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1 **Gating transitions and modulation of a hetero-octameric AMPA glutamate receptor**

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7 **Abstract**

8 AMPA glutamate receptors (AMPA receptors) mediate the majority of excitatory transmission in the
9 brain, and enable synaptic plasticity that underlies learning ¹. A diverse array of AMPAR
10 signaling complexes are established by receptor auxiliary subunits, associating in various
11 combinations to modulate trafficking, gating and synaptic strength ². However, their
12 mechanisms of action are poorly understood. Here, we determine cryo-electron microscopy
13 structures of the heteromeric GluA1/2 receptor assembled with both TARP- γ 8 and CNIH2,
14 the predominant AMPAR complex in the forebrain, in both resting and active states (at 3.2
15 and 3.7 Å, respectively). Consequential for gating regulation, two γ 8 and two CNIH2
16 subunits lodge at distinct sites beneath the ligand-binding domains of the receptor tetramer,
17 with site-specific lipids shaping each interaction. Activation leads to a stark asymmetry
18 between GluA1 and GluA2 along the ion conduction path, and an outward expansion of the
19 channel triggers counter-rotations of both auxiliary subunit pairs, that promotes the active-
20 state conformation. In addition, both γ 8 and CNIH2 pivot towards the pore exit on activation,
21 extending their reach for cytoplasmic receptor elements. CNIH2 achieves this through its
22 uniquely extended M2 helix, which has transformed this ER-export factor into a powerful
23 positive AMPAR modulator, capable of providing hippocampal pyramidal neurons with their
24 integrative synaptic properties.

1 **Main**

2 Unique amongst ionotropic glutamate receptors, AMPARs form a diverse array of signaling
3 complexes, specialized for a range of functions - from faithfully decoding high frequency
4 inputs to integration of low-frequency signals in support of synaptic plasticity³. This
5 spectrum results from a mosaic of receptor combinations, assembled from four pore-forming
6 subunits (GluA1-4) and multiple auxiliary proteins that exist in various stoichiometries and
7 exhibit distinct expression patterns in the brain^{4,5}. The predominant subunit, GluA2, dictates
8 critical functions such as ion permeation, and forms heteromeric complexes with GluA1 or
9 GluA3 in principal neurons of the forebrain^{2,6}.

10

11 Members of the transmembrane AMPAR regulatory proteins (TARPs γ 2, 3, 4, 5, 7, 8) and
12 cornichon homologue (CNIH2, 3) families are the most widely expressed auxiliary subunits
13 ². TARP γ 8 and CNIH2 are highly abundant at cortical synapses^{7,8}, and are powerful
14 AMPAR modulators^{9,10} that co-purify and synergize to determine receptor expression levels,
15 kinetics and pharmacology^{7,11-13}. Both are four-helical bundle transmembrane proteins, yet
16 they have different topologies, resulting in fundamentally different mechanisms of action.
17 Whilst engaging the receptor transmembrane domain (TMD), TARPs possess an elaborate
18 extracellular beta-sheet structure that contacts the ligand-binding domains (LBDs)¹⁴⁻¹⁶.
19 CNIHs on the other hand are mostly embedded within the membrane¹⁷, but have two
20 cytoplasmic loop elements of unknown structure that contribute to their function as cargo
21 exporters from the endoplasmic reticulum (ER)¹⁸. Hetero-auxiliary receptor complexes have
22 yet to be resolved, and therefore the stoichiometry and distinct modulatory mechanism of
23 each associating protein are poorly understood. To address these questions, we investigated
24 the architecture and functioning of the principal forebrain AMPAR complex, the GluA1/2
25 receptor assembled with TARP γ 8 and CNIH2.

1 **Recombinant GluA1/2/ γ 8/CNIH2 resembles native AMPARs**

2 We first reconstituted the hetero-auxiliary AMPAR complex and compared its properties to
3 those of native hippocampal AMPARs. CNIH2-expressing HEK-Expi293F cells were co-
4 transfected with GluA1 and a GluA2_ γ 8 (A2_ γ 8) fusion gene, to ensure the presence of two
5 γ 8 subunits¹⁵. Although AMPAR stoichiometries with four γ 8 molecules may exist¹⁹, the
6 co-existence of γ 8 and CNIH2 predominates in forebrain neurons^{7,11}. In response to rapid
7 glutamate application to excised membrane patches, γ 8 slowed desensitization rates about 2-
8 fold compared to GluA1/2 alone, and addition of CNIH2 led to much further slowing that
9 was greater than that of four γ 8 subunits alone (**Fig. 1a, b**)¹⁵. CNIH2 also further increased
10 the equilibrium current (**Extended Data Fig. 1a**). Together with previous data¹³, this
11 confirms incorporation of CNIH2 into a hetero-auxiliary complex.

12
13 We found close correspondence between responses from the recombinant complex and
14 AMPARs expressed in CA1 and CA3 pyramidal neurons, but not to those from dentate
15 granule cells or CA1 interneurons, where γ 8 and other auxiliary subunits prevail (**Fig. 1a, b**,
16 **Extended Data Fig. 1a**)²⁰. This demonstrates the diversity of AMPAR complexes, and
17 provides further evidence that CNIH2 acts to define the unique response properties of
18 pyramidal neurons, with the GluA1/2/ γ 8/CNIH2 octamer the major assortment in these cells
19^{7,11,13}. We subjected this native-like complex for structural analysis by cryo-electron
20 microscopy (cryo-EM).

22 **Trapping GluA1/2/ γ 8/CNIH2 in active and inactive states**

23 To resolve gating transitions, the complex was trapped both in an inactive state, bound to the
24 competitive antagonist NBQX, and in an active state with the agonist L-glutamate together
25 with the desensitization blocker cyclothiazide (CTZ) (**Extended Data Fig. 1b**). 3-D

1 reconstructions revealed the classic three-layered AMPAR domain architecture, with the
2 extracellular N-terminal domain (NTD) and LBD arranged as dimers of dimers, attached to
3 the ion channel of pseudo four-fold symmetry (**Fig. 1c-e, Extended Data Fig. 1c**). Focused
4 processing of the LBD-TMD, and of individual domain layers resulted in high-resolution
5 maps ranging from 3.0 Å to 3.8 Å (**Extended Data Fig. 2-4, and Extended Data Table 1**),
6 permitting us to follow gating transitions at comparable resolutions. Moreover, reconstruction
7 of particles from resting-state data lacking CNIH2, enabled us to also examine the effects of
8 CNIH2-association with the receptor (**Extended Data Fig. 2**).

9

10 **Subunit arrangement in the hetero-octamer**

11 EM-map quality permitted unambiguous assignment of the four core subunits^{15,21}, placing
12 GluA1, marked by unique N-glycans in the NTD (**Extended Data Fig. 5a**), to the two outer
13 (or AC) positions, and GluA2 to the inner (BD) positions. This arrangement dictates function
14 as the BD subunits exert a greater mechanical force on the ion channel gate than the AC
15 subunits²², with consequences on the entire complex, as we describe below. The core
16 subunits alternate around the pore with their peripheral M1 and M4 transmembrane helices
17 contributing two pairs of non-equivalent binding sites for auxiliary subunits: the A'C' sites
18 (M1_{GluA2}/M4_{GluA1}) locate beneath the LBD dimers, thus these positions are spatially more
19 restricted than the B'D' sites (M1_{GluA1}/M4_{GluA2}), beneath the LBD dimer-of dimers interface
20 (**Fig. 1c-e**). With $\gamma 8$ binding to its preferential B'D' site¹⁵, CNIH2 occupies the A'C'
21 positions, and despite its greater detergent resistance^{4,7}, CNIH2 does not displace $\gamma 8$ from the
22 receptor. This organization determines gating modulation, as each site provides differential
23 access of the auxiliaries to the gating machinery.

24

1 We obtained a complete structural model for CNIH2, including its two sequence-diverse
2 cytoplasmic loops between the M1/2 and the M3/4 helices, that were not resolved in the
3 recent CNIH3 structure ¹⁷ (**Extended Data Fig. 5f**). Lodging to the A'C' sites, the CNIH2
4 M1 and M2 helices project deeply into the cytoplasm (>20 Å) (**Fig. 1f**), forming a loop
5 element of currently unknown function. A comparison of the binding modes for CNIH2 and
6 γ8 shows that both engage the same set of residues on the receptor, yet their footprints vary
7 and differences in side chain orientations along the M1 helix are apparent (**Fig. 1g-i**,
8 **Extended Data Fig. 6a**). While TARP γ8 contacts are spread out more evenly, CNIH2
9 prominently engages two regions: the upper tier of the A'C' site, centered around GluA2
10 Cys528, and its base adjacent to GluA2 Phe546 (**Fig. 1i**). Three highly conserved N-terminal
11 phenylalanines (Phe3, -5, -8)¹⁷ slot CNIH2 into the TMD proximal to the gate (**Fig. 1g**), and
12 these are crucial for modulation, as we show below. At the base, CNIH2 interacts with
13 Arg545 and Phe546 through polar and hydrophobic contacts (**Fig. 1h**), and this region of the
14 A'C' site is shaped by lipids.

