Crystal structure of the natural anion–conducting channelrhodopsin GtACR1

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The naturally occurring channelrhodopsin variant anion channelrhodopsin-1 (ACR1), discovered in the cryptophyte algae *Guillardia theta*, exhibits large light-gated anion conductance and high anion selectivity when expressed in heterologous settings, properties that support its use as an optogenetic tool to inhibit neuronal firing with light. However, molecular insight into ACR1 is lacking owing to the absence of structural information underlying light–gated anion conductance. Here we present the crystal structure of *G. theta* ACR1 at 2.9 Å resolution. The structure reveals unusual architectural features that span the extracellular domain, retinal–binding pocket, Schiff–base region, and anion–conduction pathway. Together with electrophysiological and spectroscopic analyses, these findings reveal the fundamental molecular basis of naturally occurring light–gated anion conductance, and provide a framework for designing the next generation of optogenetic tools.

Most organisms depend on light for energy and information. Motile organisms typically capture light using rhodopsin proteins, largely classified into two groups: microbial (type I) and animal (type II)1,2, both exhibiting seven-transmembrane helices and a retinal-based chromophore, but with different effector mechanisms. Animal rhodopsins primarily work as G-protein-coupled receptors that recruit secondary messengers to control effectors such as ion channels that modulate cellular activity, whereas channel and pump microbial rhodopsins can directly provide effector functionality as transmembrane current1–2. Heterologous expression of single-component microbial opsin genes targeted to specific cells of animals defines an experimental approach (optogenetics)3 for biology, enabling control of specific cells in behaving organisms with light.

Both channel and pump-encoding opsins are established in optogenetics. Variants of the channel subtype (cation-conducting channelrhodopsins, CCRs) elicit light-triggered cation currents (usually excitatory in neurons). Indeed, light-triggered cation currents are excitatory in the natural host as well; plant behaviours initially observed by botanists more than 150 years ago (movement of single-celled algae *Chlamydomonas reinhardtii* excited by light)4 were later found to be due to CCRs, with the initially known member of this subclass (*Chlamydomonas reinhardtii* ChR1) identified as a cation channel in 20025. Many CCRs have been discovered or designed5–14, and currently available CCRs offer a palette of diversity in absorption spectrum, photocurrent magnitude, light sensitivity and on/off-kinetics12,15.

The development of inhibitory optogenetics initially lagged, but has made strides in recent years3,4,16. Light-induced neuronal inhibition with microbial opsins was first achieved with inward Cl⁻ pumps and outward H⁺ pumps such as *Natriumononas pharaonis* halorhodopsin (NpHR) and archaeorhodopsin-3 (AR3)17,18. Although widely used, these pumps move only one ion per photon (versus hundreds for channels), thereby exhibiting reduced efficacy15,16. In 2014, anion-conducting channelrhodopsins (ACRs) were created19,20 on the CCR backbone, guided by structural modelling; subsequently, in 2015, naturally occurring ACRs were isolated from chlorophyte algae21 (GtACR1 and GtACR2). The designed ACRs have been developed further22–24 and additional natural ACRs have been found by genome mining22–24. ACRs can translocate 10⁻¹⁰ ions per second22 and can exhibit 10⁻¹⁰⁻¹⁰⁰ higher light sensitivity than inhibitory pumps19–21. After the first demonstration in 2015 of ACRs as inhibitory optogenetic tools that could successfully modulate animal behaviour (with a designed ACR called iCret25), both ACR classes have been widely applied in mice, flies and fish12,21–23,27–30.

Despite progress in ACR-based inhibitory optogenetics, little is known about the structural basis of radically different ion-selectivity involved in anion conduction. Homology models of GtACR1 were built27,31–33 using the structure of the C1C2 CCR34, but precise structural information on ACRs remained completely lacking. A high-resolution crystal structure would be beneficial, not only to enhance fundamental understanding, but also to provide a foundation for expanding the toolbox of optogenetics (as rapidly resulted from the first CCR crystal structure24 in 2012).

Here we obtain and characterize the crystal structure for GtACR1 at 2.9 Å resolution. This information, together with electrophysiological and spectroscopic analyses, revealed unique natural ACR structure–function relationships that span the extracellular domain, retinal–binding pocket, Schiff base region, and anion–conduction pathway. These features advance our understanding of natural channelrhodopsin biology, and reveal a path for the design and creation of new tools for optogenetics.

