Methods

PAH[4] reconstitution into lipid vesicles. Both control lipid and PAH[4]-reconstituted lipid vesicular membranes were prepared by the film rehydration method[38]. A PC and PS lipid mixture (3 mg in total) with a molar ratio of 4:1 (mol mol^{-1}) was dissolved in chloroform. The required amounts of PAH[4] s, depending on the desired mCLRs, were calculated and dissolved in lipid chloroform solutions. The solutions were transferred into 50 ml round-bottomed flasks and gently dried using a rotary evaporator to form lipid films on the bottom of the flasks. Any residual solvent (chloroform) was removed by placing the flasks in a high vacuum chamber for 16 h. Completely dried films were rehydrated with 1 ml of rehydration buffer (10 mM HEPES, 100 mM NaCl and 0.01% (w/v) NaN$_3$ at pH 7.0) and extruded 15 times through 0.2 μm polycarbonate track-etched membranes to obtain uniformly sized lipid vesicles ~200 nm in diameter, which was confirmed by a Zetasizer Nano-ZS90 (Malvern Panalytical).

Water permeability measurement of vesicular membranes. The osmotic water permeabilities (P_f (m s^{-1})) of lipid vesicular membranes were measured using a SF-300X stopped-flow instrument (KinTek). The vesicles were abruptly exposed to a hypertonic solution (10 mM HEPES, 100 mM NaCl, 100 mM poly(ethylene glycol)$_{600}$ and 0.01% (w/v) NaN$_3$ at pH 7.0) by mixing in the stopped-flow cell to impose an outwardly directed osmotic pressure across the vesicular membranes, and the membrane permeabilities were calculated based on the amount of water efflux from the vesicles driven by the osmotic gradient (shrinking mode of vesicles) (Supplementary Fig. 6). The size change of the vesicles due to the water efflux was monitored through the side-scattering (detection angle of 90° against an incident light of 600 nm wavelength) intensity change with less than 10 ms of dead time after mixing to obtain accurate kinetic information[39]. The kinetic coefficients (k) of the size change of vesicles were obtained by fitting the scattering traces into double-exponential functions and used to calculate the osmotic permeability (P_f (m s^{-1})) of vesicular membranes (Supplementary Fig. 7), using equation (1)[21]:

$$P_f = \frac{k}{(S/V_0) \times v_w \times \Delta C_{osm}} \tag{1}$$

where k is a larger exponential coefficient of fitted double-exponential function, S is the initial surface area of the vesicles, V_0 is the initial volume of the vesicles, v_w is the molar volume of water and ΔC_{osm} is the imposed difference of osmolarity across the vesicular membranes. Note that, for single-channel permeability measurements from mCLR 0.00001 vesicles, a smaller exponential coefficient (k) of the fitted double-exponential function was used, because no apparent permeability increase was seen between the control vesicles and the mCLR 0.00001 vesicles from the first exponent. This approach was previously demonstrated and used to characterize water channels that have a low water permeability[13,40]. Accurate ΔC_{osm} values were measured using a freezing point osmometer (Model 3300, Advanced Instruments) for all the tested solutions.

FCS to evaluate the single PAH[4] channel water permeability. To calculate the single PAH[4] channel permeability, the actual number of PAH[4]s embedded in reconstituted lipid vesicles was counted using FCS. For the FCS analysis, PAH[4] s were first labelled with 5-(and-6-)-((N-(5-aminopentyl)amino)carbonyl) tetramethylrhodamine (Invitrogen) dyes using dicyclohexylcarbodiimide chemical cross-linkers (Thermo Fisher Scientific). A molar ratio of 1:8:8 of PAH[4]s, dyes and dicyclohexylcarbodiimides were dissolved in dimethyl sulfoxide (DMSO) and reacted for 16 h by stirring at room temperature. Unreacted dyes were removed via dialysis using a Slide-A-Lyzer MINI device (Thermo Fisher Scientific) for 5 d, changing fresh DMSO every 12 h. After the free dye removal by dialysis, labelled PAH[4] channels were harvested by fully evaporating the DMSO solvent inside a high-vacuum chamber for 2 d, and used for both the FCS and FRAP experiments. Dye-labelled PAH[4]s were reconstituted into lipid vesicles using a film rehydration method and the reconstituted vesicles were subsequently subjected to FCS analysis. The fluorescence intensity fluctuation of vesicles ($\delta F(t)$) in a small confocal volume was monitored using a confocal microscope equipped with a time-resolved single-photon counting module (Becker-Hickl GmbH) and the autocorrelation function ($G(t)$) was obtained based on equation (2):

