199 of the TARP (Fig. 5D).

The side-chain resolution in the TARP interaction region (Fig. 5E-F), permitted us to visualize the residues responsible for binding of recently described $\gamma 8$ -specific antagonists, which hold promise in epilepsy treatment (55, 56). Ligand specificity over other TARPs is conferred by two residues in the $\gamma 8$ TMD, V176 in TM3 and G209 in TM4, which pack against M1 of the AMPAR in the vicinity of M523 and C524 (in GluA1) (Fig. 5B). Although the specificity-determining residues in $\gamma 8$ are less bulky than those of other TARPs (Val and Gly compared to Ile and Ala), our structure does not suggest a binding pocket large enough to accommodate the modulators. Together with recent studies showing that these antagonists can bind to free $\gamma 8$ (57), larger scale conformational changes, triggered by association of liganded $\gamma 8$ with the receptor, may occur to functionally uncouple TARP and receptor.

Comparison of $\gamma 8$ with other TARP-like proteins

Superposition of $\gamma 2$ and $\gamma 8$ reveals close structural similarities, including alignment of the four transmembrane helices (RMSD 3.14 Å), and arrangement of the extracellular region (ECR), formed of the pre-TM2 helix and a five-stranded beta sheet (Fig. 1B and Fig. S9). The three loops emanating from the beta sheet, the $\beta 1$ - $\beta 2$ and $\beta 4$ -TM2 loops (both in Ex1) and the TM3- $\beta 5$ (Ex2), project towards the LBD and modulate gating kinetics (20, 21, 58) (Fig. 1C). As is the case in current $\gamma 2$ structures, these loops are incomplete in our 4.4 Å map, because of their inherent flexibility (Fig. 3A).

There are a number of marked differences between TARPs and structurally related proteins, the AMPAR auxiliary subunit GSG1L and claudins (Fig. S9). Claudins exhibit tighter packing of the four-helical bundle and a substantial reorientation of the pre-TM2 helix (51) (Fig. S9E). While in claudins, this pre-TM2 element contributes to formation of epithelial tight junction arrays, in TARPs, pre-TM2 swings toward the AMPAR M4 helix (M4 of GluA2 in A1/2- $\gamma 8$), to position the modulatory $\beta 4$ -TM2 loop beneath the LBD (44) (Fig. 5A). This ‘acidic loop’, which is enriched in negatively charged residues, has been implicated in interactions with the basic ‘KGK’ motif within the LBD lower lobe (20, 21, 58, but see 34, 59). Interestingly, TM2 is contiguous in GSG1L (60), and pre-TM2 is absent due to the lack of flexible residues giving rise to the TM2 kink (Fig. S9D), resulting in reorientation of its acidic $\beta 4$ -TM2 loop (60) and likely has consequences for AMPAR modulation by GSG1L versus TARPs.

$\gamma 8$ interactions with the GluA2 LBD

The mechanisms by which the flexible TARP loops engage the LBD to modulate gating are not completely understood (61). Dictated by the heteromeric subunit organization, the $\gamma 8$ loops will primarily engage the functionally dominant GluA2 subunit (Fig. 3B). The shorter loop of the Ex2 segment (TM3- $\beta 5$) reaches towards the LBD-TMD linkers and we see contacts with K511 in the GluA2 LBD-M1 linker (Fig. S10), while the two loops in Ex1 are expected to predominantly engage the LBD. The greater modulatory potency of $\gamma 8$ versus $\gamma 2$ has been ascribed to its distinctive $\beta 1$ - $\beta 2$ loop, which is 12 residues longer, allowing it to more easily reach the LBD (59, 62, 63).

While density for the Ex1 loops was fragmented in our 4.4 Å map, processing of the entire data set without masking (~900,000 particles) resulted in a 4.7 Å map that revealed additional signal between $\gamma 8$ and the GluA2 LBD. While the NTD layer had poor resolution due to heterogeneity, density can be seen emanating from the Ex1 $\beta 1$ - $\beta 2$ strands towards a loop (M408-R420) on the upper lobe of the GluA2 LBD (Fig. 6A). This observation suggests that $\gamma 8$ is able to reach the LBD upper lobe, with the $\beta 1$ - $\beta 2$ loop spanning a distance of ~40 Å (between C α atoms of $\gamma 8$ H197 and GluA2 K409) (Fig. 6B).