**Structure determination**

To understand the structural basis of light-activated anion conduction, we purified (Extended Data Fig. 1a) and crystallized the best-characterized natural ACR, GtACR1. To improve crystallizability, we truncated 13 C-terminal residues; the resulting construct (residues 1–282) showed similar photocurrents to full-length GtACR1 in human HEK293 cells (Extended Data Fig. 1b) and robust expression in neurons (Extended

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GtACR1 structure and comparison with C1C2 and CrChR2

GtACR1 exhibits a unique N-terminal extracellular domain (residues 4–29), a 7-transmembrane domain (residues 30–249), and a C-terminal domain (residues 250–280) (Fig. 1). In comparing GtACR1 with the CCRs C1C2 (PDB accessesions 3UG9 and 4YZ3)24,25 and CrChR223, we observed both similarities (despite relatively low sequence identities of 28% and 27%, respectively; Extended Data Fig. 3) and notable distinctions. Although there were aspects of architectural commonality between GtACR1 and C1C2 dimers and between GtACR1 and CrChR2 dimers (root mean square deviation (r.m.s.d.) values of 2.10 Å and 1.87 Å respectively over all Cα atoms), and between corresponding monomers (r.m.s.d. values of 1.62 Å and 1.39 Å), many crucial differences with GtACR1 were apparent (Fig. 2a, b).

First, although transmembrane helix 7 (TM7) of C1C2–CrChR2 protrudes approximately 18 Å from the membrane and its following C-terminal region exhibits a β-sheet (Fig. 2a, b), TM7 of GtACR1 does not protrude (resembling more pump-type rhodopsins such as bacteriorhodopsin and halorhodopsin) (Extended Data Fig. 4) and its C-terminal region displays a random coil (Fig. 2c). Further, the interface area of the GtACR1 dimer (1,315 Å²) is smaller than that for the C1C2 (2,027 Å²) or CrChR2 (1,688 Å²) dimers (Extended Data Fig. 5a–h). This property is concordant with our finding that loss of the intermolecular disulfide bridge markedly affects GtACR1 dimerization in SDS–PAGE analysis (Extended Data Fig. 5e), whereas the loss of the disulfide in C1C2–CrChR2 has minimal effect on dimerization.36–38

Third, ICL2 of GtACR1 has a β-sheet that is unique among microbial rhodopsins, extending from the protein core (Fig. 2a, b), in contrast to ICL2 of the C1C2–CrChR2 dimer, which is a random coil close to the protein core involved in dimerization.34 Notably, because of these differences in the N terminus and ICL2, the interface area of the GtACR1 dimer (1.315 Å²) is smaller than that for the C1C2 (2.027 Å²) or CrChR2 (1.688 Å²) dimers (Extended Data Fig. 5f–h). This property is concordant with our finding that loss of the intermolecular disulfide bridge markedly affects GtACR1 dimerization in SDS–PAGE analysis (Extended Data Fig. 5e), whereas the loss of the disulfide in C1C2–CrChR2 has minimal effect on dimerization.36–38

Finally, we note a feature of overall GtACR1 structure; the extracellular ends of TM1/TM2 are notably tilted compared to those of CCRs (Fig. 2a, b). These tilts remodel the extracellular vestibule, forming a novel ion-conducting pathway. This unanticipated structural feature appears of substantial importance for understanding the unique ion-conduction properties of GtACR1 (below).

Retinal-binding pocket

In all rhodopsins, retinal is covalently bound to a TM7 lysine residue, forming the Schiff base. GtACR1 and C1C2 contain similar configurations of all-trans-retinal and 15-anti-retinal43,49,50 (Fig. 2a). Here we focus on comparison with C1C2, because the 2017 CrChR2 CCR structure was almost identical to the well-studied 2012 C1C2 CCR structure (Extended Data Fig. 4c; r.m.s.d. value of 0.82 Å over all Cα atoms), and was also reported as a mixture of two states D480 and D470...
(absorbing light at 480 and 470 nm, respectively)\textsuperscript{35,36,41} making it difficult to compare to GIACR1\textsuperscript{35,36}. The GIACR1 structure reveals that most residues forming the retinal-binding pocket (RBP) are not conserved between GIACR1 and CCRs (Fig. 3a, b; Extended Data Fig. 3); in C1C2, ATR is enclosed by 16 residues (Fig. 3b), but 11 are not conserved in GIACR1 (Fig. 3a). To analyse the function of these residues, we measured absorption spectra and photocurrents in 10 mutants.

Previous studies reported that GIACR1 has five spectroscopically distinguishable intermediate states: K, L, M, N and O (with L and M as conducting states), and with opening and closing regulated by two different mechanisms (coupled fast-opening–slow-closing and slow-opening–fast-closing)\textsuperscript{31,35,36}. Confirming previous measurements, we observed that wild-type GIACR1 photocurrent peaks at \( \lambda_{\text{max}} = 514 \text{ nm} \) with biphasic decay (\( \tau_{\text{off1}} = 54 \pm 4.5 \text{ ms} \), \( \tau_{\text{off2}} = 280 \pm 25 \text{ ms} \)), and that mutant GIACR1(C102A) shows decreased \( \tau_{\text{off1}} = 32 \pm 12 \text{ s} \)\textsuperscript{31,35} (Fig. 3c, e). Notably like C102A, C102S also exhibits decreased \( \tau_{\text{eff}} \) (17 ± 2.5 s). M105A, M105I and E163Q show markedly slowed \( \tau_{\text{eff}} \) (120 ± 30 ms, 90 ± 3.9 ms and 110 ± 22 ms, respectively), suggesting that Met105 and Glu163 are involved in the slow-opening–fast-closing mechanism (Fig. 3c, e).