$$G(\tau) = \frac{\langle \delta F(t) \rangle \langle \delta F(t+\tau) \rangle}{\langle F(t)^2 \rangle} \tag{2}$$

where t is time and τ is the time lag. $G(\tau)$ is specifically related to several parameters, as in equation (3):

$$G(\tau) = \frac{1}{N} \sum_{i=1}^{M} f_i \left[\frac{1}{1+\tau/\tau_{Di}} \right] \left[\frac{1}{1+(r/z)^2 (\tau/\tau_{Di})} \right]^{1/2} \tag{3}$$

where r and z are the radius and half height of the confocal volume, τ_{Di} is the lateral diffusion time of fluorescence species i and N is the total number of fluorescent species in a confocal volume, which is the number of reconstituted vesicles (N_{ves}) in this case. N_{ves} can be simply obtained from the y-axis intercept of the autocorrelation function ($G(0) = 1/N_{ves}$). After that, the vesicles were completely

micellized by adding 10% (w/v) of non-ionic detergent octyl β-D-glucoside and the number of channels (N_{chan}) were obtained using same procedure for N_{ves} (Supplementary Figs. 8 and 9). The average number of channels per vesicle (N_{chan}/N_{ves}) was used to calculate both the channel densities in the lipid membranes and the single-channel PAH[4] permeability.

AFM imaging of PAH[4] clusters in supported bilayer membranes. AFM analyses were performed to observe the PAH[4] channel clusters in lipid bilayer membranes. Control and PAH[4]-reconstituted (mCLR 0.005) PC/PS vesicles were prepared as described above with an additional buffer composition of 3 mM CaCl$_2$. Supported bilayer membranes were prepared by rupturing vesicles (suspending diluted vesicle solutions) on freshly cleaved mica. After 1.5 h of incubation, the membranes were gently washed with water and subjected to AFM imaging in liquid (water) on a Bruker BioScope Resolve AFM. These liquid AFM measurements were conducted in the PeakForce tapping mode using a ScanAsyst Fluid plus tip with a spring constant of 0.7 N m^{-1}. The force used for both the control and PAH[4] channel samples was 750 pN or less, and the images contained a minimum of 384 pixels per line. The acquired images were plane-fit to the first order to account for the sample tilt. Multiple line scans were utilized throughout the scan to determine the size of each identified PAH[4] channels to determine the size of their domains. For PAH[4]-reconstituted membranes, island domains ~30 nm in diameter (in the adhesion mode) were identified as spreading over the entire membrane, which was not seen in the control lipid membranes (Supplementary Fig. 12).

FRAP analysis to observe lateral diffusion of PAH[4]s in lipid bilayer membranes. Two PC/PS vesicles were prepared; one was PC/PS vesicles with dye-labelled PAH[4] s (mCLR 0.005) and the other (control vesicle) was PC/PS with PAH[4]s (unlabelled, mCLR 0.005) and an additional 0.5 mol% of Texas Red 1,2-dihexadecanoly-sn-glycero-3-phosphoethanolamine (TR-DHPE) lipids. TR-DHPE-containing control vesicles were used to confirm the bilayer formation and obtain the membrane viscosity, as described below. The vesicle solutions were combined with a buffer solution to create a vesicle environment with a final concentration of 2 mM CaCl$_2$, 100 mM NaCl and 10 mM HEPES at pH 7. Supported bilayers were made by rupturing the vesicles on a cleaned glass coverslip in a polydimethylsiloxane well. After a fusion time of 10 min, the formed bilayers were rinsed with buffer (100 mM NaCl and 10 mM HEPES at pH 7.0), scratched with tweezers to remove a small section of bilayer and rinsed with the same buffer a second time. The FRAP experiments were conducted on a Nikon Eclipse TE-2000-U inverted microscope through a ×10 objective lens. A 532 nm laser (Spectra Physics) was used to bleach the labelled channel or the TR-DHPE. The bleach spot diameter was approximately 16 μm. The fluorescence intensity was monitored every 3 s for the initial 75 s, and then every 30 s for the remainder of the experiments (Supplementary Fig. 13). The fluorescent recovery curves were used to determine the diffusion coefficients of the labelled PAH[4] channel and TR-DHPE, respectively. The fluorescence intensity of the bleached spot at a given time was divided by the intensity of a non-bleached region to correct for differences in light intensity and photobleaching. The normalized fluorescence recovery, $f(t)$, was calculated using equation (4):