To test the functional consequence of disrupting this potential interaction, we introduced an N-glycan in the LBD loop at N411 in GluA2 and assayed responses to rapid application of L-glutamate. The GluA2 LBD mutant (‘E413T’) indeed exhibited desensitization kinetics and steady-state responses comparable to GluA1/2 in the absence of $\gamma 8$ and significantly

different to A1/2_γ8 wild type, but was of no effect when introduced into the GluA1 LBD (at N407 using the 'N409T' mutation) (Fig. 6C-D and Fig. S11A-C). Moreover, the GluA2 glyco-mutant retained TARP modulation when assayed in the context of γ2, which has a shorter β1-β2 loop (Fig. 6C-D and Fig. S11A-C). These data suggest that the interaction site in the LBD upper lobe is selectively required for the modulatory action of TARP γ8.

Discussion

This structure of the GluA1/2 heteromer in association with two TARP γ8 subunits provides a framework for deciphering the inner working of the primary hippocampal AMPAR (22–24, 28), which drives synaptic strengthening during LTP (64–67). The arrangement of the four core subunits, with GluA2 preferentially in the functionally dominant BD position, explains why γ8 primarily targets GluA2 in our resting-state structure. An additional major component in regulation of the hippocampal AMPAR complex is the auxiliary subunit CNIH-2 (22, 28, 34). Future work will resolve how CNIH-2 interacts with AMPAR and how this relates to TARP binding. Whether the subunit placement rules described for GluA1/2 apply to other AMPAR heteromers is another crucial question. GluA2 was detected in both AC and BD positions of the GluA2/3 receptor (25), raising the possibility that the subunit arrangement in GluA2/3 receptors could be more flexible. Placement rules, perhaps controlled by signal sequence parameters on initial assembly (33), will have consequences for gating control, modulation by auxiliary subunits and organization of the NTD layer (25), a platform for AMPAR positioning at synapses (68, 69). Lastly, we hypothesize that the asymmetric distribution of lipid-like densities, proximal to the γ8 β1 and β5 strands, is caused by TARP γ8 and may point to an additional regulatory capacity of auxiliary subunits, either by influencing the local lipid environment of synaptic receptors, or by modulation of channel properties through lipids, analogous to KARs (53).

Materials and Methods

cDNA Constructs

All constructs and mutations were produced using IVA cloning (70). cDNAs encoding rat wild-type GluA2 (flip, R/G, Q/R) and rat GluA1 (flip) were expressed from either the pRK5 or pIRES plasmids. Rat TARP γ8 was expressed from pIRES-eGFP construct. Tandem constructs (pRK5) were produced by connecting the C-terminus of GluA2 (excluding residues Gln840-Ile862) or GluA1 (excluding residues Ile836-Leu889) through a GGSGSG linker to the N-terminus of rat γ8 (residues Glu2-Lys419; 4 amino-acid deletion). These are denoted A2_γ8 and A1_γ8 respectively. GluA1 C524L and N409T and GluA2 C528L and E413T mutations were all produced on the pRK5 plasmids above. GluA1 I629A was expressed from pIRES-mCherry construct and GluA2 I633A was expressed from pIRES-eGFP construct. TARP γ2 tandems were produced as per γ8 tandems by fusing the rat γ2 coding sequence (Glu2-Arg319) to the C-terminus of GluA2 with the equivalent linker sequence.

For protein purification, the plasmids described above were modified by introducing two tags. For GluA1, a FLAG tag was inserted after the first residue of the mature protein (Ala1). For GluA2_γ8, a 3C protease site (LEVLFQGP~~G~~GS), eGFP (Val2-Lys239) and a

GGSHHHHHHH His-tag sequence were added to the C-terminus of $\gamma 8$ (Fig. S2A). pOPINE-GFP nanobody plasmid (Addgene #49172) was used for the production of the GFP nanobody (71).

Electrophysiology

HEK293T cells (ATCC: Cat# CRL-11268, RRID: CVCL_1926, Lot 58483269: identity authenticated by STR analysis, mycoplasma negative), cultured at 37°C and 5 % CO₂ in DMEM (Gibco; high glucose, GlutaMAX, pyruvate, Cat#10569010) supplemented with 10 % foetal bovine serum (Gibco) and penicillin/streptomycin, were transfected using Effectene (Qiagen) according to manufacturer protocol and plated on poly-L-lysine-coated glass coverslips. 30 μ M 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Tocris) was added to media post-transfection to avoid AMPAR-mediated toxicity.