Notably, studies of Halobacterium salinarum bacteriorhodopsin (HsBR) predict that mutation of certain residues would affect the energy barrier for the transition from K to L intermediates\textsuperscript{42} (closed to open in GIACR1\textsuperscript{31}). Thr198, which interacts with the β-ionone ring of ATR in C1C2, corresponds to Cys133 in GIACR1 (Fig. 3a, b). In HsBR and CrChR2, mutations in residues surrounding the β-ionone affect biophysical properties; for example, M118A in HsBR changes the absorption spectrum (\( \lambda_{\text{max}} \) shifting from 551 to 474 nm)\textsuperscript{43}, T159C in CrChR2 affects conductance and kinetics\textsuperscript{44}. However, the GIACR1(C133A) and GIACR1(C133R) mutants exhibited only slightly blue-shifted spectra, with kinetics and photocurrents comparable to wild-type levels (Fig. 3c, e; Extended Data Figs. 6–8). Thus, the RBP of the GIACR1 β-ionone may be unusually robust (which could also depend on additional non-conserved residues around Cys133, such as Thr134 and Phe160; Fig. 3a, b; Extended Data Fig. 3). Another interesting RBP residue is Cys237, which affects key properties including absorption, kinetics and selectivity when mutated to alanine (Fig. 3c–e; Extended Data Figs. 6–8); notably, the mutant exhibits only a single fast component of current decay (\( \tau_{\text{off1}} = 87 ± 4.1 \) ms), suggesting involvement of this residue in the slow-closing mechanism (likely along with the Cys102 residue\textsuperscript{31}, Fig. 3c–e).

**The Schiff–base region**

In C1C2, two carboxylates (TM3 Glu162, TM7 Asp292) are within 4 Å of the Schiff-base nitrogen, which forms a direct hydrogen bond with Asp292 (Fig. 4a). However, in GIACR1, the TM3 residue is Ser97, and the TM7 Asp234 has a conformation quite different from Asp292 of C1C2, possibly owing to local interactions with Tyr72 and Tyr207. Notably, the overall architecture of the Schiff–base region in GIACR1 is more similar to halorhodopsins (Fig. 4a). However, in GIACR1, there is no clear electron density, suggesting water or Cl\textsuperscript{−} within hydrogen-bonding distance of the Schiff-base (Supplementary Discussion), and presumably the protonated Schiff base forms at least a weak hydrogen bond with Asp234 (Fig. 4a). Therefore, we undertook structure-guided functional characterization of Tyr72, Tyr207 and Asp234.

First, we analysed protonation of Asp234 using ultraviolet-visible (UV-vis) and low-temperature Fourier-transform infrared (FTIR) spectroscopy. Both assays strongly suggested the protonation of Asp234 in the dark, for the following reasons: first, wild-type and D234N mutants showed almost identical UV-vis absorption spectra (Fig. 4b; Extended Data Fig. 9a); and second, the light-induced difference-FTIR spectra at 77 K showed that a peak pair at 1,740(−)/1,732(+) nm (Extended Data Fig. 9a) and second, the light-induced difference-FTIR spectra at 77 K showed that a peak pair at 1,740(−)/1,732(+) nm (Extended Data Fig. 9b). Because the wild-type \( \lambda_{\text{max}} \) of the UV-vis spectra and intensity of the FTIR peak–pair remain unchanged from pH 5–9 (Extended Data Fig. 9c), Asp234 is therefore presumed to be protonated over a wide pH range, concurrent with previous Raman spectroscopy\textsuperscript{39}.