15

16 **Annular lipids shape auxiliary subunit binding sites**

17 An abundance of lipid-like densities ensheath the ion channel, forming an annular belt
18 demarcating the membrane boundaries (inset in **Fig. 1d**). Lipids in the upper leaflet connect
19 the gate-surrounding pre-M1 helices to the M2 pore helices in the lower leaflet, their location
20 within the lateral fenestrations of the channel indicate a role in channel modulation. Other
21 lipids, denoted lower-leaflet lipid (LL) 1 and LL2, bridge between auxiliary subunits and the
22 M2 pore helix, thereby extending the reach of these modulating proteins to the ion
23 conduction path (**Extended Data Fig. 6c, d**).

24

1 The acyl chains of two lipids penetrate the lower part of the CNIH2 binding site: wedging
2 between GluA1 and CNIH2, upper-leaflet lipid (UL) 1 contacts Phe546 (**Fig. 1h**), while
3 immediately beneath, LL3 anchors CNIH2 via its Phe22 and Trp26 side chains to the base of
4 the GluA1 M4 helix (**Fig. 1i, Extended Data Fig. 6d**). These acyl chains likely influence
5 CNIH2 binding by extending the hydrophobic network at the A'C' site. This idea is
6 supported by the GluA2-CNIH3 structure (PDB 6PEQ), where some lipids are also observed
7 ¹⁷, but LL1 and LL2 are missing. As a result, CNIH3 aligns with the receptor more closely
8 than CNIH2 (**Extended Data Fig. 6e**). Hence, lipids may regulate CNIH-modulation of
9 AMPARs.

10

11 **The GluA2 subunit dominates gating**

12 Receptor activation is triggered by glutamate closing the LBD 'clamshells' ²³ (**Extended**
13 **Data Fig. 5b-e**), leading to separation of the lower lobes within an LBD heterodimer and
14 rearrangement of the LBD-TMD linkers to open the gate ^{14,16}. This state transition is well
15 resolved. Focusing on the outer gate formed by the M3 transmembrane helices, M3 linker
16 tension ruptures polar contacts between GluA1 Arg624 and GluA2 Arg628, a position
17 determining gating kinetics ²⁴, and breaks a stacking interaction between GluA1 Arg624 and
18 Phe623 in GluA2 (**Fig. 2a, b**). These changes are stabilized by reorganization of the gating
19 linkers, and are accompanied by opening of the channel gate formed by hydrophobic contacts
20 facing the pore axis along the conserved **SYTANLAAFLT** motif (**Fig. 2b, c**).

21

22 Gate dilation is more pronounced between the two opposing GluA2 subunits than between
23 GluA1, and this continues into the selectivity filter, formed by the M2 pore loops midway in
24 the conduction path (**Fig. 2c**). At the Q/R site (Arg586), which determines Ca²⁺ flux and
25 channel conductance at the apex of the M2 pore loop ^{25,26}, activation widens the filter

1 entrance selectively between the GluA2 subunits (**Extended Data Fig. 7a, b**). This
2 asymmetric widening extends down to GluA2 Cys589, midway in the filter and to the M2
3 pore helices, which separate by $> 1 \text{ \AA}$ in GluA2 but not GluA1, and is apparent at the
4 cytoplasmic face of the pore, the site modulated by polyamines²⁷ (**Extended Data Fig. 7c, d,**
5 **e**). We note that binding of CNIH2, which increases channel conductance²⁸, does not cause a
6 visible expansion of the selectivity filter under resting-state conditions (**Extended Data Fig.**
7 **7b-d**).

8

9 Receptor activation is also accompanied by an anti-clockwise twist of the AMPAR TMD
10 helices, when looking down the pore from the extracellular side, and a marked expansion in
11 the upper part of the channel that surrounds the gate (**Extended Data Fig. 8a**). This dilation
12 is greater between opposing GluA2 subunits, than between GluA1, and includes the
13 channel's peripheral helices (pre-M1, M1 and M4) and both pairs of auxiliary subunits (**Fig.**
14 **2d**).

15

16 **Gating transitions of core and auxiliary subunits**

17 Dictated by its architecture, receptor activation differentially impacts the interactions between
18 core and auxiliary subunits. On activation, the GluA2 M3 gate helix pushes its Phe623 and
19 Leu624 side chains against its pre-M1 helix, triggering channel dilation (**Fig. 2b, d,**
20 **Supplementary Video 1**). Pre-M1 couples LBD closure to channel opening, and shapes
21 gating kinetics throughout iGluRs²⁹⁻³¹. At the same time, the GluA2 M1 and M3 linkers
22 closely align and approach the TARP extracellular (β 4) loop, which is simultaneously
23 contacted by the GluA2 LBD through its 'KGK' motif³² (**Extended Data Fig. 8b**). GluA1
24 does not experience these large changes, as its M3 linkers are pulled upward (left panel in
25 **Fig. 2c**), a characteristic of AC subunits^{14,16}. As a result, the GluA1 M3 helices minimally

1 contact pre-M1 on activation, which underlies the asymmetric expansion of the channel (**Fig.**
2 **2d**). We also observe newly formed contacts between the GluA1 M4 linkers (at Thr780) with
3 the GluA2 M3 gate on activation, a change that is not apparent with GluA1 M3. Together,
4 these rearrangements lead to enhanced engagement of the GluA2 subunit by CNIH2 and $\gamma 8$.

5

6 **Counter-rotations of the auxiliary subunit pairs promote 2-fold symmetry**

7 Expansion of the channel, triggered by M3 gate opening, transmits to both pairs of auxiliary
8 subunits, leading to global structural changes (**Fig. 2d**): The TARP extracellular beta-sheet
9 segment bends away from the pore axis; its TMD sector undergoes a right-handed twist
10 around its vertical axis, while CNIH2 turns left-handedly by $\sim 4^\circ$ (**Fig. 2d-f**). As a net result,
11 the GluA2 side of the channel (between the GluA2 M1 and M4 helices) widens on activation,
12 while the GluA1 side (between GluA1 M1 and M4) contracts (**Extended Data Fig. 8c, d**),
13 contributing to the two-fold symmetry-switch upon opening. The counter-rotations of the
14 two auxiliaries are likely driven by their different topologies, and is of major consequence, as
15 the left-handed twist of CNIH2 follows the rotation of the channel and thereby will stabilize
16 this active conformation, with an open pore.

17

18 Moreover, rotations of the auxiliary subunits are accompanied by tilts in the vertical plane,
19 bringing them into closer reach of cytoplasmic portions of the active receptor
20 (**Supplementary Video 2**). We observe formation of contacts between the $\gamma 8$ M4 helix and
21 the start of the GluA1 M1/2 cytoplasmic loop (**Fig. 2e**, inset in **Extended Data Fig. 8c**),
22 while the cytoplasmic part of the CNIH2 M2 helix tilts towards the pore (**Fig. 2f**). This
23 behaviour is also evident by normal mode analysis (**Supplementary Video 3**)^{33,34}, where
24 both twisting and pivoting of CNIH2 is apparent in a number of modes. Hence, activation

1 facilitates interaction of both auxiliaries with the AMPAR intracellular portion, which is
2 currently unresolved.

3

4 **Mechanism of CNIH2 modulation**

5 Dictated by their architecture, CNIHs will exert AMPAR modulation exclusively at the TMD
6 or cytoplasmic levels. Three conserved phenylalanines (Phe3, -5, -8) slot CNIH2 (and
7 CNIH3¹⁷) into the upper tier of the A'C' binding site (**Fig. 1g, i**). When mutated individually
8 to leucine, all three mutants weakened CNIH2's modulation of GluA2 kinetics, and reduced
9 the equilibrium response, but did not affect complex formation or receptor trafficking (**Fig.**
10 **3a, Extended Data Fig. 9a-d**). Simultaneous mutation of all three residues to leucine
11 reduced trafficking and, consequently, gating modulation (**Fig. 3a, Extended Data Fig. 9a-**
12 **d**), highlighting this interaction as an important modulatory site.

13

14 However, the N-terminus, including Phe3, -5, -8, is conserved in CNIH1 (**Fig. 3b**), which
15 also associates with the receptor and traffics to the cell surface, but modulates gating only
16 weakly (**Fig. 3c, Extended Data Fig. 9a-c, e**)^{28,35}. Yet, CNIH1 lacks an elongated N-
17 terminal segment in the M2 helix (termed 'M2-N') present in both modulatory subunits,
18 CNIH2 and CNIH3 (**Fig. 3b, d**). M2-N is formed by a kink in M2, created by a conserved
19 proline (Pro70), that 'uncouples' M2-N from the rest of the helix, thus enabling its tilting
20 towards the pore on activation (**Fig. 2f, Supplementary Videos 2, 3**). Structural flexibility of
21 M2-N is exemplified by its different kink angle between CNIH2 and CNIH3 (PDB 6PEQ).
22 The CNIH2 M3/4 loop, with a potential cholesterol binding site in its vicinity (**Extended**
23 **data Fig. 7f**), straddles M2-N, thereby impacting its dynamics (**Fig. 3d**).