Outside-out patches (or lifted whole-cells for IA mutant analysis), voltage clamped at -60 mV, were subject to fast application of L-glutamate (10 mM unless otherwise stated) using a two-barrel theta glass tube controlled by a piezoelectric translator (Physik Instrumente). Signals were acquired using the MultiClamp 700B or Axopatch-200B amplifier (Axon Instruments), digitised using a Digidata 1440A interface and recorded with pClamp 9 or 10 (Molecular Devices). Extracellular solution contained (in mM) NaCl (145), KCl (3), CaCl₂ (2), MgCl₂ (1), glucose (10), and HEPES (10), adjusted to pH 7.4 using NaOH. Borosilicate glass electrodes (1.5mm o.d., 0.86mm i.d., Science Products GmbH), pulled with a PC-10 vertical puller (Narishige) with tip resistance of 2-5 M Ω , were filled with internal solution containing (in mM) CsF (120), CsCl (10), EGTA (10), ATP-sodium salt (2), HEPES (10), and spermine (0.1), adjusted to pH 7.3 with CsOH.

When recording heteromeric responses, 20 μ M IEM 1925 dihydrobromide (Tocris) was added to extracellular solutions to block currents contributed by GluA2-lacking receptors. For heteromeric recordings, the I/V relationship of glutamate responses (from -100mV to 100mV) was recorded, rectification index (RI: $(I_{40mV} - I_{0mV}) / (I_{-60mV} - I_{0mV})$) calculated, and cells with a RI of <0.6 were discarded. Desensitization entry was determined by 500 ms glutamate application and the first 200 ms was fitted with a two-exponential function to obtain the (weighted) time constant. Recovery from desensitization was tested using a two-pulse protocol consisting of a desensitizing 500 ms pulse followed by a 10 ms pulse at increasing time intervals. Recovery curves were initially fitted with the Huxley-Hodgkin equation, which produced an exponent value close to 1, (as previously described (72)), suggesting a single rate-limiting transition in desensitization recovery. Consequently, all recovery rates were determined by monoexponential fit. Steady-state current was measured 200 ms after peak and expressed as a percentage of the peak current amplitude. Deactivation kinetics were measured by 2 ms glutamate application and responses were fitted with biexponential decay to acquire the weighted deactivation time-constant. Resensitization was recorded by 10 s glutamate applications and quantified using the percentage of peak amplitude that recovers between 200 ms and 10 s post peak amplitude. Whole-cell peak amplitude was determined from the mean peak response of 5 consecutive sweeps, and is

presented as box and whisker plots depicting 5 to 95 percentile range. All other data are presented as mean value $\pm$ standard error of the mean.

Protein production

GFP nanobody was produced in *Escherichia coli* BL21-Gold and purification was carried out as previously described (71). GFP nanobody was coupled to CNBr Activated Sepharose 4b beads (GE) following manufacturer protocols. For the production of the A1/2_γ8 complex, DNAs coding for GluA1_FLAG and GluA2_γ8_eGFP were transfected into HEK-Expi293^{TM} cells (Thermo Fisher Scientific) grown in Expi293^{TM} expression media (Gibco). For transfection, 33 ml of Expi293^{TM} media containing 0.55 mg of GluA1_FLAG-pRK5 and 0.55 mg of GluA2_γ8_eGFP-pRK5 were mixed with 33 ml of Expi293^{TM} media containing 3 mg of PEI-max (Polyscience), incubated for <15 min at room temperature and added to 1 l of cells (density of 2×10^6 cells/ml). To block AMPAR currents, media was supplemented with ZK200775 (2 nM) and kynurenic acid (0.1 mM). 36-48 h post-transfection, cells were harvested and lysed for 1.5 h in buffer containing 25 mM Tris pH 8, 150 mM NaCl, 0.75 % digitonin (w/v) (Sigma), 5 μM NBQX, 1 mM PMSF, 1x PI (Roche). Insoluble material was removed by ultracentrifugation (41,000 rpm, 1h, rotor 45-50 Ti) and the lysate was incubated with anti-GFP beads for 2 hours. Beads were washed with 0.1 % digitonin and the protein was eluted by digestion with 3C protease (the first elution was done overnight at 4 °C and the second elution was done for 4 h at 4 °C). Eluted fractions contained A1/2_γ8 heterotetramers and GluA2_γ8 homotetramers (Fig. S2B). Pooled fractions were incubated with FLAG magnetic beads (Sigma), washed with 0.1 % digitonin, and the A1/2_γ8 heterotetramer was eluted with 0.15 mg/ml 3xFLAG peptide (Fig. S2B). Eluted fractions were pooled and concentrated to ~0.4 mg/ml in 25 mM TRIS, pH 8, 150 mM NaCl and 0.1 % digitonin (w/v).