$$f(t) = \frac{I_t - I_0}{I_i - I_0} \tag{4}$$

where I_t is the normalized spot intensity at time t, I_0 is the normalized spot intensity immediately after bleaching and I_i is the average spot intensity prior to bleaching.

The recovery as a function of time was fit to an exponential equation to obtain the mobile fraction, A, as well as the time of half recovery, $\tau_{1/2}$, shown in equations (5) and (6):

$$f(t) = A(1 - e^{-\tau t}) \tag{5}$$

$$\tau_{1/2} = \frac{\ln 2}{\tau} \tag{6}$$

The lateral diffusion coefficient, D_{lat}, was calculated using equation (7), where ω is the half-width of the Gaussian laser beam:

$$D_{lat} = \frac{0.88 \times \omega^2}{4 \times \tau_{1/2}} \tag{7}$$

Measurement of activation energy of water permeation through PAH[4] channels. Water permeability measurements of both the control and channel-reconstituted lipid membranes were repeated at different temperatures (between 5 and 25 °C) to measure the activation energy (E_a) of water permeation through the channels as well as the lipid membrane matrix (Supplementary Fig. 14). Measurements were performed under the swelling mode for this part of the study. Channel-mediated water permeabilities at each temperature were obtained by subtracting the control lipid membranes' permeability from the channel-reconstituted membranes' permeability. Water permeability (P_f (μm s^{-1})) and the E_a of water permeation is related by the Arrhenius equation[14,22,23]:

$$P_f = Ae^{-E_a/RT} \tag{8}$$

where A is a pre-exponential constant, R is the ideal gas constant and T is the temperature (K). E_a can simply be extracted from a slope of the Arrhenius plot, as shown in equation (9) (Fig. 2d):

$$\ln P_f = \ln A - \frac{E_a}{R}\frac{1}{T} \tag{9}$$

Cl^- ion permeability measurement. Measurements of the Cl^- permeability of both the control and PAH[4]-reconstituted lipid vesicular membranes were performed using stopped-flow fluorescence experiments. Among several halide ion sensitive colloidal quenching dyes, lucigenin dyes were selected as the measurement probes as they have the highest Stern–Volmer constant value among commercial dyes ($K_{SV} = 390\,M^{-1}$ for chloride ions in aqueous solutions) and, therefore, the highest sensitivity to a Cl^- concentration change[41]. To measure accurately the Cl^- influx into vesicular membranes, the K_{SV} of lucigenin inside ~200 nm PC/PS vesicles was first determined (95 M^{-1} for intravesicular environments (Supplementary Fig. 15)). The excitation wavelength was set as 455 nm using a monochromator and photomultiplier tube (PMT) module was equipped with a BrightLine fluorescence 534/30 emission filter. Lucigenin-encapsulating vesicles prepared with a Cl^- free buffer (10 mM HEPES, 100 mM KNO_3, 10 μM valinomycin and 0.01% (w/v) NaN_3 at pH 7.0) were abruptly exposed to different Cl^- gradients in the stopped-flow measurement cell (for example, to impose a 20 mM Cl^- gradient, the same volumetric amount of Cl^--containing buffer (10 mM HEPES, 40 mM KCl, 60 mM KNO_3, 10 μM valinomycin and 0.01% (w/v) NaN_3 at pH 7.0) as that of the vesicle solution was mixed in the measurement cell) and the fluorescence intensity change was monitored. Note that potassium ionophore valinomycin was added to the vesicles beforehand to mitigate charge imbalance across the membrane, and therefore prevent an underestimation of the Cl^- permeability if an electrical potential developed as a consequence. The initial fluorescence kinetic information $(dF/Ft|_{t=0})$ is quite important for an accurate evaluation of the Cl^- permeability and stopped-flow traces (Supplementary Fig. 16). More than ten traces of fluorescence intensity change were recorded for each measurement and the averaged trace was used for the analysis. The vesicular membrane mole flux of Cl^- ions (j_{Cl}) at each tested Cl^- concentration condition was calculated using equation (10):