24

1 To test the role of M2-N in AMPAR modulation, we inserted this segment from CNIH2 into
2 CNIH1. The resulting CNIH1 chimera strongly gained modulatory activity, substantially
3 slowing GluA2 desensitization and increasing the equilibrium response (**Fig. 3c, Extended**
4 **data Fig.9e**), thus highlighting a critical role for CNIH2 interactions with cytoplasmic
5 receptor elements. Given the presence of positively charged side chains projecting from M2-
6 N towards the negatively charged M1/2 receptor loops (**Fig. 3d**), these two cytosolic
7 elements are strong candidates to interact and enable this regulation.

8

9 **Conclusion**

10 Based on these data, we propose the following mechanism underlying CNIH2 modulation of
11 AMPARs (**Fig. 3e**): Association with CNIH2 stabilises the active-state conformation of pore
12 helices, through tight local contacts between Phe3,-5,-8 and gate-surrounding receptor
13 residues. Opening of the M3 gate triggers a left-handed rotation of CNIH2, that promotes the
14 open state by stabilising the expanded A'C' site helices and M2 pore loop, with a dilated
15 pore. Full modulation requires additional contacts between M2-N and cytosolic receptor
16 elements, which may further maintain the dilated ('active') channel conformation, slowing
17 relaxation of the rotated upper regions back to the closed conformation. This scheme
18 accounts for residual modulation by CNIH1, which supports the active state locally through
19 its conserved N-terminus, but in lacking M2-N, is unable to maintain the fully active-state
20 conformations strongly.

21

22 Dictated by their position in the octameric complex and by topological differences, these two
23 auxiliary subunits act in concert to modulate AMPARs. While TARPs engage the LBDs and
24 the M1&M3 gating linkers through their extracellular portion, CNIH2 &3 act through their
25 distinct helical rearrangements and, now resolved, intracellular M1/2 loop element. These

1 appendages, unique to each auxiliary subunit, stabilise transient gating conformations, and
2 determine the rotational behavior of CNH2 and γ 8 to enact global structural changes. These
3 mechanisms combine to build a relatively slowly deactivating AMPAR complex, providing
4 hippocampal pyramidal neurons with the required synaptic properties to temporally
5 coinciding inputs.

6

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13

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25 **Author contributions:** IHG conceptualized and supervised the study, and wrote the paper with
26 input from JFW. DZ performed protein purification, cryo-EM data collection, data processing
27 and model building. JFW and OC designed and performed electrophysiological experiments.
28 PMM and JFW performed confocal imaging and data analysis.

29 **Data and materials availability:** Cryo-EM density and PDB models have been submitted to
30 the PDB and EMDB.

31

Fig. 1

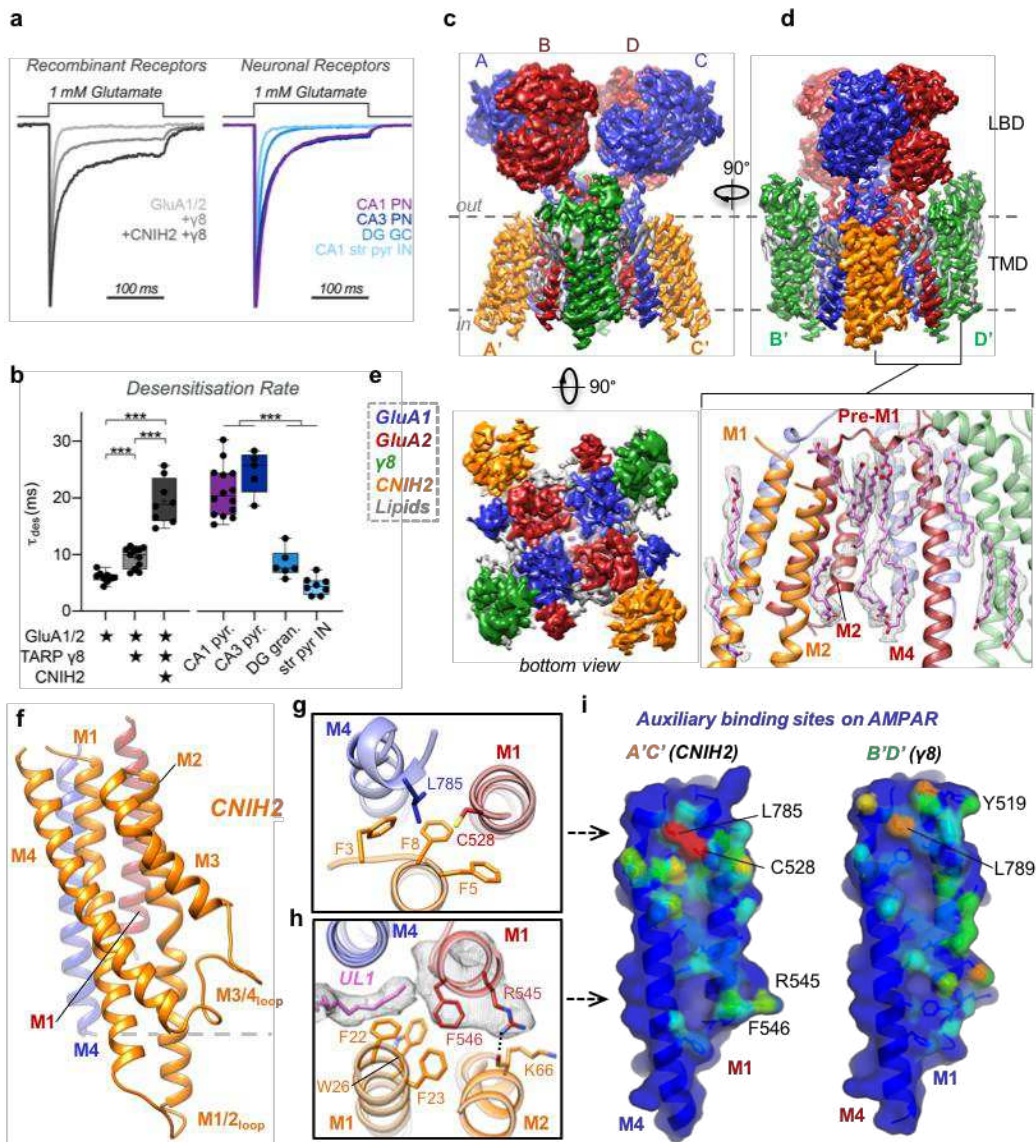


Fig. 1: Physiology and architecture of the GluA1/2_ γ 8 /CNIH2 complex.

a,b, Auxiliary subunits slow desensitization rates of recombinant receptors (**a**, left). Native AMPAR responses show diverse properties (**a**, right), with A1/2_ γ 8 +CNIH2 recapitulating CA1 & CA3-like kinetics (**b**, weighted τ_{des} (ms), mean \pm SEM). *Recombinant receptors*: GluA1/2: 6.01 ± 0.31 , n=9; + γ 8: 9.29 ± 0.53 , n=12; +CNIH2+ γ 8: 19.71 ± 1.27 , n=9. *Neuronal receptors*: CA1 pyramidal: 21.03 ± 1.19 , n=14; CA3 pyramidal 24.51 ± 1.69 , n=5; DG granule cell: 8.38 ± 1.01 , n=6; CA1 stratum pyramidale interneurons: 4.48 ± 0.55 , n=8;

1 Welch's ANOVA tests with Dunnett's multiple comparisons test – Recombinant: $W(2,15.11)$
2 = 60.68, $p < 0.0001$; Neurons: $W(3,11.60) = 76.28$, $p < 0.0001$.

3 **c-e**, Cryo-EM maps of the AMPAR octamer, depicting the LBD and TMD domain layers.
4 Core subunits positioned to AC/BD, and auxiliary subunits associated with A'C'/B'D' sites
5 are shown. **(c)** Front view, depicting $\gamma 8$ located at B'D' sites. **(d)** Side view, offering a view
6 of CNIH2 at A'C' sites, beneath an LBD dimer. Inset: annular lipids concentrating at side of
7 the TMD, beneath the pre-M1 helix. **(e)** Bottom view, highlighting CNIH2 binding to GluA2
8 transmembrane helices M1-3 (red), and $\gamma 8$ contacting the GluA1 M1-3 helices (blue). **f**,
9 Model of CNIH2, including the M1/2 and M3/4 cytosolic loops, docking to its binding site
10 ($M1_{GluA2}$ [red] $M4_{GluA1}$ [blue]). **g**, CNIH2 Phe3, -5, -8 slotting into the top tier of its binding
11 site close to Cys528 (GluA2) and Leu785 (GluA1). **h**, CNIH2 contacts at the bottom of the
12 binding site are mediated by Phe23 and Lys66; a lipid (UL1) penetrates the A'C' site and
13 interacts with Phe546 (GluA2). **i**, A'C' (left) and B'D' (right) site surface representation.
14 $M1_{A2}$ and $M4_{A1}$ residues contacted by CNIH2 are coloured depending on the number of
15 atoms contributing to the interaction (red: high, blue: low). Contacts were counted using the
16 'findNeighbors' command from *ProDy*³⁸, with a cutoff of 4.5 Å between heavy atoms.
17

1 **a**, View onto a GluA1/GluA2 LBD dimer and associated M3 gating helices (depicted in bold)
2 in the resting state. **b**, Side view onto the M3 gate with open (bold colour) and resting (soft
3 colour) states overlain, depicting rearrangement of the M3 linkers and reorientation of M3
4 side chains relative to pre-M1. **c**, Comparison of GluA1 and GluA2 conduction path; resting
5 state (grey) is superimposed on the active state (colour). Divergence from the closed gate is
6 apparent at the top of M3, and is more pronounced in GluA2 (C α distances between opposing
7 T621 (GluA1), and T625 (GluA2) indicated). Also shown is the selectivity filter with the Q/R
8 site at its apex. **d**, Top view onto the closed- (grey) and open-state (colour) models at the
9 level of the M3 gate. The dilation of the gate-surrounding helices is shown and measured
10 between opposing pre-M1 helices. **e**, Side view showing outward expansion of the upper part
11 of GluA1 M1 and M3 and the γ 8 β -sheet, accompanied by a slight inward movement of the
12 γ 8 M4 helix towards the GluA1 M12 loop. **f**, CNIH2 undergoes a $\sim 3^\circ$ pivot ('tilt') on
13 activation, moving its M12 loop closer to the pore axis.

