Rhodopsin-cyclases for photocontrol of cGMP/cAMP and 2.3 Å structure of the adenylyl cyclase domain

Ulrike Scheib¹, Matthias Broser¹, Oana M. Constantin², Shang Yang³, Shiqiang Gao³ Shatanik Mukherjee¹, Katja Stehfest¹, Georg Nagel³, Christine E. Gee² & Peter Hegemann¹

The cyclic nucleotides cAMP and cGMP are important second messengers that orchestrate fundamental cellular responses. Here, we present the characterization of the rhodopsin-guanylyl cyclase from Catenaria anguillulae (CaRhGC), which produces cGMP in response to green light with a light to dark activity ratio >1000. After light excitation the putative signaling state forms with $\tau = 31$ ms and decays with $\tau = 570$ ms. Mutations (up to 6) within the nucleotide binding site generate rhodopsin-adenylyl cyclases (CaRhACs) of which the double mutated YFP-CaRhAC (E497K/C566D) is the most suitable for rapid cAMP production in neurons. Furthermore, the crystal structure of the ligand-bound AC domain (2.25 Å) reveals detailed information about the nucleotide binding mode within this recently discovered class of enzyme rhodopsin. Both YFP-CaRhGC and YFP-CaRhAC are favorable optogenetic tools for non-invasive, cell-selective, and spatio-temporally precise modulation of cAMP/cGMP with light.

¹Institute for Biology, Experimental Biophysics, Humboldt-Universität zu Berlin, 10115 Berlin, Germany. ²Institute for Synaptic Physiology, Center for Molecular Neurobiology Hamburg, University Medical Center Hamburg-Eppendorf, 20251 Hamburg, Germany. ³Department of Biology, Institute for Molecular Plant Physiology and Biophysics, Biocenter, Julius-Maximilians-University of Würzburg, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany. These authors contributed equally: Christine E. Gee, Peter Hegemann. Correspondence and requests for materials should be addressed to C.E.G. (email: christine.gee@zmnh.uni-hamburg.de) or to P.H. (email: hegemann@rz.hu-berlin.de)
The cyclic nucleotides cAMP and cGMP are ubiquitous second messengers that regulate essential cellular processes including basic metabolism, gene expression, differentiation, proliferation, and cell survival. Spatial and temporal segregation of cyclic nucleotides and their effectors allows precise coordination of a multitude of signaling pathways. Despite the enormous impact of cyclic nucleotides many questions about their exact roles remain unanswered. Pharmacological approaches (e.g., forskolin, IBMX) do not allow cell specific manipulation of cyclic nucleotides in tissue and lack precision in space and time, limitations that can be overcome using light-activated enzymes.

The advent of optogenetic tools has allowed precise spatiotemporal control of cellular processes. For example, non-invasive control of intracellular [cAMP] is achieved using the soluble photoactivatable adenyl cyclases from Euglena (ePAC) or Beggiatoa (bpAC). However, the cytoplasmic localization, and slow off-kinetics of these flavin-based enzymes (bpAC τ off = 12 s) are disadvantageous characteristics to study fast cyclic nucleotide signaling near cellular membranes. Recently, BeRhGC (a.k.a. BeGC, a C. anguillulae rhodopsin directly connected via a linker to a guanylyl cyclase domain, was discovered in the aquatic fungus Blastocladiella emersonii. BeRhGC converts GTP into cGMP in many cell types upon green light stimulation whereas it is totally inactive in the dark. Upon excitation the putative rhodopsin signaling state is formed within 8 ms and decays after 100 ms, whereas the enzyme’s activity declines with a τ off of ~300 ms. BeRhGC is therefore, only moderately resistant to high light intensities and bleaches in continuous light.

In this study, we further characterize the RhGC from the fungus Catenaria anguillulae (CaRhGC), which is another member of the chitin-walled Blastocladiomycota, and the adenyl cyclase CaRhAC resulting from the point mutations E497K, C566D in Xenopus oocytes and hippocampal neurons. We additionally present the crystal structure of the ligand-bound adenyl cyclase domain (CaAC) at 2.25 Å resolution, which reveals the mechanistic basis for the change from cGMP to cAMP production. The rhodopsin domain from Catenaria is more photostable than that from Blastocladiella, and the signaling state persists longer, both of which might be highly desirable traits for optogenetic applications. YFP-CaRhGC together with the YFP-CaRhAC mutant, expand the optogenetic toolbox allowing control of cAMP/cGMP signaling within milliseconds close to cellular membranes.