$$j_{Cl} = \frac{d[mole]}{S \times dt} = \frac{V}{S}\frac{d[Cl^-]}{dt}\bigg|_{t=0} = \frac{-V}{S \times K_{SV} \times F_0}\frac{dF}{dt}\bigg|_{t=0} \tag{10}$$

where S and V are the surface area and volume of the vesicles, $[Cl^-]$ is molar concentration of Cl^- ions inside vesicles, K_{SV} is the determined Stern–Volmer constant of the lucigenin dyes inside the vesicles (~ 95 M^{-1}), F_0 is the fluorescence intensity under Cl^--free conditions (fluorescence intensity at $t = 0$). The Cl^- permeability coefficient (B_{Cl} (cm s^{-1})) of the membranes was obtained from slope of the j_{Cl} plot as a function of the Cl^- concentration gradients using equation (11) (Fig. 2f):

$$B_{Cl} = \frac{dj_{Cl}}{d[Cl^-]} \tag{11}$$

The single PAH[4] channel Cl^- permeability coefficient ($B_{Cl,PAH}$ (cm^3 s^{-1})) was calculated based on the channel density in the lipid membranes obtained from the FCS analyses (equation (12)):

$$B_{Cl,PAH} = \frac{d(j_{Cl,PAH,lipid} - j_{Cl,lipid})}{d[Cl^-]} \times \frac{1}{\sigma_{PAH}} \tag{12}$$

where $j_{Cl,PAH,lipid}$ is the PAH-reconstituted membrane Cl^- mole flux, $j_{Cl,lipid}$ is the bare lipid membrane Cl^- mole flux and σ_{PAH} is the number of PAH[4] channels per unit membrane area.

Current-to-voltage measurement of PAH[4]-reconstituted lipid membranes using the DIB system. A DIB set-up was used to characterize the ionic transport properties of PAH[4] and PAP[5] channels (as a control) in lipid bilayer membranes. The lipid membranes were formed between two aqueous droplets anchored to wire-type electrodes in a transparent reservoir filled with oil, as described elsewhere[28,29]. In brief, the set-up consisted of two lipid-encased aqueous droplets that hung from Ag/AgCl wires (Goodfellow) in oil (1:2 mixture of hexadecane (≥99%, Sigma) and decane (≥95%, Sigma)). When the droplets were brought into contact, the lipid tails from each droplet interacted with each other at the interface to expel oil and form an oil-depleted hydrophobic centre (that is, a thinned lipid bilayer). To help the droplets anchor to the Ag/AgCl wires, we coated their ball-ended tips with a 1% agarose gel solution. To confirm the lipid bilayer formation, which is reflected as an increase in membrane capacitance, we supplied a 10 Hz, 10 mV triangular wave to the electrodes using a function generator (Hewlett-Packard 3314A). Owing to the capacitive nature of the membrane, the resulting current response was square-like. As the area of the thinned lipid membrane grew, the peak-to-peak current amplitude increased until it reached a steady state. To generate the I–V relationships, we applied a 0.01 Hz, 50 mV sinusoidal voltage waveform and recorded the induced current. In parallel, to monitor the changes in the membrane we acquired images of the droplets from

below through a ×4 objective lens of an Olympus IX51 inverted microscope using a QI Click charge-coupled device. All the current recordings were made using an Axopatch 200B patch clamp amplifier and Digidata 1440 data acquisition system (Molecular Devices). For all the measurements, the droplets and measurement probes were placed under a lab-made Faraday cage to minimize noise from the environment.