14

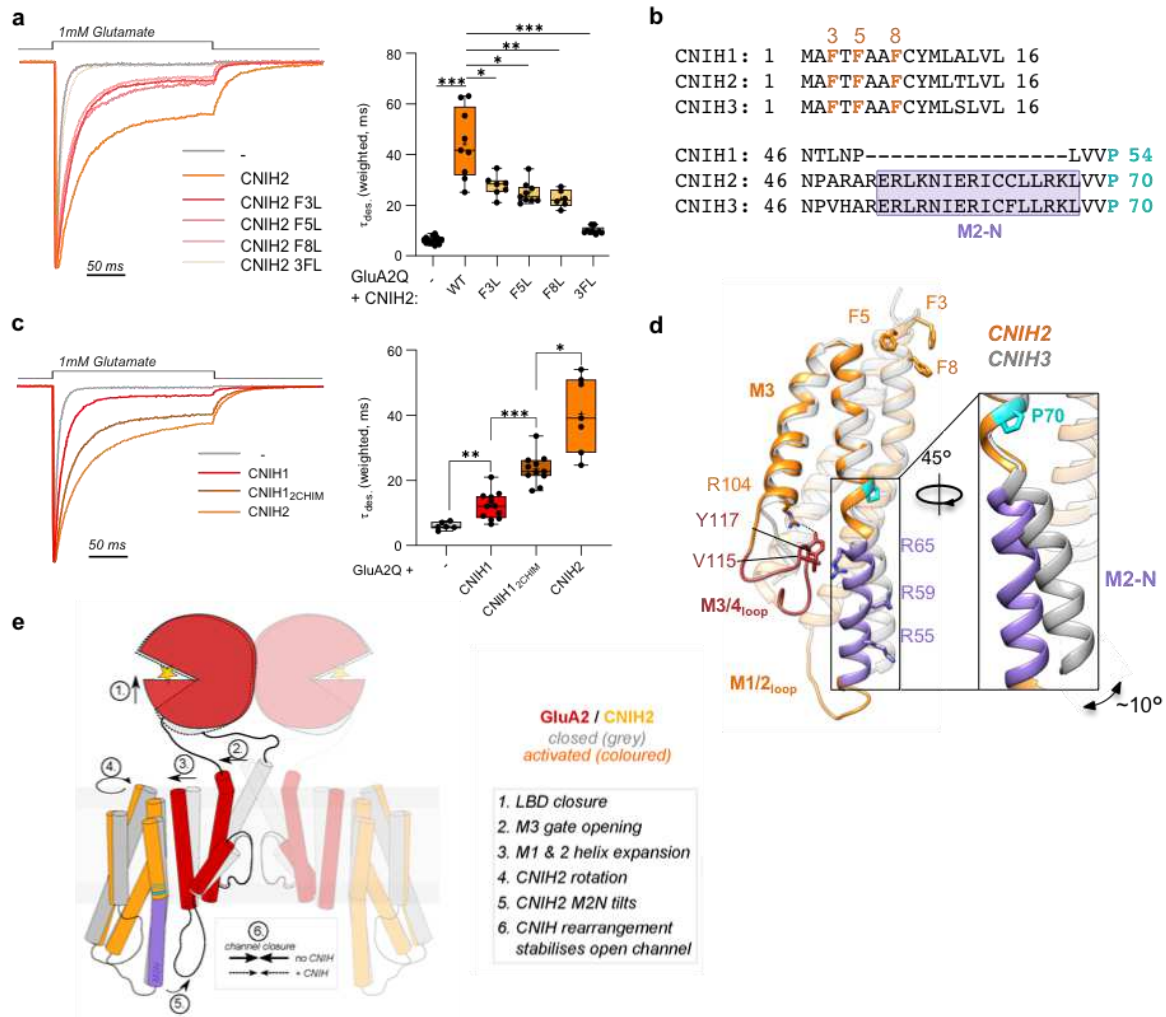


Fig. 3: Mechanism of AMPAR modulation by CNIH2.

a, Phe to Leu mutations of the CNIH2 N-terminus reduces modulation of GluA2Q

desensitization kinetics: weighted τ_{des} (ms), mean \pm SEM – GluA2 alone: 6.16 ± 0.35 , n=15;

CNIH2: 44.32 ± 4.58 , n=9; F3L: 27.73 ± 1.58 , n=7; F5L: 24.85 ± 1.43 , n=9; F8L: $22.57 \pm$

1.40 , n=6; 3FL: 10.07 ± 0.46 , n=9; Welch's ANOVA with Dunnett's multiple comparisons

test: $W(5,17.83) = 85$, $p < 0.0001$. **b**, Sequence alignment of CNIH1-3 (from mouse),

highlighting the conserved phenylalanines (F3, F5, F8) in the N-terminal region and the

region around M2-N, where CNIH1 lacks 16 residues. Pro70 is shown in cyan. **c**, The CNIH2

M2-N helix contributes to modulation of GluA2 kinetics, demonstrated by a gain-of-function

when transplanted onto the weakly modulating CNIH1: weighted τ_{des} (ms), mean \pm SEM –

1 GluA2 alone: 6.03 ± 0.47 , n=6; CNIH1: 12.22 ± 1.20 , n=12; CNIH1_{2chimera}: 23.48 ± 1.39 ,
2 n=11; CNIH2: 40.45 ± 4.32 , n=7; Welch's ANOVA with Dunnett's multiple comparisons
3 test: $W(3,15.30) = 64.09$, $p < 0.0001$. **d**, Superposition of CNIH2 (orange) and CNIH3 (grey;
4 PDB: 6PEQ), using the top of their M1 and M2 helices for alignment. CNIH2 M2-N (purple)
5 kinks at Pro70 (cyan) and diverges from CNIH3 by $\sim 10^\circ$ (inset); N-terminal phenylalanines
6 are also indicated. An interaction with the M3/4 loop through Val115 and Arg65 is shown,
7 Arg55 and Arg59 project toward the pore axis. **e**, Molecular mechanism underlying CNIH2
8 modulation of the AMPAR.

9
10

1 **Methods**

2 cDNA constructs

3

4 All cDNA constructs were produced using IVA cloning ³⁷. To achieve heteromeric
5 AMPAR expression, GluA1 (rat cDNA sequence, flip isoform) was co-expressed with GluA2
6 (rat cDNA sequence, flip isoform, R/G edited, Q/R edited), expressed from the pRK5 vector.
7 For TARP-containing complex recordings, GluA2 was expressed in a tandem configuration
8 (denoted GluA2_γ8) with TARP γ8 (rat cDNA sequence) by cloning the TARP γ8 coding
9 sequence (Glu2 - Lys419) at the C-terminus of the GluA2 coding sequence (Val1 to Ser839),
10 in the pRK5 vector, separated by a Gly-Gly-Ser-Gly-Ser-Gly linker sequence. pN1-EGFP
11 (Clontech) was used for visualisation of transfected cells. CNIH2 (rat) was expressed either
12 from a stable cell line (heteromeric AMPAR recordings, see below), or from either a pRK5 or
13 a pBOS vector, with an HA-tag at the extreme C-terminus. A chimera of CNIH1 and CNIH2
14 (CNIH1_{2CHIM}) was made by inserting 16 amino acids from CNIH2 (51-
15 RERLKNIERICLLRK-66) into CNIH1 at position P50-L51.

16

17 Organotypic slice preparation

18

19 All procedures were carried out under PPL 70/8135 in accordance with UK Home Office
20 regulations. Experiments conducted in the UK are licensed under the UK Animals (Scientific
21 Procedures) Act of 1986 following local ethical approval.