However, surprisingly, electrophysiology revealed that D234N nearly abolishes the photocurrent (Fig. 4d). Generally, the effects of aspartate–to-asparagine mutation are small when aspartate is protonated, but in the uniquely configured GIACR1 Schiff-base environment involving close apposition of Asp234, the small difference between aspartate-hydroxyl and asparagine-amino could rearrange the hydrogen-bond network around the Schiff base and thus disturb light-induced conformational changes. This concept is supported by difference FTIR spectra...
hydrogen-bond network of the Schiff-base region thus appears essential
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Second, extracellular vestibules of GIACR1 differ markedly from C1C2. C1C2 has two extracellular vestibules (EV1 and EV2) but only EV2 is connected to the ion-conducting pathway; EV1 is occluded by hydrogen bonding among Glu95, Glu136 and Glu140 (extracellular constriction site 1, ECS1) (Fig. 5b). However, Glu136 and Glu140 are not conserved in GIACR1, and the extracellular-side TM1 and TM2 are markedly tilted, as described above (Figs. 2c, 5a; Extended Data Fig. 3). Thus, pore size becomes much larger, and EV1 becomes connected to the GIACR1 pore-pathway. Furthermore, in contrast to EV1, EV2 of GIACR1 is disconnected because of interactions among Tyr81, Arg94 and Glu223 (extracellular constriction site 2, ECS2) (Figs. 5a, 6a), indicating that EV1 serves as the primary anion-entry pathway in GIACR1. Third, the anion-conducting pathway of GIACR1 is opened not only towards the extracellular side but also intracellularly. In C1C2, although the cation-conducting pathway is opened towards the extracellular side, the cytoplasmic side is occluded intracellularly. In C1C2, the channel is thus maintained in a closed state only by the CCS (Figs. 5, 6c). In C1C2, the CCS is formed by Ser102, Glu129 and Asn297. These three residues are conserved in GIACR1 (Gln46, Glu68 and Asn239) and its Gln46 on TM1 forms an additional hydrogen bond with Asn239, thereby further stabilizing the CCS. To test the function of these residues, we prepared 10 mutants of Glu46, Glu68 and Asn239, and measured activity by patch-clamp analysis. All Glu68 and Asn239 mutants exhibited smaller photocurrents, and Q46A showed comparable photocurrents but depolarized reversal-potential (Fig. 6d, e). Thus, all three CCS residues are important for anion-channel function, but with different roles: Glu68 and Asn239 for conductance, and Gln46 for selectivity.

**Discussion**

This high-resolution view into the inner workings of GIACR1 reveals that CCRs and natural ACRs share certain overall features, but also exhibit highly informative differences (especially in the architecture of the GIACR1 anion-conducting pathway, with exchange of one extracellular vestibule for another). The GIACR1 closed-state pore is also remarkable, almost entirely open with the exception of a single central constriction formed by Gln46, Asn239, Ser43 and Glu68; anions can be released intracellularly via the open conduction pore formed by Ala61, Leu108 and Thr249 (Figs. 5a, 6b). Thus, these data provide the first,

in the amide-I region at 170 K and 200 K (Fig. 4e); the intensity of negative bands at 1,640 and 1,655 cm$^{-1}$ decreases in D234N, revealing that the conformational change of transmembrane helices in D234N is significantly smaller than in the wild type, just as with D234N, the nearby Y207F mutation also causes loss-of-function (Fig. 4d). Considering that Phe207 naturally occurs in fully functional C1C2 and even in other natural ACRs including GIACR2 and the ZipACR variant with divergent sequences (Extended Data Fig. 3), the precisely arranged hydrogen-bond network of the Schiff-base region thus appears essential for channel activity.
to our knowledge, crystal structure of any channelrhodopsin revealing an open intracellular pore pathway.

Integration of structural, electrophysiological and spectroscopic analyses uncovered unique features of the Schiff base relevant to ChR (and halorhodopsin and bacteriorhodopsin) evolution. As in HsHR, a TM7 aspartate is coordinated by two tyrosine residues in GtACR1, and the TM3 glutamate in the CCR C1C2 is instead represented in both GtACR1 and HsHR by a neutral hydrophilic residue (Fig. 4a). Furthermore, a TM2 tyrosine (Tyr72, uniformly conserved among pump-type halorhodopsins and bacteriorhodopsins) is present in GtACR1 (and is almost 100% conserved among natural ACRs; Extended Data Fig. 3) but is dispensable for function; Y72F changes neither conductance (Fig. 4d) nor kinetics of the M-intermediate rise or decay (Extended Data Fig. 6d), characterized by fast or slow kinetics, respectively. This differs from bacteriorhodopsin, in which Y57F accelerates formation of the M-intermediate. Because CCRs have replaced this residue (Extended Data Fig. 3), an evolutionary model is suggested in which natural ACRs such as GtACR1 evolved from light-driven Cl⁻ pumps, and CCRs subsequently arose from natural ACRs via surface electrostatic remodelling.

Further insight into the mechanism and development of anion conduction arises from the consideration of another unusual feature of the Schiff-base region: charge distribution. In the dark, the Schiff-base nitrogen is protonated and therefore requires a mechanism to stabilize the positive charge. In GtACR1, Glu68 and Asp234 provide the only carboxylates within 6 Å of this Schiff-base nitrogen (approximately 5.4 Å and 3.5 Å, respectively), but FTIR analyses indicate that both are also protonated in the dark (Fig. 4d, e). Cl⁻ does not have a charge-stabilization role either, as Cl⁻ is not bound to the Schiff-base region in GtACR1 (Fig. 4a; unlike in HsHR). One possible explanation is that strongly polarized water could bind to the Schiff base (behaving as a hydroxyl ion, as proposed in HsBR and mutants; Supplementary Discussion), and another possibility is that the partial-negative charge of the nearby Asp234 carboxyl is sufficient to weakly stabilize the positively charged Schiff base. As a result, the net charge in the Schiff-base region may represent the achievement of perhaps the most challenging evolutionary step in the adaptation to facilitate anion conduction (alongside the acquisition of positive surface electrostatic potential throughout the pore and vestibules; Fig. 4): namely, partial local positivity despite the obligatory negative nature of the Schiff base counterion.