Results

Characterization of CaRhGC in oocytes and rat neurons.

Comparing the amino acid sequences of CaRhGC and BeRhGC (a.k.a. CaCyclop and BeCyclop, or BeGC1 and CaGC1) revealed 77% identity (Supplementary Fig. 1). Most differences occur within the N-terminal extension (i.e., amino acids 72–170), which is predicted to harbor 1–2 extra helices upstream of the 7 transmembrane helices that characterize the rhodopsins (Fig. 1a, Supplementary Figs. 1 and 2). A second region of variation occurs in helices 4 and 5 including the helix 4/5-loop. Additionally, we found a higher probability for coiled-coil formation of the N-terminal helix-1 of CaRhGC compared to BeRhGC (Supplementary Tables 1 and 2). To localize the N terminus, we inserted the rhodopsin domain of CaRhGC (aa 1–425) between the two fragments of a split YFP. The observed YFP fluorescence confirmed that the extra N-terminal segment spans the membrane in such a way that the N terminus is positioned intracellularly (Fig. 1b).

We co-expressed full-length CaRhGC and the cGMP-sensitive cyclic nucleotide-gated A2 channel from rat olfactory neurons (CNG(cAMP), K1/2 cAMP = 36 μM, K1/2 cGMP = 1.3 μM) in Xenopus oocytes and recorded inward photocurrents in response to 2 second green light pulses (Fig. 1c). The photocurrents were light intensity-dependent with half maximal saturation for the slope (EC50) at 0.027 mM mm−2 (Fig. 1d) and declined after light-off with an apparent τ off = 9.2 ± 1.7 s (n = 8, 0.12 mM mm−2). CaRhGC is highly selective for GTP and no photocurrents were recorded from oocytes co-expressing CaRhGC with the cAMP-sensitive CNGA2 channel (C460W/E583M, CNG(cAMP), K1/2 cAMP = 0.89 μM, K1/2 cGMP = 6.2 μM) (Fig. 1c), which also excludes that CaRhGC itself is conductive.

To quantify cyclic nucleotide concentrations, cGMP and cAMP were determined in oocyte lysates using an enzyme-linked immunosorbent assay (ELISA) (Fig. 1e, f). In CaRhGC-expressing cells the dark concentration of cGMP remained unchanged at 0.4 pmol/oocyte, whereas illumination for 1 min with green light increased cGMP to 120 pmol/oocyte. To facilitate future studies in host cells, including neuronal networks, we labeled CaRhGC N-terminally with YFP, which did not change enzyme activity. In contrast, C-terminal YFP tagging caused the enzyme to be partially active in the dark increasing cGMP concentration 10-fold (Fig. 1e). For all CaRhGC variants, CAMP concentrations were unaffected (Fig. 1f), confirming the GTP selectivity of CaRhGC.

Hippocampal neurons expressing YFP-CaRhGC, CNG(cAMP) and mtSapphire had normal morphology and normal whole-cell voltage responses to current injection (Fig. 2a–c). Flashes of green light repeatedly evoked transient currents through the cGMP-sensitive channels (Fig. 2c). The photocurrents evoked by strong green light in YFP-CaRhGC-expressing neurons were similar in size to the photocurrents recorded from neurons expressing BeRhGC (Fig. 2d, e; Supplementary Table 3, Supplementary Fig. 3a, b). Interestingly, without the YFP-tag photocurrents were of similar amplitude but evoked in only 30% of neurons expressing CaRhGC (Fig. 2d, e; Supplementary Table 3). We attempted to add a C-terminal mycHis tag to YFP-CaRhGC and this also had a negative impact on reliability (Supplementary Table 3). But whether codons were optimized for human or mouse expression was unimportant (Supplementary Table 3).