22

23 Organotypic slice cultures were prepared as described previously ³⁸. Briefly, hippocampi from
24 P6-8 C57/Bl6 mice were isolated in high-sucrose Gey's balanced salt solution containing (in
25 mM): 175 sucrose, 50 NaCl, 2.5 KCl, 0.85 NaH₂PO₄, 0.66 KH₂PO₄, 2.7 NaHCO₃, 0.28 MgSO₄,
26 2 MgCl₂, 0.5 CaCl₂ and 25 glucose at pH 7.3. Hippocampi were cut into 300 μm thick slices
27 using a McIlwain tissue chopper and cultured on Millicell cell culture inserts (Millipore Ltd)
28 in equilibrated slice culture medium (37°C/5% CO₂). Culture medium contained 78.5%
29 Minimum Essential Medium (MEM), 15% heat-inactivated horse serum, 2% B27 supplement,
30 2.5% 1 M HEPES, 1.5% 0.2 M GlutaMax supplement, 0.5% 0.05 M ascorbic acid, with
31 additional 1 mM CaCl₂ and 1 mM MgSO₄ (all from Thermo Fisher Scientific; Waltham, MA).
32 Medium was refreshed every 3–4 days. Recordings were performed at 7-14 days *in vitro*.

33

1 Recombinant cell electrophysiology

2
3 For heteromeric AMPAR experiments, suspension HEK-Expi293FTM cells (ThermoFisher
4 Scientific) were cultured in Expi293TM expression media (Gibco) and were transfected using
5 PEI Max with a DNA to PEI ratio at 1:3. GluA1, GluA2 (with or without TARP γ 8 tethered
6 via a flexible linker, as described before ¹⁵ and EGFP plasmids were transfected at a 2:8:1
7 stoichiometry to aid heteromeric receptor production, and identification of transfected cells.

8
9 For homomeric AMPAR experiments, GluA1 or GluA2Q (pIRES2-mCherry or pIRES2-
10 EGFP) and CNIH2 (pRK5 or pBOS) plasmids were transfected at a 1:2 ratio using Effectene
11 (QIAGEN) into adherent HEK293T cells (*ATCC: Cat# CRL-11268, RRID: CVCL_1926, Lot*
12 *58483269: identity authenticated by STR analysis, mycoplasma negative*). Cells were cultured
13 at 37°C and 5% CO₂ in DMEM (Gibco; high glucose, GlutaMAX, pyruvate, Cat#10569010)
14 supplemented with 10% foetal bovine serum (Gibco) and penicillin/streptomycin. Transfected
15 cells were recorded from 36 hours post-transfection. Cells were plated on poly-L-lysine coated
16 glass coverslips approximately 12 hours before recording, to allow outside-out patch recording
17 and 30 μ M 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX;
18 Tocris, Cat#1044) was added to media post-transfection to avoid AMPAR-mediated toxicity.

19
20 Borosilicate glass electrodes (1.5mm o.d., 0.86mm i.d., Science Products GmbH), pulled with
21 a PC-10 vertical puller (Narishige) with tip resistance of 2 to 6 M Ω , were filled with internal
22 solution containing (in mM) CsF (120), CsCl (10), EGTA (10), HEPES (10), and spermine
23 (0.1), adjusted to pH 7.3 with CsOH. Surface membrane patches were acquired by gentle
24 excision after achieving the whole-cell patch clamp configuration. Patches were voltage
25 clamped at -60 mV, and subject to fast application of 1 mM L-glutamate using two-barrel theta
26 glass tube controlled by a piezoelectric translator (Physik Instrumente or Burleigh), allowing
27 solution exchange in around 200 μ s (open tip response). Signals were acquired using the
28 MultiClamp 700B or Axopatch 200B amplifier (Axon Instruments), digitised using a Digidata
29 1440A interface and recorded with pClamp10 (Molecular Devices). Extracellular solution
30 contained (in mM) NaCl (145), KCl (3), CaCl₂ (2), MgCl₂ (1), glucose (10), and HEPES (10),
31 adjusted to pH 7.4 using NaOH. 20 μ M IEM 1925 dihydrobromide (Tocris, Cat#4198) was
32 added to the extracellular solution to limit the contribution of homomeric AMPAR complexes
33 to heteromeric recordings.

34

1 Current/voltage relationships were recorded for AMPAR responses with holding potentials of
2 -100 mV to +100 mV in 20 mV steps. To ensure heteromeric receptor analysis, patches with a
3 rectification index of less than 0.5 were discarded. Recordings were not corrected for the liquid
4 junction potential. Rectification index was calculated as follows:

$$RI = \frac{I_{+40\text{ mV}} - I_{0\text{ mV}}}{I_{-60\text{ mV}} - I_{0\text{ mV}}}$$

7
8 Desensitization entry was determined from the first 150-200 ms after the peak response, which
9 was fitted with a two-exponential function to obtain the (weighted) time constant.
10 'Equilibrium' responses were denoted as the percentage of peak current remaining after 200
11 ms.

$$\text{Equilibrium current} = \frac{I_{200\text{ ms}}}{I_{\text{peak}}} \times 100\%$$

14
15 Signal acquisition and data analysis were performed using pClamp10.

16 17 Neuronal cell electrophysiology

18
19 Cultured organotypic hippocampal slices were submerged in artificial cerebrospinal fluid
20 (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose, 1
21 sodium pyruvate, 4 CaCl₂ and 4 MgCl₂ at pH 7.3 and saturated with 95% O₂/5% CO₂. Neuronal
22 surface patches were acquired from the cell soma as detailed above for recombinant cell
23 patches, using borosilicate pipettes containing (in mM): 135 CH₃SO₃H, 135 CsOH, 4 NaCl, 2
24 MgCl₂, 10 HEPES, 4 Na₂-ATP, 0.4 Na-GTP, 0.15 spermine, 0.6 EGTA, 0.1 CaCl₂, at pH 7.25.
25 Agonist was applied to neuronal patches using a piezo-driven theta-barrelled perfusion setup,
26 with aCSF containing (in mM): 140 NaCl, 3.5 KCl, 1 MgCl₂, 2.5 CaCl₂, 10 HEPES, 10 glucose,
27 1 sodium pyruvate, 2 NaHCO₃, at pH 7.3. 100 μM D-APV (Tocris, Cat #0106) was added to
28 the extracellular solution of both barrels to prevent the contribution of NMDA receptor
29 currents. 1 mM L-glutamate was added to one barrel for fast-agonist application (as above). A
30 subset of hippocampal CA1 pyramidal neuron patches were recorded using the same extra- and
31 intracellular solutions as recombinant cell electrophysiology, and no difference in kinetic

1 parameters analysed was observed between recording configurations, therefore these datasets
2 were pooled and presented together.

3
4 CA1 and CA3 pyramidal, and dentate gyrus (DG) granule cells were identified by cell
5 morphology and location in tissue. *Stratum radiatum* interneurons (IN) were selected for
6 analysis as a population of interneuronal cells that can be identified by location, rather than
7 requiring a genetic driver line, and consisted of neurons located between *stratum pyramidale*
8 and *stratum lacunosum-moleculare* in the CA1 region of organotypic slices. Cell identity was
9 not confirmed by morphological or other analysis *post-hoc*, and therefore likely consists of a
10 heterogeneous combination of IN cell types.

11 12 Generation of CNIH2-1D4-HA stable cell line

13
14 A CNIH2-1D4-HA stable expression cell line was generated using a lentiviral expression
15 system following an established protocol³⁹. A 1D4 tag followed by an HA tag were included
16 at the extreme C-terminus of rat CNIH2 with a TEV cleavage site, separated by a ‘GGG’ linker
17 sequence. The CNIH2 gene together with the two tags were synthesized and cloned into pHR
18 vector. pHR-CNIH2-ID4-HA was co-transfected with psPAX2 and pMD2.G into HEK293T
19 Lenti-X cells (Takara/Clontech, Cat# 632180). 72 hours after transfection, lentiviral particles
20 were harvested from the media and used to infect HEK-Expi293F cells. 48 hours after
21 infection, fresh Expi293TM expression media (Gibco) was added, cells were surface-labelled
22 with an APC-conjugated anti-HA antibody (Miltenyi Biotec, Cat# 130-098-404, 1:50) and
23 sorted by flow cytometry, collecting HA-positive and DAPI-negative cells. Positive cells were
24 then scaled up for A1/A2_{γ8}/C2 complex expression.