To advance our understanding of the molecular mechanism of light-gated anion conduction, additional studies (including structural resolution of natural or designed ACRs in fully open or intermediate states) will be required. This initial high-resolution structural information provides a framework for the further development of ACR-based optogenetic tools—for example, the creation of kinetic, spectral and selectivity variants that maintain the advantages of the GtACR1 backbone including strong photocurrents, just as the initial CCR structure allowed the development of new classes of optogenetic functionality. Further insights into the evolutionary and functional relationships among different channelrhodopsin family members will continue to arise from the solution of structures that correspond to kinetic, spectral and selectivity variants, advancing basic understanding of this remarkable class of natural protein.

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**Fig. 5** | Ion-conducting pathways of GtACR1 and C1C2. a, b, Ion-conducting pathways of GtACR1 (a) and C1C2 (b). The surface is coloured by the electrostatic potential calculated using PDB accession 2PQR for both GtACR1 and C1C2. Green, purple and orange-dashed circles represent the extracellular constriction site (ECS), intracellular constriction site (ICS) and central constriction site (CCS), respectively. IV, intracellular vestibule.

**Fig. 6** | Constriction sites of GtACR1. a, The ECS separating EV1 and EV2. Hydrogen bonds are shown as dashed lines. b, Initial glimpse of a patent intracellular conduction pathway for a light-activated channel; architecture of the GtACR1 intracellular ion exit pore leading to the intracellular vestibule (IV). c, The CCS architecture: sole constriction site in the pore, which separates the extracellular and intracellular vestibules. d, Current densities of mutants in residues comprising the CCS. Note the importance of residues E68 and N239 for photocurrents. Data are mean and s.e.m. n = 9 for WT, 5 for Q46A, E68A, E68T and E239A, and 4 for the rest. *P < 0.05, **P < 0.01, one-way ANOVA followed by Dunnett’s test. e, Comparison of reversal potentials. Note the signature of increased cation flux (depolarized reversal potential), consistent with disrupted pore selectivity. Data are mean and s.e.m. n = 10 for WT, 6 for Q46A and Q46C, 5 for E68A and 4 for the rest. *P = 0.014, one-way ANOVA followed by Dunnett’s test.

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METHODS
Sample sizes were determined based on previous literature and best practices in the field; no statistical methods were used to predetermine sample size. No experiments in animals were conducted in this paper and hence experiments were not randomized or blinded.

Cloning, protein expression and purification. The crystallization construct of GaCR1 was generated with several features to enhance protein purification and crystallization. The flexible 13 amino acids at the C terminus were truncated after Gly282. A Flag tag followed by the 3C protease cleavage site was added to the N terminus and an enhanced GFP (eGFP) with a His6 tag and the 3C site was attached to the truncated C terminus via the 3C cleavage site. The finalized GaCR1 crystallization construct was expressed in Sf9 cells using the BestBac (Expression Systems) baculovirus system. Cell cultures were grown to a density of 4 × 10^6 cells ml^-1, infected with GaCR1 baculovirus, and shaken at 27°C for 18 h. Then, 20 μl all-trans retinal (ATR) (Sigma) was supplemented to the culture and incubation continued for 42 more hours, and cell pellets were collected and stored at −80°C. To purify GaCR1, the pellets were lysed with a hypotonic lysis buffer (20 mM HEPES pH 7.5, 1 mM EDTA and protease inhibitors). The cell debris was then homogenized with a glass dorer in a solubilization buffer (1% n-dodecyl-β-D-maltopyranoside (DDM), 0.06% cholesteryl hemisuccinate tris salt (CHS), 20 mM HEPES pH 7.5, 500 mM NaCl, 20% glycerol, 10 mM imidazole and protease inhibitors) and solubilized for 2 h in 4°C. The insoluble cell debris was removed by centrifugation (38,000g; 25 min), and the supernatant was mixed with the Ni-NTA agarose resin (Qiagen) for 2 h in 4°C. The Ni-NTA resin was collected in a glass chromatography column, washed with 20 column volumes of a wash buffer (0.5% DDM, 0.01% CHS, 200 mM HEPES pH 7.5, 500 mM NaCl, 20% glycerol and 20 mM imidazole) and eluted in a wash buffer supplemented with 250 mM imidazole. The Ni-NTA eluent was then supplemented with 2 mM CaCl2 and was loaded over anti-Flag M1 resin over 1 h. The protein was then washed with a Flag wash buffer (0.05% DDM, 0.01% CHS, 200 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol and 200 mM CaCl2) and eluted with a Flag elution buffer (0.05% DDM, 0.01% CHS, 200 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 0.2 mg ml^-1 Flag peptide and 3 mM EDTA). After the cleavage of the Flag tag and eGFP-His6 by His6-tagged 3C protease, the sample was reloaded onto the Ni-NTA column to capture the cleaved eGFP-His6. The flow-through containing GaCR1 was collected, concentrated and purified through gel-filtration chromatography in a final buffer (100 mM NaCl, 20 mM HEPES pH 7.5, 0.05% DDM and 0.01% CHS). Peak fractions were pooled and concentrated to 30 mg ml^-1 (Extended Data Fig. 1b).