MD simulations. All atom MD simulations were performed using the MD program NAMD2[42]. PAH[4] channels (25) were arranged in a 5×5 array and embedded into a 14.4×14.4 nm^2 patch of a pre-equilibrated POPC (1-palmitoyl-oleoyl-sn-glycerophosphocholine) lipid bilayer membrane. The lipid patch was generated from the CHARMM-GUI membrane builder[43] and equilibrated for approximately 400 ns. Lipid molecules that overlapped with the channels were removed. The system was then solvated with the water[44] using the Solvate plugin of VMD. Sodium and chloride ions were added using the Autoionize plugin of VMD to produce an electrically neutral solution of 0.6 M salt concentration. The resulting system measured 14.4×14.4×10 nm^3 and contained approximately 170,000 atoms. Next, we created three conformations of PAH[4] dimers, namely lateral, orthogonal and inverted, each of which comprised two units of single PAH[4] channels (Supplementary Information gives details). We embedded 25 PAH[4] dimers in a regular 5×5 array into a 20×20 nm^2 patch of a pre-equilibrated POPC lipid bilayer membrane. We solvated the structure and added ions to create a charge-neutral 0.6 M solution of NaCl in each system. The resulting system measured 20×20×10 nm^3 and contained approximately 353,000 atoms. We created four such systems, three of which contained dimer arrays with different conformations and one in which the water molecules were placed inside an array of orthogonal PAH[4] dimers. Choosing the most conductive dimer conformation from these simulations, we created a pre-assembled cluster structures using the self-docking protocol developed in OptMAVEn-2.0[36]. Three different thermodynamically stable conformations of PAH[4] clusters were generated and embedded into a 14.4×14.4 nm^2 patch of a pre-equilibrated POPC lipid bilayer membrane. Each structure was solvated by a 0.6 M NaCl solution. Each system measured 14.4×14.4×10 nm^3 and contained approximately 172,000 atoms.

The assembled systems were subjected to energy minimization using the conjugate gradient method that removed steric clashes between the solute and solvent atoms. After the energy minimization, the systems were subjected to a short equilibration at a constant number of atoms (N), constant pressure ($P = 1$ bar) and constant temperature ($T = 303$ K). This was an NPT ensemble with harmonic restraints on all the non-hydrogen atoms of PAH[4] channels with respect to their initial positions (with spring constants of 1 kcal mol^{-1} Å^{-2}). Subsequently, the harmonic restraints were released completely, and the system was equilibrated free of any restraints.

The initial all-atom configuration of the AQP1 system was built starting from the all-atom structure of bovine AQP1, Protein Data Bank entry 1j4n[17]. The structure was embedded into a POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) lipid bilayer membrane as described in our previous study[45]. For the sake of comparison, the AQP1 system was simulated using exactly the same simulation protocols as for the PAH[4] systems with a 200 ns simulation time.

All the MD simulations were performed using periodic boundary conditions and the particle mesh Ewald method to calculate the long-range electrostatic interactions in NAMD2[42]. A Nose–Hoover Langevin piston[46,47] and Langevin thermostat were used to maintain a constant pressure and temperature in the system[48]. CHARMM36 force-field parameters[49,50] described the bonded and non-bonded interactions among the PAH[4] channels, lipid bilayer membranes, water and ions along with NBFIX corrections for non-bonded interactions[51,52]. A 8–10–12 Å cutoff scheme was used to calculate van der Waals and short-range electrostatics forces. All the simulations were performed using 1 fs time steps to integrate the equation of motion. The SETTLE algorithm[53] was applied to keep the water molecules rigid, whereas the RATTLE algorithm[54] constrained all other covalent bonds that involved hydrogen atoms. For each system, 300–400 ns equilibrium MD simulations were performed, which gave rise to transmembrane water permeation through irregular dendritic paths between the PAH[4] structures. The coordinates of the system were saved at the interval of a 4.8 ps simulation. The analysis and post-processing the simulation trajectories were performed using VMD and CPPTRAJ[55].