Interestingly, the kinetics of the guanylyl cyclases from Blastocladiella and Catenaria were quite different (Fig. 2f, Supplementary Fig. 3c, d). The time to photocurrent onset was significantly shorter in neurons expressing CaRhGC than in neurons expressing BeRhGC (median time to onset CaRhGC 23 ms, BeRhGC 120 ms, p = 0.0029, Kruskall–Wallis). In addition, the slope was greater indicating that the cGMP concentration increases faster in neurons expressing the Catenaria guanylyl cyclase than it does in neurons expressing the cyclase from Blastocladiella (Fig. 2f). Moreover, the CaRhGC photocurrent decay was much faster in neurons than in oocytes (τ off ~0.2 s, Supplementary Fig. 3d), suggesting that phosphodiesterases rapidly degrade cGMP in neurons. The YFP-CaRhGC photocurrent amplitudes and slopes were graded with the light intensity with an EC50 of 0.7 mW mm−2 (Fig. 2g, h). No photocurrents were evoked in neurons transfected with CaRhGC or YFP-CaRhGC and CNG(cAMP), confirming that the specificity for producing cGMP is unchanged in neurons (Fig. 2d, e; Supplementary Table 3).

Spectroscopic properties of the Ca rhodopsin domain.

To assess the spectral properties of CaRhGC, we purified the recombinant rhodopsin fragment CaRh (amino acid residues 1 to 396) from insect cells (Sf9). Dark-adapted CaRh showed a typical unstructured rhodopsin spectrum with a maximum at 540 nm (D360, Fig. 3a). Bright green light (530 nm) converted D360 into a light-adapted species with slightly shifted absorption maximum
(L$_{538}$) (inset Fig. 3a), but caused very little bleaching even after long exposure$^{17}$. To characterize CaRh photocycle intermediates, absorption changes were recorded from 100 ns to 10 s after stimulation with 10 ns 530 nm laser flashes (Fig. 3b, c). Evolution-associated difference spectra (EADS) of the intermediates and their life times (τ values) were extracted using a global fit routine (Fig. 3b). We first detected an early red-shifted K-like photocycle intermediate (K$_{600}$) that rose faster than our time resolution and decayed at pH 7.5 with τ = 0.81 µs into a blue-shifted intermediate (L$_{1450}$), which was not observed in the BeRh photocycle$^{13}$. EADS and the extracted time trace at 458 nm (Fig. 3b) revealed accumulation of a second L$_{2450}$ state with τ = 397 µs, which converted into the proposed signaling state M$_{380}$ with τ = 31 ms (compared to 8 ms for BeRhGC$^{13}$). The temporal dependence of the absorbance changes at key wavelengths (Fig. 3c), which refer to the photocycle intermediates, revealed a slower decay of the M$_{380}$ state (τ = 571 ms) compared to BeRh (τ = 100 ms)$^{13}$. In summary, green light converts CaRh D$_{540}$ to a red-
shifted K600-like intermediate that reverts via two blue-shifted states L1450, L2450 and the putative signaling state, M380, back to the dark state D540 (Fig. 3d).

Enzymatic characterization of solubilized RhGCs and CaGC.

The high photo-stability of CaRh encouraged us to purify recombinant full-length CaRhGC from insect cells and to determine the kinetic parameters of the enzyme (Fig. 4a). A $K_M$ value of 6.1 mM, $v_{\text{max}}$ of 821 µmol cGMP min$^{-1}$ µmol protein$^{-1}$ and a $k_{\text{cat}}$ of 410 min$^{-1}$ was determined at pH 7.5 where the protein is most active (Fig. 4b, Table 1). The light intensity used was below the EC$_{50}$ determined from the oocyte recordings. For illuminated BeRhGC the $K_M$, maximal velocity, and turnover ($K_M = 0.92$ mM, $v_{\text{max}} = 129$ µmol cGMP min$^{-1}$ µmol protein$^{-1}$, $k_{\text{cat}} = 64$ min$^{-1}$) were all lower than those measured for CaRhGC.