Crystalization. Purified GaCR1 protein was crystallized using the lipidic cubic phase (LCP) method as described previously^{34}. Protein was mixed with monopalmitolein (Nu-chek) at a weight ratio of 1:1 (protein:lipid) using a coupled syringe mixing device. Then, 20–25 nl protein–LCP mixture drops were accurately dispensed on a 96-well sandwich plate and overlaid by 500 nl of precipitant solution by a micro liquid handling device. Then, 20–25 nl precipitant solution was carefully overlaid by 500 nl of precipitant solution. Crystals were harvested using Micromeshes (MiTeGen), then transferred using micropipettes and were transferred in 2 mM CaCl2 precipitant solution by a micro liquid handling device. Then, 20–25 nl precipitant solution was carefully overlaid by 500 nl of precipitant solution. The sample was then flash-cooled in liquid nitrogen above 10°C. Crystals were then transferred to a 10 × 10 mm2, at a wavelength of 1.033 Å. Small wedge X-ray diffraction data were collected using a Bruker Kappa Apex II diffractometer equipped with an Oxford Cryostream 700 cryostream ( Oxford Cryostreams ) mounted in a Bio-Rad FTS-40 spectrometer (instrumental resolution of FTIR is 2 cm^-1). For the formation of photo-intermediates at 77 K, samples were illuminated at 500 nm (interference filter) from a 1-kW halogen-tungsten lamp for 2 min and photo-reversed with >600 nm light (R-62 cut-off filter, Toshiba) for 1 min. For each measurement of photo-intermediates at 170 K and 200 K, samples were illuminated with >500 nm light (Y-52 cut-off filter, Toshiba) for 1 min. For each measurement of photo-intermediates, 40 identical recordings at 77 K and 7 K and 170 K and 200 K were averaged.

Measurement of UV absorption spectra. Protein absorbance spectra were measured using an Infinite M1000 microplate reader (Tecan Systems Inc.) using 96 well plates (ThermoFisher scientific). The GaCR1 samples were suspended in a buffer containing 100 mM NaCl, 0.05% DDM, 0.01% CHS, and 20 mM sodium citrate, sodium acetate, sodium cacodylate, HEPES, Tris, CAPSO or CAPS. pH was adjusted from 4 to 10 by the addition of NaOH or HCl. Spectra recorded spectra value was averaged from 20 measurements from a single session.

Reporting summary. For complete transparency, this reporting summary is available in the Nature Research Reporting Summary linked to this paper.

Data availability. The protein coordinate and atomic structure factor have been deposited in the Protein Data Bank (PDB) under accession number 6CSM. The raw diffraction images have been deposited in the SBGrid Data Bank repository (ID: 569). All other data are available from the corresponding authors upon reasonable request.