Cryo-EM grid preparation and data collection

Protein was incubated with 50 μM NBQX for at least 30 min on ice prior to grid preparation. Quantifoil Cu 1.2/1.3 grids (300 mesh) coated with an ultrathin layer of amorphous carbon (2 nm-thin from Quantifoil or home-made) were glow-discharged for 7 seconds at 0.35 mA. 3 μl of sample were applied to the grids for 60 seconds, blotted for 2.5-3.5 seconds and plunged-frozen in liquid ethane with a FEI Vitrobot Mark IV equilibrated at 4 °C and 100 % humidity. Cryo-EM data were acquired at Diamond Light Source (DLS) electron Bio-Imaging Centre (eBIC) on an FEI Titan Krios IV operated at 300 keV, with a K2 Summit detector (Gatan) operated in counting mode, a GIF Quantum energy filter (slit width 20 eV) and a Volta phase plate. Three datasets were collected using EPU, with 5006 micrograph movies in total. Each movie contained 35 movie frames collected at a dose rate of 4.5 e⁻/pixel/sec over 14 seconds, resulting in a total dose of ~ 32 e⁻ per Å². The magnification used was 105,000x resulting in a pixel size of 1.40 Å on the specimen.

Cryo-EM data processing and model building

Data were processed using RELION 3.0 (73). Stage drift correction, beam induced motion correction and dose-weighting were performed with MOTIONCORR2 (74) using 5 x 5 patches. CTF correction and phase shift estimation were performed with Gctf 1.06 (75). Defocus values ranged from -0.2 μm to -1.4 μm. Phase plate position was changed every 45

images. Auto-picking was performed using a Gaussian blob, and particles were extracted in a box of 220 pixels. For each of the three datasets we performed 2 or 3 rounds of 2D classification with particles binned to a 50-pixel box, before re-extracting and performing a last round of classification at full pixel size. For 3D classification we created an *ab initio* model starting from 12,000 particles, which was low-pass filtered to 40 Å. For Dataset 1, we performed two rounds of 3D classification that allowed us to remove models that were not showing AMPAR-like features and particles that were too close together. All AMPAR models showed clear TMD and LBD sectors, whereas the third layer, corresponding to the two NTD dimers, was heterogeneous (Fig. S3). We pooled the particles containing quality TMD and LBD features and performed refinement in C2 symmetry with a mask that excluded the NTD layer. We performed a similar 3D classification and refinement approach for the three datasets, yielding three maps for TMD/LBD at 5.0 Å, 4.4 Å and 4.8 Å resolution, respectively (Fig. S3). Particles from the three datasets were pooled together and refined with C2 symmetry, obtaining a map at 4.3 Å resolution, which was not showing side-chain density (1,057,013 particles). Two steps of 2D classification (without alignment) allowed us to further clean the dataset, obtaining a refined model at 4.3 Å resolution showing improved features (899,088 particles). This set of particles was further classified to obtain a full-length receptor map and a TMD/LBD receptor map (Fig. S3). The full-length map was obtained after a step of 3D classification with alignment in 5 classes, followed by a refinement (using C2 symmetry), a step of 2D classification, and a second step of refinement (C2 symmetry). 183,811 particles contributed to the final model, which had an overall resolution of 6.3 Å after gold-standard resolution estimation in RELION (FSC = 0.143). Local resolution was calculated with RELION with the model displaying a resolution ranging from ~5 Å in the TMD core to ~7.5 Å in the NTD (Fig. S4). The TMD/LBD map was obtained after two rounds of 3D classification without alignment (C1 symmetry), which allowed us to isolate a subset of 114,730 particles that were subjected to refinement (C2 symmetry). During refinement, the low-pass filter effect of the Wiener filter in the regularized likelihood optimization algorithm was relaxed through the use of a regularization parameter ($T = 2$). This allowed the refinement algorithm to consider higher spatial frequencies in the alignment of the individual particles. Nevertheless, both half-reconstructions were kept completely separate, and the final resolution estimate (at the post-processing stage in RELION) was based on the standard Fourier shell correlation (FSC) between the two unfiltered half-reconstructions. The overall resolution of the resulting map was 4.4 Å (lower than the 899,088 particle dataset) with high-quality density, especially at the TMD region, which reached a local resolution of ~3.4 Å, while ~6.1 Å was achieved at the top of the LBD layer (local resolution calculated in RELION) (Fig. S4). The 899,088 particles were also refined without mask in order to investigate the density of the $\gamma 8$ loops, which were poorly defined in the TMD-LBD map. We refined the particles in C2 symmetry, obtaining a model at 4.7 Å resolution, which allowed us to identify an interaction between the $\gamma 8$ Ex1 and the LBD upper lobe (Met408-Arg420) (Fig. 6 and Fig. S3). Density for the NTD was very poor, highlighting the heterogeneity of this region.