25 26 Expression and purification of A1/A2_{γ8}/C2

27
28 Constructs of FLAG-tagged GluA1(flip) and GluA2(flip)-TARPy8-eGFP tandem (pRK5) used
29 in this paper are the same as previously reported¹⁵. GluA1 and GluA2-TARPy8 were co-
30 transfected into the CNIH2-1D4-HA stable expression cell line at a ratio of 1:1. To prevent
31 AMPA-mediated excitotoxicity, AMPAR antagonists ZK200775 (2 nM, Tocris, Cat# 2345)
32 and kynurenic acid (0.1 mM, Sigma, Cat# K335-5G) were added to the culture medium. 36-44
33 hours post-transfection, cells were harvested and lysed for 3 hours in lysis buffer containing:

1 25 mM Tris pH 8, 150 mM NaCl, 0.6 % digitonin (w/v) (Sigma, Cat# 300410-5G), 5 μ M
2 NBQX, 1 mM PMSF, 1 \times Protease Inhibitor (Roche, Cat# 05056489001). Insoluble material
3 was then removed by ultracentrifugation (41,000 rpm, 1 hour, rotor 45-50 Ti) and the clarified
4 lysate incubated with anti-GFP beads for 3 hours. After washing with glyco-diosgenin (GDN)
5 (Anatrace, Cat# GDN101) buffer (25 mM Tris pH 8, 150 mM NaCl, 0.02% GDN) the protein
6 was eluted from the beads by digestion with 1 mg/ml 3C protease at 4 $^{\circ}$ C overnight. Eluted
7 fractions were incubated with FLAG beads (Sigma, Cat# A2220) for 1.5 hours and washed 3
8 times with GDN buffer. Finally, the complex was eluted using 0.15 mg/ml 3 \times FLAG peptide
9 (Millipore Cat# F4799) in GDN buffer. Eluted fractions were pooled and concentrated to \sim 3
10 mg/ml for cryo-EM grid preparation.

11

12 Cryo-EM grid preparation and data collection

13

14 Cryo-EM grids were prepared using a FEI Vitrobot Mark IV. For the resting state complex,
15 protein was incubated with 100 μ M NBQX for at least 30 min on ice before freezing. For the
16 active state complex, protein was first incubated with 300 μ M Cyclothiazide (CTZ, Tocris,
17 Cat# 0713) for at least 30 min on ice and then quickly mixed with 1 M L-glutamate stock
18 solution to a final concentration of 100 mM prior to loading onto the grids. Quantifoil Au
19 1.2/1.3 grids (300 mesh) were glow-discharged for 30 s at 0.35 mA before use. 3 μ l sample
20 was applied to the grids, blotted for 4.5-5 s at 4 $^{\circ}$ C with 100 % humidity and plunge-frozen in
21 liquid ethane. Cryo-EM data were collected on an FEI Titan Krios operated at 300 kV,
22 equipped with a K3 detector (Gatan) and a GIF Quantum energy filter (slit width 20 eV).
23 Movies at 1.5-2.5 μ m underfocus were taken in counting mode with a pixel size of 1.1 \AA /pixel.
24 A combined total dose of 50 $e/\text{\AA}^2$ was applied with each exposure and 50 frames were recorded
25 for each movie. Two datasets of resting state were collected using SerialEM with 10462 movies
26 in total, and two datasets of active state were collected using EPU2 with 8246 movies in total.

27

28 Cryo-EM data processing and model building

29

30 Dose-fractionated image stacks were first motion-corrected using MotionCor2⁴⁰. Corrected
31 sums were used for CTF estimation by GCTF⁴¹. All further data processing was performed
32 with RELION 3.1⁴². Automatic particle picking was performed using a Gaussian blob and
33 particles were binned to 4.4 \AA /pixel and extracted in a box of 80 pixels. 2 to 3 rounds of 2D
34 classification were carried out to remove particles not showing AMPAR-like features. For the

1 following 3D classification, emd-20332 was used as initial model to further eliminate low-
2 quality particles. Following data clean-up, particles were re-centered, scaled up to 2.2 Å/pixel
3 and re-extracted in a box of 160 pixels. Another 3D classification focused only on the LBD-
4 TMD and C2 symmetry was applied on the re-extracted data set with EMD-20330 as an initial
5 model. After this round of classification, CNIH2 containing and CNIH2-free particles were
6 separated into two datasets with 3D refinement applied on each set. Refined particles were
7 scaled to the original 1.1 Å/pixel size, and refined with C2-symmetry followed by post-
8 processing. Four datasets were processed independently in the beginning, and particles at the
9 same states were joined together before CTF refinement and Bayesian polishing. The final
10 refinement was carried with side-splitter to achieve maps at 3.2 Å (CNIH2 containing) and 3.6
11 Å (CNIH2 free) resolution in the resting state and 3.7 Å (CNIH2 containing) in the active state
12 (individually based on the standard Fourier shell correlation (FSC) 0.143). To further improve
13 map resolution, we applied masked refinement on TMD or LBD region independently by
14 continuing old run from the last iteration of the global LBD-TMD refinement. Local resolution
15 was also estimated by RELION3.1. To help with model building, we used EMDA
16 (<https://www2.mrc-lmb.cam.ac.uk/groups/murshudov/content/emda/emda.html>) to generate
17 composite maps of LBD-TMD from the mask refinement. However, the density observed at
18 the CNIH region was weaker than that of the receptor. In order to further enhance the density
19 for model building, symmetry expansion was applied on the aligned particles from TMD
20 reconstruction. A masked classification without alignment was then performed to select CNIH
21 stable particles. To process the active state data, a smaller mask and higher T value were
22 required for the focused classification. The following focused refinement was found to improve
23 the density of the CNIH region in both resting and active states. For reconstruction of the NTD
24 in the resting state, after scaling particles back up to 2.2 Å/pixel, 3D classifications against the
25 full-length receptor were performed and particles with a stable NTD signal were selected and
26 subjected to masked refinement with a soft mask placed only on the NTD. CTF refinement and
27 Bayesian polishing were also performed to attain a final 3.5 Å map.

28

29 Model building and refinement were performed using Coot⁴³, REFMAC5⁴⁴ and PHENIX⁴⁵
30 real-space refinement. For modelling, the GluA1/A2-γ8 complex (PDB 6QKC and 6QKZ) and
31 CNIH3 in GluA2-CNIH3 complex (PDB 6PEQ) were used as starting points. The individual
32 coordinates were first rigid-body fitted into the map using UCSF chimera
33 (<http://www.rbvi.ucsf.edu/chimera>) and then automatically refined by REFMAC5. After this,
34 manual refinement through Coot was performed, followed by PHENIX real-space refinement,

1 to further refine the geometry. External restraints generated from LBD crystal structure 3TKD
2 were used to further improve the model quality in the LBD region. For lipid building, EMDA
3 bestmap was used to enhance the lipid signal in the maps. Model validation was performed
4 with MolProbity ⁴⁶. All graphics figures in the paper were prepared using UCSF Chimera or
5 PyMOL (<http://www.pymol.org>). Pore radius was calculated using a plugin version of HOLE
6 ⁴⁷ in Coot.

7 8 Normal mode analysis

9 Normal modes were calculated and visualized using the ANM server ³⁴ and the *ProDy* API ⁴⁸
10 In both cases, the protein was treated as an elastic network model as previously described ³³.
11 Briefly, each residue is represented as one bead placed at the C α position and interactions were
12 treated as uniform harmonic springs within a cutoff distance of 15 Å. This simple
13 representation allows fast analytic calculation of the most energetically favourable modes of
14 motion via normal mode analysis. Calculations were facilitated using the *SignDy* module
15 within *ProDy* ⁴⁹, which enabled easier alignment of the active and resting structures and
16 comparisons of their modes to each other and the transition between them, which were
17 calculated using the correlation cosine overlap between vectors. Movies were created using
18 PyMOL version 1.8.2.0 and Fiji ⁵⁰.

19 20 Flag Immunoprecipitation of CNIH homologues and CNIH2 mutants

21
22 All CNIH homologues, the CNIH12 chimera and CNIH2 mutants were HA-tagged at the
23 extreme C-terminus and cloned into the pRK5 vector. FLAG-tagged GluA2 was co-transfected
24 alongside CNIH constructs. 10 ml of HEK-Expi293F cells was used for each transfection. 40
25 hours post-transfection, cells were harvested and resuspended in 1 ml lysis buffer (25 mM Tris
26 pH 8, 150 mM NaCl, 0.6 % digitonin (w/v)). After a 2 hour incubation, cell lysate was
27 centrifuged at 21000 \times g for 30 min and the clarified supernatant was taken as the input sample.
28 10 μ l FLAG beads was added to the input and incubated for 1.5 hours. Following incubation,
29 FLAG beads were pelleted and washed 5 \times with GDN buffer (25 mM Tris pH 8, 150 mM NaCl,
30 0.02% GDN). To elute samples the beads were then boiled in SDS sample buffer for 5 mins
31 and run on a 4-12% Bis-Tris gel (Invitrogen, Cat# NW04127BOX). Rabbit GluR2 polyclonal
32 antibody (Millipore, Cat# AB1768-1) and rabbit HA antibody (Sigma, Cat #6908) were used
33 to detect the GluA2 and CNIHs signal, respectively.