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Extended Data Fig. 1 | Crystallography. a, Size exclusion chromatogram of the purified GiACR1 protein used for crystallography. Similar results were seen in more than 20 independent experiments. b, Electrophysiology of full-length GiACR1 (left) and the final crystallization construct (right); whole-cell voltage-clamp recordings in five cells held at −70 mV, with 513 nm light at 1.0 mW mm\(^{-2}\) irradiance delivered with timing as shown with green-coloured bars, while cells were held at resting potentials from −95 mV (lowest trace) to +5 mV (uppermost trace) in steps of 10 mV. Similar results were seen in 3–5 cells from each group, and no significant difference was seen in resting potential, input resistance, reversal potential or photocurrent magnitude. c, Confocal images of cultured hippocampal neurons expressing full-length GiACR1 (left) and the final crystallization construct (right). Note the markedly reduced aggregation of the truncated construct. d, Crystals of GiACR1. Similar crystals were generated in more than 200 experiments. e, Lattice packing of GiACR1 crystals, viewed parallel to the x axis (left) and the y axis (right). f, Different amino acid configurations at different chains within the asymmetric unit of GiACR1. g, C-terminal interactions among different chains within the asymmetric unit of GiACR1.
Extended Data Fig. 2 | Structural analysis of GtACR1. a, 2Fo − Fe maps (blue mesh, contoured at 1σ) for the retinal-binding pockets of chains A–D. b, 2Fo − Fe maps (blue mesh, contoured at 1σ) for the lipid molecules. c, 2Fo − Fe maps (blue mesh, contoured at 1σ) and Fo − Fe maps (green and red meshes, contoured at 3.0σ and −3.0σ, respectively) for the Schiff base region of chains A–D. Water molecules are shown as red spheres. d, Table describing data collection and refinement statistics of GtACR1. Dataset was collected from 80 crystals. Values in parentheses are for the highest-resolution shell.
Extended Data Fig. 3 | Structure-based sequence alignment of microbial opsin genes. The sequences are GtACR1 (GenBank accession AKN63094.1), GtACR2 (GenBank AKN63095.1), ZipACR (GenBank APZ76709.1), PsuACR (GenBank KP92074.1), the chimaeric channelrhodopsin between CrChR1 and CrChR2 (C1C2, PDB code 3UG9)34, CrChR1 (GenBank 15811379), CrChR2 (GenBank 158280944), ChR1 from Volvox carteri (VcChR1, UniProtKB B4Y103), ChR1 from V. carteri (VcChR2, UniProtKB ID: B4Y105), Chrism (GenBank ID: AH02126.1), ChR from Tetraselmis striata (TsChR, GenBank ID: KF992089.1), HsBR (PDB code 1C3W)59, HsHR (PDB code 1E12)48, and Krokinobacter eikastus rhodopsin 2 (KR2, PDB code 3X3B)60. The sequence alignment was created using PROMALS3D 61 and ESPript 362 servers. Secondary structure elements for GtACR1 are shown as coils and arrows. ‘TT’ represents turns. Cysteine residues forming intermolecular and intramolecular disulfide bridges are highlighted in green and yellow, respectively. The residues of retinal-binding pockets are coloured pink. The residues in the Schiff base region are coloured cyan. The residues forming the ECS2 and CCS are coloured orange and blue, respectively.
Extended Data Fig. 4 | Structural comparison among GtACR1, HsBR, HsHR, C1C2 and CrChR2. a, b, Side view and extracellular view of the superimposed transmembrane regions of GtACR1 (blue) and HsBR (cyan) (a), GtACR1 (blue) and HsHR (beige) (b), C1C2 (green) and CrChR2 (yellow) (c). The ATRs are shown as stick models, and are coloured orange (GtACR1), salmon (HsBR), light-yellow (HsHR), green (C1C2) and yellow (CrChR2).
Extended Data Fig. 5 | Interactions between N- and C-terminal regions and the 7-TM domain. a, Interactions between the C-terminal region and the 7-TM domain. Hydrogen bonds are shown by dashed lines. b, Fluorescent size-exclusion chromatography traces of the full-length GtACR1 (1–295), the crystallized construct (1–282), and the C-terminal truncated construct (∆C: 1–253), showing possible importance of the C terminus in proper folding and/or stability. Similar results were observed in three independent experiments. c, Interactions between the N-terminal region and the ECL1. Hydrogen bonds are shown by dashed lines. d, Fluorescent size-exclusion chromatography traces of wild-type and C-to-S mutants of GtACR1. Labels indicate estimated elution positions of the aggregate, GtACR1–eGFP, and free eGFP; C-to-S mutants show decreased (<1/3) expression compared to the wild type. Similar results were observed in three independent experiments. e, Stained SDS–PAGE gel image of wild-type and N-terminal 6-amino-acid-truncated GtACR1 in the presence and absence of reducing reagent (β-mercaptoethanol); the wild type runs as a mixer of monomer and dimer in β-mercaptoethanol, whereas N-terminal-truncated GtACR1 stays monomeric even in the absence of β-mercaptoethanol. This experiment was performed once, but similar experiments with different concentrations of β-mercaptoethanol were performed three times, all with similar results. f–h, Dimer interfaces of GtACR1 (f), C1C2 (g) and CrChR2 (h) viewed at two angles from the side; note reduced interface area (outlined) for GtACR1. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 6 | Conductances, reversal potentials, absorption spectra and kinetics of wild-type GtACR1 and mutants.