**Fig. 2** YFP-CaRhGC produces cGMP and not cAMP in hippocampal neurons. **a**, **b** Confocal images of neurons 8 days after electroporation with DNA encoding YFP-CaRhGC and mtSapphire; **a** maximum intensity projection, excitation 405 nm (mtSapphire); **b** top: maximum projection, excitation 515 nm (YFP); bottom: single plane. scale bars 10 µm. **c** Top: Whole-cell response to current injections from −400 pA to 400 pA in 100 pA steps. Bottom: The first, second and fifth currents evoked by repeated green light flashes (530 nm, 0.3 mW mm$^{-2}$, 100 ms, inter-stimulus interval 40 s). **d** Sample currents evoked by a 2 s green light pulse (530 nm, 27.3 mW mm$^{-2}$) in neurons expressing YFP-CaRhGC, CaRhGC or the guanylyl cyclase from Blastocladiella emersonii (BeRhGC) together with CNG(cGMP) or the cAMP-sensitive CNGA2 (CNG(cAMP)) channels. Green bar: light application, 2 s. **e** Peak photocurrents (p) and the sustained response (s) recorded from neurons expressing YFP-CaRhGC, CaRhGC, or BeRhGC and one of the CNG channels. Shown are individual data points, median and 25–75% interquartile range. n = 12, 12, 4, 17, 17, 6, 13, 7 left to right; **p = 0.0001, p = 0.0006, *p = 0.009; Mann-Whitney test, peak vs sustained response of YFP-Ca/Ca/BeRhGC + CNG(cGMP). Median peak current YFP-CaRhGC −0.79 nA, CaRhGC −1.3 nA, BeRhGC −0.6 nA; median sustained current YFP-CaRhGC −0.25 nA, CaRhGC −0.24 nA, BeRhGC −0.11 nA. **f** Detail of photocurrent onset from neurons expressing YFP-CaRhGC, CaRhGC or BeRhGC, and CNG(cGMP). Graph shows individual data points, median and interquartile range. n = 12, 17, 12, median slope YFP-CaRhGC = −8.7 pA ms$^{-1}$, CaRhGC = −7.2 pA ms$^{-1}$, BeRhGC = −2.2 pA ms$^{-1}$. **g** Sample currents recorded from a neuron expressing YFP-CaRhGC + CNG(cGMP) when stimulated with green light. **h** Light intensity-response relationship for YFP-CaRhGC + CNG(cGMP) fitted with a quadratic equation. Photocurrents were normalized to the maximum current recorded for each neuron. n = 17. RhGC DNA was electroporated at 10 ng µl$^{-1}$, CNG channel DNA at 25 ng µl$^{-1}$ and mtSapphire DNA at 5 ng µl$^{-1}$.
CaGC, from *E. coli* constitutively active (Table 1, Supplementary Fig. 5). Guanine base and C566 should coordinate the C6-ketogroup hydrogen bond with the exocyclic 2-amino group, and N1 of the tiadyl cyclases indicated that the glutamate E497 should form a comparison of BeRhGC and CaRhGC to other type III nucleotide swap the substrate selectivity from GTP to ATP. Sequence that allow fast spatio-temporal control of cAMP, we sought to generation of rhodopsin-adenylyl cyclases. Due to the importance of cAMP signaling and the demand for optogenetic tools that allow fast spatio-temporal control of cAMP, we sought to swap the substrate selectivity from GTP to ATP. Sequence comparison of BeRhGC and CaRhGC to other type III nucleotidyl cyclases indicated that the glutamate E497 should form a hydrogen bond with the exocyclic 2-amino group, and N1 of the guanine base and C566 should coordinate the C6-ketogroup (Fig. 5a, Supplementary Fig. 6). In adenylyl cyclases, these positions are held by a lysine and an aspartate (or threonine), which anchor the adenine base through interactions with the ring nitrogen N1 and the amino group N6, respectively. Similar to previous studies (e.g., refs. 18,20), we mutated E497 to K and C566 to D and expressed the N-terminal YFP-tagged constructs in oocytes. After 3 days, oocytes were illuminated or kept in darkness, and cyclic nucleotide concentrations in lysates were quantified by ELISA using the YFP fluorescence as expression marker.

The mutations indeed changed both BeRhGC and CaRhGC into adenylyl cyclases, which we named BeRhAC and CaRhAC respectively (alternative naming: CyclOp-PACs). Similar to Trieu et al.14, we found that in the dark BeRhAC increased resting cAMP 5× (18.5 ± 2.4 pmol/oocyte vs. 3.2 ± 1.1 pmol/oocyte non-injected oocytes) and upon illumination cAMP only increased a further 9× (Fig. 5b). In oocytes expressing CaRhAC, the dark cAMP was only 2.6× higher than in the non-injected oocytes (dark 7.9 ± 2.6 pmol/oocyte) and cAMP increased 31× when exposed to light (150 ± 28.2 pmol/oocyte, Fig. 5b). For both RhACs, no cGMP increase was detected (Fig. 5c). To reduce the dark activity of BeRhAC, we mimicked the nucleotide binding pocket of membrane anchored adenylyl cyclases (tmAC C2) by introducing four additional point mutations between aa 564 and 568 (Fig. 5a). Dark activity was not detected in oocytes expressing the E497K, 564-QYDIW-568 variants BeRhAC-6× and CaRhAC-6×, but light-induced cAMP production was reduced as well (Fig. 5b).