For the TMD/LBD map, model building and refinement were performed using Coot (76) and REFMAC5 (77), using the CCP-EM suite of programs (78). For modelling, the $\gamma 2$ in the GluA2- $\gamma 2$ complex (PDB code 5WEO), the GluA2 LBD (PDB 3UA8) and the

transmembrane domain in the GluA2- γ 2 complex (PDB 5KK2) were taken as starting points. The individual coordinates were rigid-body fitted in Chimera (79). Sequence assignment and initial manual modifications were performed in Coot, prior to jelly-body refinement in REFMAC5. Alternating rounds of manual modifications and restraint refinement were performed in Coot and REFMAC5 respectively. ProSMART (80) was used to generate restraints in order to maintain secondary structure in regions of lower local resolution. Atomic B-factors were reset to 40 Å² prior to refinement. Clear density for lipid-like molecules were found in the map, however, their identity could not be discerned based exclusively on the cryo-EM density. We therefore modelled all as single acyl chain lipids, taking the 1-oleoyl-R-glycerol as a representative general lipid. Overfitting in refinement was monitored throughout using the FSC_{work}/FSC_{test} (81) (Fig. S6E). The final model had good geometry (Table S1) with validation performed in Coot, Molprobity (82) and EMRinger (83). The model fit to the map was assessed using Phenix (84) using a global model-vs-map FSC correlation (Fig. S6). Dimensions of the pore were calculated from the TMD/LBD protein model using the program HOLE (85). Sequence alignment was created using PROMALS3D (86). For the full-length model 1, rigid body fitting of the TMD/LBD model obtained above and single chains for the GluA1 NTD (PDB 3SAJ) and GluA2 NTD (PDB 3H5V) was performed in Chimera (79).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Lukas Helfinger for experimental work at early stages of this project. We acknowledge James Krieger, Radu Aricescu and members of the Greger lab for comments on the manuscript, as well as Kris Birchall for examining the TARP/receptor drug binding site, Maria Carvalho for analyzing pore dimensions of A1/2- γ 8 using the HOLE program and Sjors Scheres for helpful comments regarding cryo-EM data processing. Thanks to Olga Perisic for providing the HEK293-ExpiTM transfection protocol, Giuseppe Cannone and Shaoxia Chen for support at the LMB EM facility, Jake Grimmett and Toby Darling for maintaining the LMB computer cluster, Paul Elmsley for help in modelling, Diamond for access and support of the Cryo-EM facilities at the UK national electron bio-imaging centre (eBIC), proposal EM17434, funded by the Wellcome Trust, MRC and BBSRC, and Kyle Dent for his support during data collection. The cryo-EM density maps representing the full-length 1 and TMD/LBD models have been deposited in the Electron Microscopy Data Bank with accession numbers EMD-4575 and EMD-4572 respectively. The corresponding coordinates have been deposited in the Protein Data Bank with PDB codes 6QKZ and 6QKC respectively.