1 Immunostaining

2

3 Suspension HEK-Expi293F™ cells were transfected with GluA2Q (pRK5) and CNIH (pRK5)
4 at a stoichiometry of 1:1 and settled on poly-L-lysine coated 12 mm glass coverslips (Corning)
5 36 hours later. Live-labelling of surface CNIH was performed at 48 hours post-transfection by
6 incubation with rabbit anti-HA (Sigma, Cat# H6908; 1:200) for 20 min at room temperature
7 (RT) in Expi293™ expression media (Gibco). Cells were washed 3× in medium and once in
8 PBS before fixation in 4% paraformaldehyde and 4% sucrose for 10 min at RT. Subsequently,
9 cells were washed in PBS before permeabilisation in 0.1% Triton X-100 (Fisher Bioreagents)
10 and blocking in 1% bovine serum albumin (BSA; Fisher Bioreagents) and 10% normal goat
11 serum (NGS; Sigma) in PBS for 30 min. Permeabilised cells were then incubated sequentially
12 in primary and secondary antibodies prepared in 1% BSA and 10% NGS for 2 hours at RT and
13 washed in PBS after each incubation. Total CNIH was detected using mouse anti-HA
14 (BioLegend, Cat# 901501; 1:500) and total GluA2 was detected using guinea pig anti-GluA2
15 (Synaptic Systems, Cat# 182 105; 1:500), followed by incubation in corresponding secondary
16 antibodies: goat anti-rabbit IgG Alexa Fluor (AF) 568 (Invitrogen, Cat# A-11036; 1:500), goat
17 anti-mouse IgG AF 488 (Invitrogen, Cat# A-11029; 1:500) and goat anti-guinea pig IgG AF
18 647 (Invitrogen, Cat# A-21450; 1:500). Coverslips were mounted in ProLong Diamond
19 Antifade Mountant (Invitrogen) and left to cure in the dark for 48 hours at RT.

20

21 Images were acquired with a Leica TCS SP8 confocal inverted microscope using Leica
22 Application Suite X software. Z-stacks (5x 1 µm steps) of whole cells were acquired using a
23 63x oil-immersion objective. 6 images were taken per coverslip and repeated from 3
24 independent preparations. Equivalent laser power settings were applied across all experimental
25 conditions. Z-stacked images were averaged and the fluorescence intensity was measured from
26 individual cells using ImageJ (Fiji) and normalised for cell area and background fluorescence.
27 Brightness and contrast settings were kept consistent across all images.

Figures

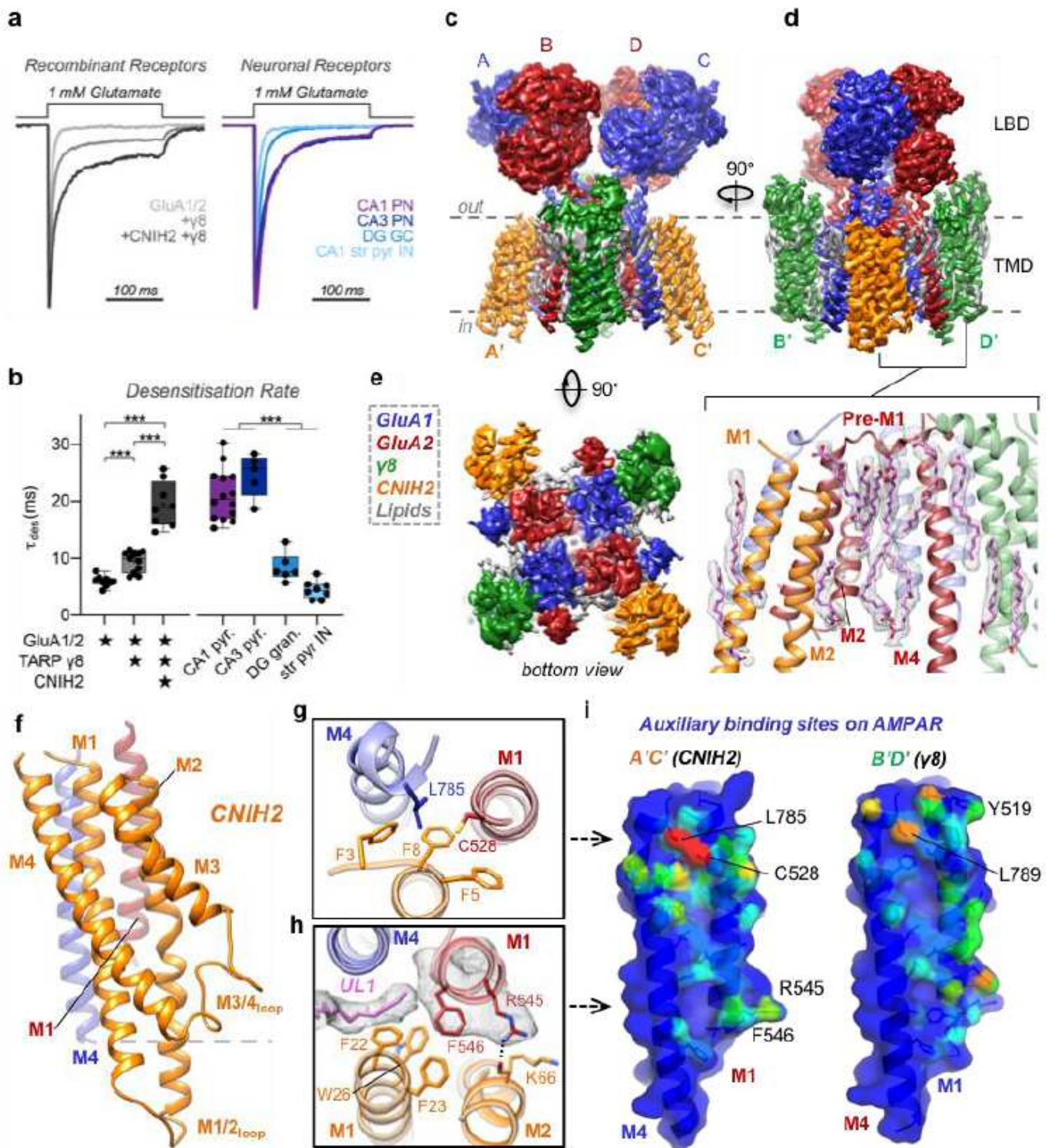


Figure 1

Physiology and architecture of the GluA1/2_ γ 8 /CNIH2 complex. a,b, Auxiliary subunits slow desensitization rates of recombinant receptors (a, left). Native AMPAR responses show diverse properties (a, right), with A1/2_ γ 8 +CNIH2 recapitulating CA1 & CA3-like kinetics (b, weighted τ_{des} (ms), mean \pm

SEM). Recombinant receptors: GluA1/2: 6.01 ± 0.31 , $n=9$; + $\gamma 8$: 9.29 ± 0.53 , $n=12$; +CNIH2+ $\gamma 8$: 19.71 ± 1.27 , $n=9$. Neuronal receptors: CA1 pyramidal: 21.03 ± 1.19 , $n=14$; CA3 pyramidal 24.51 ± 1.69 , $n=5$; DG granule cell: 8.38 ± 1.01 , $n=6$; CA1 stratum pyramidale interneurons: 4.48 ± 0.55 , $n=8$; Welch's ANOVA tests with Dunnett's multiple comparisons test – Recombinant: $W(2,15.11) = 60.68$, $p < 0.0001$; Neurons: $W(3,11.60) = 76.28$, $p < 0.0001$). c-e, Cryo-EM maps of the AMPAR octamer, depicting the LBD and TMD domain layers. Core subunits positioned to AC/BD, and auxiliary subunits associated with A'C'/B'D' sites are shown. (c) Front view, depicting $\gamma 8$ located at B'D' sites. (d) Side view, offering a view of CNIH2 at A'C' sites, beneath an LBD dimer. Inset: annular lipids concentrating at side of the TMD, beneath the pre-M1 helix. (e) Bottom view, highlighting CNIH2 binding to GluA2 transmembrane helices M1-3 (red), and $\gamma 8$ contacting the GluA1 M1-3 helices (blue). f, Model of CNIH2, including the M1/2 and M3/4 cytosolic loops, docking to its binding site (M1GluA2 [red] M4GluA1 [blue]). g, CNIH2 Phe3, -5, -8 slotting into the top tier of its binding site close to Cys528 (GluA2) and Leu785 (GluA1). h, CNIH2 contacts at the bottom of the binding site are mediated by Phe23 and Lys66; a lipid (UL1) penetrates the A'C' site and interacts with Phe546 (GluA2). i, A'C' (left) and B'D' (right) site surface representation. M1A2 and M4A1 residues contacted by CNIH2 are coloured depending on the number of atoms contributing to the interaction (red: high, blue: low). Contacts were counted using the 'findNeighbors' command from ProDy 38, with a cutoff of 4.5 \AA between heavy atoms.