Characterization of the adenylyl cyclases. The purified CaAC domain had an activity similar to CaGC (Table 1, Supplementary Fig. 7). GTP did not serve as a substrate but was able to antagonize the production of cAMP (Supplementary Fig. 7). As for the GCs, we observed that, detergent solubilized full-length CaRhAC was de-stabilized, showing substantial dark activity and multiple bands on protein immunoblots (Supplementary Fig. 8). We therefore used membranes from oocytes expressing the YFP-tagged RhACs and CaRhGC for further in vitro enzymatic characterization. N-terminally tagged YFP-CaRhGC had lower activity than the full-length construct, but higher activity than the CaAC (Supplementary Fig. 8).

Generation of rhodopsin-adenylyl cyclases. Due to the importance of cAMP signaling and the demand for optogenetic tools that allow fast spatio-temporal control of cAMP, we sought to swap the substrate selectivity from GTP to ATP. Sequence comparison of BeRhGC and CaRhGC to other type III nucleotidyl cyclases indicated that the glutamate E497 should form a hydrogen bond with the exocyclic 2-amino group, and N1 of the guanine base and C566 should coordinate the C6-ketogroup (Fig. 5a, Supplementary Fig. 6). In adenylyl cyclases, these positions are held by a lysine and an aspartate (or threonine), which anchor the adenine base through interactions with the ring nitrogen N1 and the amino group N6, respectively. Similar to previous studies (e.g., refs. 18,20), we mutated E497 to K and C566 to D and expressed the N-terminal YFP-tagged constructs in oocytes. After 3 days, oocytes were illuminated or kept in darkness, and cyclic nucleotide concentrations in lysates were quantified by ELISA using the YFP fluorescence as expression marker.
cGMP turnover in the dark than CaRhGC-YFP as expected (Table 2). In the light, cGMP turnover increased >1000×, which is comparable to the activity measured for BeRhGC (BeCyclOp) using the same assay12. For YFP-RhAC from both organisms, a light-driven cAMP turnover of ~40 min−1 and a light/dark activity ratio of ~200 was determined (Table 2). The lower activity of the RhACs compared to RhGCs and the lower dark activity of YFP-CaRhAC compared to YFP-BeRhAC confirmed the results from oocyte lysates (Table 2, Fig. 5b). The oocyte membrane assay also confirmed the reduced dark activities of YFP-RhACs-6x and increased light/dark activity ratios. Cyclic GMP was not produced by any RhAC mutants and conversely, cAMP was not produced by CaRhGCs (Table 2).

We tested several versions of CaRhAC in hippocampal neurons. YFP-CaRhAC with only the E497K,C566D mutations and no C-terminal mycHis tag was superior, producing light-induced cAMP mediated currents in all transfected neurons (Fig. 6, Supplementary Table 3, Supplementary Figs. 3, 9, 10).

Table 1 Enzymatic parameters of purified RhGCs and truncated cyclases

| Substrate | BeRhGC (light−dark) | BeGC | CaRhGC (light−dark) | CaGC | CaAC (E497K, C566D) |
|-----------|---------------------|------|---------------------|------|---------------------|
| GTP       | 0.98                | 0.99 | 0.92 ± 0.27         | 1 ± 0.18 | 0.98 ± 0.18 |
| Hill fit R² | 0.99                | 0.96 | 2.16 ± 0.30         | 1.92 ± 0.19 | 2.16 ± 0.30 |
| Vₘₐₓ (cNMP (μmol min⁻¹ mgprotein⁻¹)) | 128.5 ± 13.4 | 128.5 ± 13.4 | 11.64 ± 5.67 | 63.0 ± 1.54 | 63.0 ± 1.54 |
| kₗ (s⁻¹ M⁻¹) | 64.3                | 64.3 | 410.9               | 67.80 | 67.80 |
| kₗ/kₐ (s⁻¹ M⁻¹) | 1.1                 | 1.1  | 6.85                | 1.13  | 1.13  |
| Molecular weight (kDa) | 522 nm, 0.010 mM mm⁻² | 522 nm, 0.010 mM mm⁻² | 70.6 | 21.5 | 70.6 |