Data availability
The datasets that support the finding of this study are available in ScholarSphere repository with the identifier(s) (https://doi.org/10.26207/ykbm-r806).

References
38. Woodle, M. C. & Papahadjopoulos, D. [9] Liposome preparation and size characterization. *Methods Enzymol.* **171**, 193–217 (1989).
39. Latimer, P. & Pyle, B. E. Light scattering at various angles: theoretical predictions of the effects of particle volume changes. *Biophys. J.* **12**, 764–773 (1972).
40. Tong, J., Canty, J. T., Briggs, M. M. & McIntosh, T. J. The water permeability of lens aquaporin-0 depends on its lipid bilayer environment. *Exp. Eye Res.* **113**, 32–40 (2013).

41. Biwersi, J., Tulk, B. & Verkman, A. S. Long-Wavelength chloride-sensitive fluorescent indicators. *Anal. Biochem.* **219**, 139–143 (1994).
42. Phillips, J. C. et al. Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802 (2005).
43. Jo, S., Kim, T., Iyer, V. G. & Im, W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem.* **29**, 1859–1865 (2008).
44. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. L. Comparison of simple potential function for simulating liquid water. *J. Chem. Phys.* **79**, 926–935 (1983).
45. Decker, K. et al. Selective permeability of truncated aquaporin 1 in silico. *ACS Biomater. Sci. Eng.* **3**, 342–348 (2017).
46. Feller, S. E., Zhang, Y., Pastor, R. W. & Brooks, B. R. Constant pressure molecular dynamics simulation: the Langevin piston method. *J. Chem. Phys.* **103**, 4613–4621 (1995).
47. Martyna, G. J., Tobias, D. J. & Klein, M. L. Constant pressure molecular dynamics algorithms. *J. Chem. Phys.* **101**, 4177–4189 (1994).
48. Sindhikara, D. J., Kim, S., Voter, A. F. & Roitberg, A. E. Bad seeds sprout perilous dynamics: stochastic thermostat induced trajectory synchronization in biomolecules. *J. Chem. Theory Comput.* **5**, 1624–1631 (2009).
49. Best, R. B. et al. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone ϕ, ψ and side-chain χ1 and χ2 dihedral angles. *J. Chem. Theory Comput.* **8**, 3257–3273 (2012).
50. Klauda, J. B. et al. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *J. Phys. Chem. B* **114**, 7830–7843 (2010).
51. Yoo, J. & Aksimentiev, A. New tricks for old dogs: improving the accuracy of biomolecular force fields by pair-specific corrections to non-bonded interactions. *Phys. Chem. Chem. Phys.* **20**, 8432–8449 (2018).
52. Yoo, J. & Aksimentiev, A. Improved parametrization of Li^+ Na^+, K^+, and Mg^{2+} ions for all-atom molecular dynamics simulations of nucleic acid systems. *J Phys Chem Lett* **3**, 45–50 (2011).
53. Miyamoto, S. & Kollman, P. A. SETTLE: an analytical version of the SHAKE and RATTLE algorithm for rigid water models. *J. Comput. Chem.* **13**, 952–962 (1992).
54. Andersen, H. C. RATTLE: a 'velocity' version of the SHAKE algorithm for molecular dynamics calculations. *J. Comput. Phys.* **52**, 24–34 (1983).
55. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graph. Model* **14**, 33–38 (1996).

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Author contributions

W.S., H.J., A.A. and M.K. conceived and designed the research. W.S. and Y.-x.S. performed the experiments with the assistance of J.S.N., C.L., C.B.H., Y.-M.T., M.F., M.E.P. and J.-l.H. in specialized analytical tools. H.J. and R.C. performed the computer simulations. W.S., H.J., R.C., C.D.M., P.S.C., R.J.H., S.A.S., J.-l.H., A.A. and M.K. analysed the data. W.S., H.J., R.C., A.A. and M.K. co-wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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