Funding: This work was supported by grants from the Medical Research Council to I.H.G. (MC_U105174197). J.G.-N. is funded by Heptares Therapeutics.

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One Sentence Summary

Cryo-EM reveals the architecture of the predominant AMPA receptor heteromer in the forebrain.

Figure 1. Positioning of AMPAR subunits in the GluA1/2 heteromer.

(A) Schematic of an AMPAR tetramer (subunits A-D) depicting the four domain layers (NTD - N-terminal domain, LBD - ligand-binding domain, TMD - transmembrane domain, CTD - C-terminal domain). The AMPAR is two-fold symmetric with non-equivalent 'pore-proximal' (blue) and 'pore-distal' (red) positions. TARP auxiliary subunits can locate at two distinct sites denoted A'C' (light green) or B'D' (dark green). The inset (right) depicts the subunit arrangements as viewed from the LBD level.

(B) Schematic of TARP $\gamma 8$, depicting transmembrane helix (TM, cylinder) topology and arrangement of extracellular loops 1 and 2 (Ex1 & Ex2) into a beta sheet (arrows).

(C) Deactivation kinetics of GluA1/2 receptors in response to 2 ms of 10 mM L-glutamate are slowed when $\gamma 8$ is co-expressed or in tandem with the AMPAR, independently of the tandem fusion (deactivation τ (ms); A1:A2: $\gamma 8$, 1.53 ± 0.18 , $n=8$; A1:A2_ $\gamma 8$, 1.51 ± 0.16 , $n=8$; A1_ $\gamma 8$:A2, 1.43 ± 0.12 , $n=7$; A1:A2, 0.89 ± 0.14 , $n=6$; One-way ANOVA with Tukey's multiple comparison test, $p=0.0316$). Peak-scaled responses, scale bar = 3 ms.

(D) GluA1 I629A and GluA2 I633A mutations (denoted 'IA', or starred) impair current responses of homomeric receptors (10 mM L-glutamate peak amplitude (pA) (median, 25 and 75 percentiles reported, 5 to 95 percentiles depicted as whiskers); GluA1, 1991.3 (1323.5 to 3214.8), $n=19$; GluA1 I629A, 5.1 (3.3 to 7.1), $n=22$; Mann-Whitney test, $p<0.0001$; GluA2, 3932.0 (2989.0, 5080.9), $n=20$; GluA2 I633A, 59.9 (29.7, 129.1), $n=19$; Mann-Whitney test, $p<0.0001$). Scale bars = 400 pA & 20 ms.

(E) In a GluA1/2 heteromeric context, GluA2 IA mutation impairs channel gating significantly more than GluA1 IA (Peak amplitude (pA); GluA1/2, 1520.9 (1017.5, 2467.3), $n=48$; GluA1_{IA}/A2, 538.2 (254.2, 823.3), $n=48$; GluA1/A2_{IA}, 103.5 (38.4, 366.6), $n=42$; GluA1_{IA}/A2_{IA}, 14.5 (7.2, 29.7), $n=18$; Kruskal-Wallis test with Dunn's multiple comparisons test, $p<0.0001$, all conditions are significantly different to each other, however only statistical significance of A1_{IA}/A2 versus A1/A2_{IA} comparison is shown for clarity), demonstrating preferential pore-distal location of GluA2 in the heteromeric receptor. Scale bar = 20 ms & 400 pA.

(F) 'CL' mutation of M1 helix (yellow star) abolishes TARP-modulation of AMPAR gating. Two subunit arrangements 'GluA1 pore-proximal' and 'GluA2 pore-proximal' are possible. In a GluA1/2_ $\gamma 8$ receptor, mutagenesis will prevent TARP-modulation when included in pore-proximal, but not pore-distal subunits.

(G) Sample traces for 500 ms application of 1 mM L-glutamate to A1/A2_ $\gamma 8$ with or without 'CL' mutation (star). Peak-scaled responses, scale bar = 100 ms, and magnified inset scale bar = 10 ms.