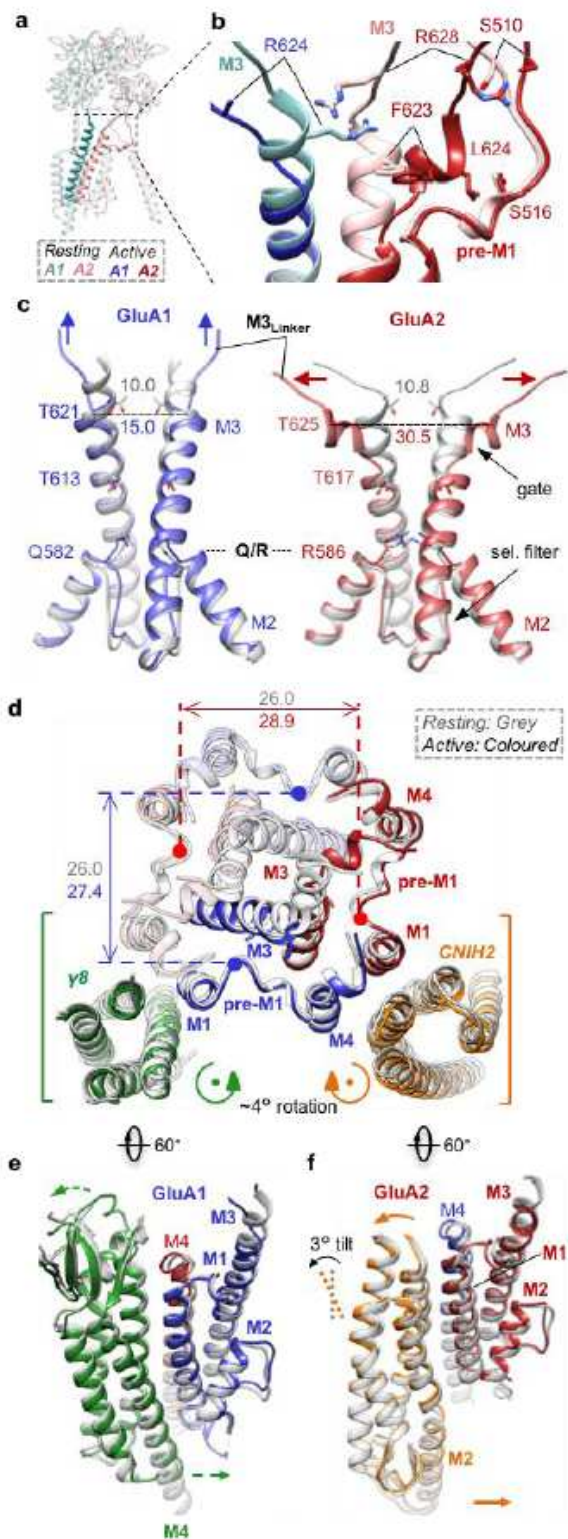


Figure 2

Gating transitions of the A1/2_γ8/C2 octamer. a, View onto a GluA1/GluA2 LBD dimer and associated M3 gating helices (depicted in bold) in the resting state. b, Side view onto the M3 gate with open (bold colour) and resting (soft colour) states overlaid, depicting rearrangement of the M3 linkers and reorientation of M3 side chains relative to pre-M1. c, Comparison of GluA1 and GluA2 conduction path; resting state (grey) is superimposed on the active state (colour). Divergence from the closed gate is apparent at the top of

M3, and is more pronounced in GluA2 (Ca distances between opposing T621 (GluA1), and T625 (GluA2) indicated). Also shown is the selectivity filter with the Q/R site at its apex. d, Top view onto the closed- (grey) and open-state (colour) models at the level of the M3 gate. The dilation of the gate-surrounding helices is shown and measured between opposing pre-M1 helices. e, Side view showing outward expansion of the upper part of GluA1 M1 and M3 and the $\gamma 8$ β -sheet, accompanied by a slight inward movement of the $\gamma 8$ M4 helix towards the GluA1 M12 loop. f, CNIH2 undergoes a $\sim 3^\circ$ pivot ('tilt') on activation, moving its M12 loop closer to the pore axis.

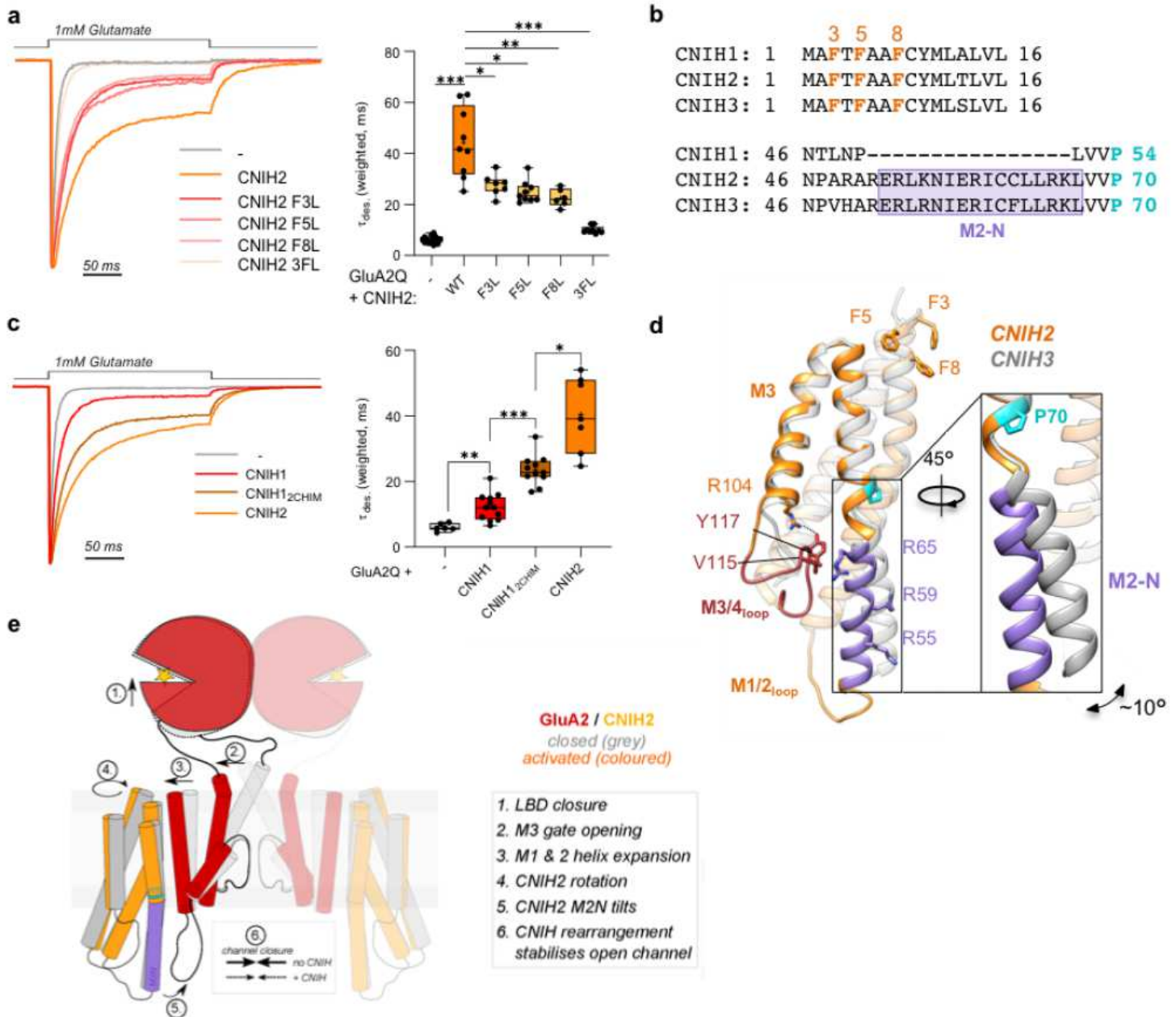


Figure 3

Mechanism of AMPAR modulation by CNIH2. a, Phe to Leu mutations of the CNIH2 N-terminus reduces modulation of GluA2Q desensitization kinetics: weighted τ_{des} (ms), mean \pm SEM – GluA2 alone: 6.16 ± 0.35 , $n=15$; CNIH2: 44.32 ± 4.58 , $n=9$; F3L: 27.73 ± 1.58 , $n=7$; F5L: 24.85 ± 1.43 , $n=9$; F8L: 22.57 ± 1.40 ,

n=6; 3FL: 10.07 ± 0.46 , n=9; Welch's ANOVA with Dunnett's multiple comparisons test: $W(5,17.83) = 85$, $p < 0.0001$. b, Sequence alignment of CNIH1-3 (from mouse), highlighting the conserved phenylalanines (F3, F5, F8) in the N-terminal region and the region around M2-N, where CNIH1 lacks 16 residues. Pro70 is shown in cyan. c, The CNIH2 M2-N helix contributes to modulation of GluA2 kinetics, demonstrated by a gain-of-function when transplanted onto the weakly modulating CNIH1: weighted τ des (ms), mean \pm SEM – GluA2 alone: 6.03 ± 0.47 , n=6; CNIH1: 12.22 ± 1.20 , n=12; CNIH12chimera: 23.48 ± 1.39 , n=11; CNIH2: 40.45 ± 4.32 , n=7; Welch's ANOVA with Dunnett's multiple comparisons test: $W(3,15.30) = 64.09$, $p < 0.0001$. d, Superposition of CNIH2 (orange) and CNIH3 (grey; PDB: 6PEQ), using the top of their M1 and M2 helices for alignment. CNIH2 M2-N (purple) kinks at Pro70 (cyan) and diverges from CNIH3 by $\sim 10^\circ$ (inset); N-terminal phenylalanines are also indicated. An interaction with the M3/4 loop through Val115 and Arg65 is shown, Arg55 and Arg59 project toward the pore axis. e, Molecular mechanism underlying CNIH2 modulation of the AMPAR.

Supplementary Files

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