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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	CryoEM: SerialEM(v3.6)
Data analysis	CryoEM Image Analysis Software: MotionCor2(v1.1.0), GCTF(v0.5), DoGPicker(v1.0), Relion(v2.0), EMAN2(v2.12), BlocRes(v2.0); Atomic Modeling and Visualization: COOT(v0.8.6), Phenix(v1.13), Molprobity(v4.4), Chimera(v1.11); Molecular Dynamics: VMD(v1.9.3), NAMD(v2.12), Solvate(v1.0.1); Mass Spectrometry: pLink(v1.07), Xcalibur(v2.2), MASCOT Daemon(v2.5)

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

Data

Policy information about availability of data

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

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

CryoEM density maps (pre-processed and post-processed) and associated masks have been deposited to the Electron Microscopy Data Bank (EMD-9116).

Coordinates for Cx46 and Cx50 atomic models have been deposited to the Protein Data Bank (6MHQ, 6MHY). The original multi-frame micrographs have been deposited to EMPIAR (EMPIAR-10212).

Field-specific reporting

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

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

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

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

Sample size	CryoEM sample size were not predetermined. An initial dataset of 261,206 particles (Dataset 1) was collected, and an additional dataset of 398,066 particles (Dataset 2) was further used to assess the effects of particle number on the achievable resolution and/or resolvability of heterogeneous features during 3D classification. The initial size of dataset 1 was estimated based on the particle density observed in test images. The increased size of dataset 2 vs. dataset 1 did not improve the global resolution of the resulting CryoEM maps, indicating the size of the particle datasets were sufficiently sampled.
Data exclusions	Single particle image data was excluded based on the absence of high-resolution features (e.g. alpha-helical transmembrane domains), which were conditions that had been pre-established based on expected structural homology to connexin-26.
Replication	All attempts at replication were successful. This included processing two independent datasets obtained from unique particles in dataset 1 and dataset 2, and by processing with alternative CryoEM image analysis software (CisTEM(v1.0)).
Randomization	Single particle image data was split randomly into two groups and processed in the same way to calculate Fourier-shell correlation coefficients, in accordance to Gold Standard Methods. Samples were not further allocated into groups, outside of what is performed by the computational image analysis programs used in this work.
Blinding	Investigators were not blinded during data acquisition or analysis. Blinded studies in this case were not possible because the investigator performing the experiments and analysis also contributed to isolation of the specimen being analyzed.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study	
☒	☐	Unique biological materials
☐	☒	Antibodies
☒	☐	Eukaryotic cell lines
☒	☐	Palaeontology
☒	☐	Animals and other organisms
☒	☐	Human research participants

Methods

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

Antibodies

Antibodies used	Antibodies were purchased from outside vendors. Primary antibodies directed against the n-terminal domain of Cx46 (rabbit, poly-clonal) where purchased from Acris (AP11570PU-N, lot SH021017A) and used for western blot analysis at a dilution of 1:500. Antibodies directed against the proximal c-terminal domain of Cx50 (mouse, poly-clonal) where purchased from Santa Cruz (sc-50432, lot C0107), and used for western blot analysis at a dilution of 1:2,000.
Validation	The Cx46 antibody was validated by the manufacturer against the human isoform using ELISA and Immunohistochemistry. The Cx50 antibody was validated by the manufacturer by western blot analysis of connexin-50 expression in 293T and TK-1 cells, and whole cell lysates from rat brain tissue. Both antibodies were further validated by in-house western blot analysis using lens fiber cell lysates (sheep).