Values are mean ± sem.
Neurons expressing YFP-BeRhAC-6× or YFP-CaRhAC-6× frequently had multiple nuclei and were difficult to record from. Hippocampal neurons expressing YFP-CaRhAC had normal morphology and membrane properties (Fig. 6a–c). The YFP fluorescence appeared to be associated with the plasma membrane and intracellular membranes (Fig. 6b). Green flashes repeatedly evoked transient currents through the cAMP-sensitive channels. The rise and decay of the currents was slower than for the YFP-CaRhGC evoked currents in Fig. 2c (Fig. 6d, e (inset), Supplementary Table 3, Supplementary Figs. 3d, 9b, d). Neurons expressing YFP-CaRhAC alone or together with CNG(cGMP) had no or small photocurrents (Fig. 6e, f, Supplementary Table 3, Supplementary Fig. 9). As hippocampal neurons have endogenous hyperpolarization-activated and cyclic nucleotide-gated channels (HCN)21, at least part of these residual currents are likely due to activation of endogenous channels by cAMP. The cAMP induced photocurrents were light intensity-dependent with an EC50 of 0.6 mW mm−2 (Fig. 6g, h).

### Table 2 In vitro production of cAMP and cGMP by RhACs in oocyte membranes

|                      | cGMP turnover in darkness (min⁻¹) | cGMP turnover in light (min⁻¹) | L/D | cAMP turnover in Dark (min⁻¹) | cAMP turnover in Light (min⁻¹) | L/D |
|----------------------|----------------------------------|--------------------------------|-----|-------------------------------|-------------------------------|-----|
| YFP-CaRhGC (20 °C)   | 0.028 ± 0.007                    | 71 ± 10.7                      | 2500 | ND                           | ND                             | —   |
| CaRhGC-YFP (20 °C)   | 0.11 ± 0.04                      | 120 ± 9.3                      | 1100 | ND                           | ND                             | —   |
| YFP-CaRhAC (20 °C)   | ND                               | ND                             | <0.001 | 0.7 ± 0.13                  | 0.14 ± 0.01                  | 39.4 ± 5.6 |
| YFP-BeRhAC-6× (20 °C)| ND                               | ND                             | <0.001 | 0.7 ± 0.13                  | 0.14 ± 0.01                  | 33 ± 5.6 |
| YFP-BeRhAC-6× (37 °C)| ND                               | ND                             | <0.001 | 0.7 ± 0.13                  | 0.14 ± 0.01                  | 33 ± 5.6 |
| YFP-CaRhAC-6× (20 °C)| ND                               | ND                             | 740   | 1.72 ± 0.36                  | 0.7 ± 0.13                  | 280  |
| YFP-CaRhAC-6× (37 °C)| ND                               | ND                             | 740   | 1.72 ± 0.36                  | 0.7 ± 0.13                  | 280  |

Values are mean ± standard deviation.

RhAC E497K C566D, RhAC-6× E497K 564QYDIW568, ND not detectable.
constructs retained the highest sensitivity to green light in neurons (Supplementary Fig. 11).

Crystal structure of the adenylyl cyclase domain. To gain structural information about the class of enzymes rhodopsin and the nucleotide binding mode, we tried to crystallize both the wild-type full-length and the isolated enzyme domains of CaRhGC and CaRhAC in presence of NTP analogs. While crystallization of the full-length CaRhGC failed, we produced highly diffracting crystals of the GC domain (aa 443–626, molecular replacement based on PDB: 4P2F). Similar to Kumar et al., our GC structures either revealed a monomeric or a non-functional dimeric arrangement, connected by an artificial disulfide bridge. Since the structures did not contain a bound substrate analog, they were not considered further. Crystals of CaAC in complex with ATPαS diffracted to a maximum resolution of 2.25 Å. In contrast to our GC structures, CaAC crystallized as a homodimer in an anti-parallel orientation forming two symmetric catalytic sites within the protein–protein interface, as expected from other adenylyl and guanylyl cyclase structures. The structure was solved by molecular replacement using our high-resolution structure (1.2 Å) of monomeric CaGC, which allowed us to refine the structure to an R factor of 18.2% and free R factor of 22.4% (Table 3). The CaAC exhibits the classical nucleotidyl cyclase type III fold with a central 7-stranded β-sheet shielded by 3 helices. The nomenclature of the secondary structure elements are used according to Zhang et al. (Supplementary Fig. 12b).