a–c, Photocurrents (a), reversal potentials (b) and absorption spectra (c) of wild-type GtACR1 and ten mutants of the retinal-binding pocket. \( \lambda_{\text{max}} \) values are listed in the table (c, bottom). Photocurrents are measured in whole-cell voltage-clamp recordings held at \(-70\) mV, with 513 nm light at 1.0 mW \( \text{mm}^{-2} \) irradiance. Data are mean and s.e.m.; \( n = 9 \) for WT, 6 for C102A, 5 for C105A, M105A, C133A, C153A, E163A and C237A, and 4 for the rest. *\( P < 0.05 \), **\( P < 0.01 \), one-way ANOVA followed by Dunnett’s test. Reversal potentials are measured with identical light stimulation while cells were held at resting potentials from \(-95\) mV to \(+15\) mV in steps of 10 mV. Data are mean and s.e.m. \( n = 10 \) for WT and C237A, 6 for E163A and E163Q, 5 for C102A, M105A, C133A and C153A, and 4 for the rest. **\( P = 0.0022 \), one-way ANOVA followed by Dunnett’s test. Spectra measurement was performed in two independent trials, with wild type as a positive control. d, Comparison of fast closing (left) and slow closing (right) coefficients of wild-type and Y72F mutant GtACR1. Data are mean and s.e.m. \( n = 10 \) for WT and 5 for Y72F. *\( P = 0.7 \) for both graphs, two-tailed t-test.
Extended Data Fig. 7 | Current–voltage (I–V) relationships of wild-type GtACR1 and mutants. The I–V relationship between −95 mV and +15 mV was determined from the single current amplitude at the indicated potentials. Each measurement is normalized to the current amplitude measured at −25 mV. Data are mean and s.e.m. n = 10 for WT and C237A, 8 for E223A, 6 for Q46C, E163A and E163Q, 4 for E68S, E68T, C102S and M105I, and 5 for the rest.
Extended Data Fig. 8 | Representative traces of the $I-V$ measurement of wild-type GtACR1 and mutants. Voltage clamp traces corresponding to the $I-V$ relationships in Extended Data Fig. 7 between $-95$ mV and $+15$ mV.
Extended Data Fig. 9  | Spectroscopic characterization of wild-type GtACR1 and the D234N mutant.  

a, Absorption spectra of wild-type GtACR1 (top left) and the D234N mutant (top right) measured from pH 3.0 to 10.0. The $\lambda_{\text{max}}$ value at each pH is listed in the table (bottom). 

| pH  | $\lambda_{\text{max}}$ (nm) |
|-----|-----------------------------|
|     | WT  | pH3 | pH4 | pH5 | pH6 | pH7 | pH8 | pH9 | pH10 |
| -----|-----|-----|-----|-----|-----|-----|-----|-----|-------|
| D234N| 442 | 514 | 514 | 514 | 514 | 514 | 514 | 514 | 514   |

b, Difference FTIR spectra of wild-type GtACR1 and the D234N mutant measured at 77 K, 170 K and 200 K. 

c, Difference FTIR spectra of wild-type GtACR1 in the 1,690–1,770 cm$^{-1}$ region measured at pH 5.0, 7.0 and 9.0. Forty identical recordings at 77 K and seven identical recordings at 170 K and 200 K were averaged.
Extended Data Fig. 10 | Comparison of surface electrostatic potential of GtACR1 and C1C2. a, b, Electrostatic potential surfaces of GtACR1 (a) and C1C2 (b) viewed from four angles. The surface is coloured on the basis of the electrostatic potential contoured from $-15 \text{kT}$ (red) to $+15 \text{kT}$ (blue). c, d, Representation of positively charged amino acids (lysine and arginine residues) in GtACR1 (c), and negatively charged amino acids (aspartate and glutamate residues) in C1C2 (d).
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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).

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- The statistical test(s) used AND whether they are one- or two-sided
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- 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
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- 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: JBlue-Ice for X-ray data collection, and Clampex 10.6 for electrophysiological data collection.

Data analysis: Crystallography : KAMO, XDS, XSCALE, MoRDa, Refmac5, Phenix, Coot, MR-rosetta, Cuemol. Electrophysiology : pClamp 10, Prism 7, Spectroscopy data : Prism 7

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The protein coordinate and atomic structure factor have been deposited in the Protein Data Bank (PDB) under accession number 6CSM. The raw diffraction images have been deposited in the SBGrid data repository (ID: S69). All other data are available from corresponding authors upon reasonable request.
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Life sciences study design

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

Sample size
Sample sizes were determined based on prior literature and best practices in the field; no statistical methods were used to predetermine sample size. The sample size for electrophysiology recordings is typically four or greater measurements.

Data exclusions
No data were excluded.

Replication
All attempts at replication were successful.

Randomization
Animal experiments were not performed in this study, so no randomization was needed.

Blinding
Animal experiments were not performed in this study, so Investigators were not blinded to the experiment.

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 |

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Sf9 cells were purchased from expression systems, and HEK293 cells were purchased from Thermo Fisher.

Authentication
Cells have been authenticated by the vendors.

Mycoplasma contamination
Cells were not tested for mycoplasma contamination.

Commonly misidentified lines
Cells are not listed in the database.

(See ICLAC register)