(H) TARP-dependent slowing of desensitization is prevented by CL mutation of GluA1 (C524L), but not GluA2 (C528L) (desensitization τ (ms); A1:A2_ $\gamma 8$, 10.1 ± 0.6 , $n=11$; A1_{CL}:A2_{CL}_ $\gamma 8$, 6.6 ± 0.5 , $n=8$; A1:A2_{CL}_ $\gamma 8$, 10.2 ± 0.4 , $n=7$; A1_{CL}:A2_ $\gamma 8$, 6.4 ± 0.2 , $n=6$; one-way ANOVA with Tukey's multiple comparison test, $p<0.0001$), which is reflected in the magnitude of steady-state AMPAR currents

(I) (Steady-state response as % of peak amplitude; A1:A2_ $\gamma 8$, 3.82 ± 0.49 , $n=11$; A1_{CL}:A2_{CL}_ $\gamma 8$, 1.55 ± 0.59 , $n=8$; A1:A2_{CL}_ $\gamma 8$, 3.76 ± 0.67 , $n=7$; A1_{CL}:A2_ $\gamma 8$, 2.37 ± 0.57 , $n=6$; One-way ANOVA with Tukey's multiple comparison test, $p=0.02$).

Figure 2. Cryo-EM structure of the full-length GluA1/2_γ8 receptor complex.

(A) Cryo-EM density map of the full-length GluA1/2_γ8 receptor (B-factor sharpening = -350). The structural model was obtained by rigid-body fitting of individual receptor domains into the density map (GluA1 in blue, GluA2 in red, γ8 in green). A top view onto the ‘NTD layer’ is shown above the receptor, highlighting the tetramer interface formed by the two GluA2 NTDs.

(B) Model depicting front view of the full-length GluA1/2_γ8 heteromer. The three domain layers, NTD, LBD and TMD, are indicated, and stippled lines indicate the boundaries of the membrane.

(C) Side view of the receptor model, visualizing the tetrameric interface in the NTD layer, a GluA1/GluA2 LBD heterodimer and two γ8 auxiliary subunits docked at the B’D’ positions.

(D) Slice of the 3D cryo-EM map at the TMD region showing transmembrane helices of GluA1 (blue), GluA2 (red) and γ8 (green).

(E) Detail of the cryo-EM density at the GluA1-specific glycosylation site on the NTD. At A/C subunits, protruding density corresponds to a GluA1-specific glycan site (Asn45).

(F) The B/D subunit (GluA2) shows no extra density for a glycan.

Figure 3. Cryo-EM map of the GluA1/2_ $\gamma 8$ TMD-LBD region.

(A) Cryo-EM map (4.4 Å) of the TMD/LBD/ $\gamma 8$ portion of the A1/2_ $\gamma 8$ complex (B-factor sharpening = -150) with GluA1 (blue), GluA2 (red) and $\gamma 8$ (green) showing lipid-like densities demarcating the upper membrane boundary (orange). Color-scheme is maintained throughout the figure. The competitive antagonist NBQX is located in the agonist-binding site of the LBD.

(B) Top view of the TMD-LBD map showing the two LBD heterodimers forming the ‘gating ring’ in a characteristic two-fold symmetric “closed-state” conformation. TARP $\gamma 8$ is positioned beneath GluA2 subunits.

(C) Detail of the M3 gate helices of the TMD region, below the LBD layer, highlighting the position of GluA1 I629 and GluA2 I633 (spheres).

(D) Details of the selectivity filter forming the inner pore constriction. Side view onto the filter showing the Q/R site residues on top of the pore loop and the adjacent +1 Q (GluA1

Q583, GluA2 Q587). The cysteines at the +3 position (GluA1 C585, GluA2 C589) surround a strong density within the pore. The polyamine-binding aspartates (GluA1 D586, GluA2 D590) are also indicated. The selectivity filter is surrounded by the M3 gating helices.

(E) Top view, showing the orientation of the Q/R site residues (GluA1 Q582, GluA2 R586) and the +1Q side chains interacting with tryptophans projecting from the M3 helices (GluA1 W602, GluA2 W606).

Figure 4. Lipid-like densities associate with the TMD region of GluA1/2_ $\gamma 8$.

(A) Model of the TMD region (as cartoon) with five modeled acyl chains (L1 to L5, as sticks in orange) filling the cavity between $\gamma 8$ TM4 and GluA1 M4, and a further lipid molecule (L6) locating to the perimeter of $\gamma 8$, between the $\gamma 8$ TM1 and TM2 helices. Cryo-EM density for lipid-like molecules is shown as a mesh.

(B) Detailed view of lipids L4 and L5. The density for L4 projects into the fenestration between M1 and M3, approaching the apex of the M2 loop of the GluA1 subunit. Side chains interacting with the potential lipid molecules are represented as sticks.

(C) TMD slice (right) of the cryo-EM map of the TMD/LBD model (left), with clear density for the GluA1, GluA2 and $\gamma 8$ helices and the 6 acyl groups color-coded as in (A).

(D) Bottom view of the A1/2_ $\gamma 8$ TMD sector with the modeled lipids displayed as orange spheres.

Figure 5. Interaction of $\gamma 8$ with the GluA1/2 TMD region.

(A) Ribbon representation of the A1/2_ $\gamma 8$ TMD-LBD model. Numbers and dashed-lines represent the points of view of the AMPAR- $\gamma 8$ interaction site as displayed in panels (B), (C) and (D).

(B) Top view of the AMPAR- $\gamma 8$ interaction region, with the cryo-EM map overlaid. Important interacting residues are shown as sticks. This slice through the density was generated at the level of $\gamma 8$ residues V176 (TM3) and G209 (TM4) to offer a view onto the ‘pocket’ occupied by antiepileptic drugs.

(C) Side view of the interaction between $\gamma 8$ (green) and the receptor. Docking of the TARP results in helical reorganization, highlighted by overlaying M1 of the TARP-free (A’C’) site (grey helices) with the TARP-bound B’D’ site (colored helices). The helical shift between M4 helices (~ 1.5 Å) is shown. A bottom view is depicted inset.

(D) TARP binding results in M1 side chain reorganizations (obtained by overlaying M1 helices from the A’C’ and B’D’ sites, as described in panel (C)). GluA1 Y519 and M523 move to interact with the TARP, while E520 forms a hydrogen bond with Y199 of $\gamma 8$ (depicted in magnified inset).

- (E) Side view of the interaction between $\gamma 8$ TM3 helix (green) and GluA2 M4 helix (red), with interacting side chains displayed as sticks and contoured with the cryo-EM density.
- (F) Interaction between $\gamma 8$ TM4 helix (green) and GluA1 M1 helix (blue), with interacting side chains displayed as sticks and contoured with the cryo-EM density.

Figure 6. A unique TARP $\gamma 8$ interaction site on the GluA2 LBD controls receptor gating.

(A) Full-length 2 cryo-EM map showing the additional density between the upper lobe of GluA2 and the $\gamma 8$ $\beta 1$ - $\beta 2$ loop in Ex1 (represented with B= -170 and using a low-threshold). Magnified inset contains TMD/LBD model as a cartoon representation, showing GluA2 (red) and $\gamma 8$ (green).

(B) Surface representation of the TMD/LBD model, depicting the likely location of $\beta 1$ - $\beta 2$ loop residues (dashed line) projecting towards the GluA2 LBD upper lobe loop. Location of the glyco-mutation for disruption of this interaction is depicted as a star.

(C) Current responses of TARP-associated A1/2 receptors evoked by 500 ms 1 mM L-glutamate application both with (light green) and without (dark green) GluA2 E413T (glyco-) mutation to introduce N-glycosylation (on N411) at the predicted interaction site. Glycosylation selectively affects the rate of entry into desensitization of TARP $\gamma 8$ but not TARP $\gamma 2$ -associated A1/2 receptors. Peak-scaled responses, scale bar = 10 ms.

(D) GluA2 glyco-mutation speeds the rate of desensitization entry of A1/2_ $\gamma 8$ to that of TARP-free A1/2 receptors. In contrast, glyco-mutation of GluA1 in A1/2_ $\gamma 8$ or GluA2 in an A1/2_ $\gamma 2$ assembly do not affect desensitization entry kinetics (desensitization τ (ms);

A1/A2 (grey bar), 6.27 ± 0.33 , $n=5$; A1/A2_γ8, 9.68 ± 0.46 , $n=25$; A1/A2_{glyco}-γ8, 7.36 ± 0.30 , $n=15$; A1_{glyco}/2_γ8, 10.71 ± 0.51 , $n=5$; A1/A2_γ2, 10.14 ± 0.72 , $n=10$; A1/A2_{glyco}-γ2, 9.71 ± 1.05 , $n=6$; One-way ANOVA with Sidak's multiple comparison test, $p=0.0005$).