Fig. 6 YFP-CaRhAC rapidly produces cAMP in hippocampal neurons. Two photon images of a neuron expressing YFP-CaRhAC (YFP-BerRhGC E497K C566D) and mtSapphire (a) or YFP-CaRhAC alone (b); a maximum intensity projection, excitation 800 nm, scale bar 50 μm; b top: maximum projection, excitation 950 nm; bottom: single plane YFP fluorescence 950 nm of same neuron, scale bar 10 μm. c Whole-cell response to current injections of a hippocampal neuron expressing YFP-CaRhAC plus the cAMP-sensitive CNGA2 channel (CNG(cAMP) C460W/E583M). Current injections from -400 pA to 400 pA in 100 pA steps. d The first, second and fifth currents evoked by repeated green light flashes (530 nm, 0.3 mW mm⁻², 100 ms, interval 20 s). e Sample currents evoked by strong green light (27.3 mW mm⁻²) in neurons expressing YFP-CaRhAC together with CNG(cGMP), CNG(cAMP) or alone. Green bar: light application, 2 s. Insert shows the onset and initial slope of the YFP-CaRhAC + CNG(cAMP) photocurrents in comparison to photocurrents from a CaRhGC + CNG(cGMP) expressing neuron. f Comparison of the maximum peak or slope of photocurrents recorded from neurons expressing YFP-CaRhAC and one of the CNG channels or by itself. Shown are individual data points, median and interquartile range, n = 10, 6, 10 left to right. *p = 0.023, **p = 0.0016, Kruskal-Wallis test vs YFP-CaRhAC + CNG(cAMP). g Sample currents recorded from a neuron expressing YFP-CaRhAC + CNG(cAMP) when stimulated with green light of different intensity. h Light intensity–response relationship for YFP-CaRhAC + CNG(cAMP) fitted with a quadratic equation. Photocurrents were normalized to the maximum current recorded for each neuron. n = 8. DNA encoding rhodopsin-adenylyl cyclases was electroporated at 25 ng μl⁻¹, CNG channel DNA at 25 ng μl⁻¹ and mtSapphire DNA at 5 ng μl⁻¹.

The nomenclature of the secondary structure elements are used according to Zhang et al. (Supplementary Fig. 12b).

**ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-04428-w**

8 NATURE COMMUNICATIONS | (2018) 9:2046 | DOI: 10.1038/s41467-018-04428-w | www.nature.com/naturecommunications
Within the crystal lattice the CaAC homodimers formed an intertwined helical superstructure, an unusual packing that leads to a large unit cell (8426 Å³) with 8 homodimers per cell. In proximity to the ribose-3′-OH, two metal ions (A and B) have been detected for other adenyl cyclases. While ion A has been associated with the abstraction of a proton from 3′-OH, ion B was found to bind the substrate through coordination of Pβ-Pγ. In CaAC the metal B site is occupied by a metal ion, which was assigned to calcium based on the coordination number and distances, while ion A is absent. Ca²⁺ is octahedrally coordinated by the conserved residues D457* and D501*, the backbone carbonyl of I458*, the oxygens of the β and γ phosphates and a less well-defined weakly bound water. Both aspartates provide only one of their oxygens to bind the metal. The second oxygen of D457* forms a further contact to R545*, whereas D501* forms a hydrogen bond with the ribose.

Apart from binding to calcium, the terminal β-γ phosphates are located close to helix α1* and forms hydrogen bonds with T462*, F461* and N460* of the adjacent loop. The γ phosphate orients towards R545*, a residue suggested to aid the exit of the formed pyrophosphate.

In particular, the orientation of the conserved arginine (R577 in CaAC), located on a4 (Fig. 7d, f), towards Pa was claimed to be essential to stabilize the additional negative charge of the a-phosphate after the ribose-3′-OH attack. In CaAC this arginine (R577) (Fig. 7f) is found to be flexible and electron density for its side chain is not present in all protein biochemical analysis showed a similar inhibitory potential for both (Supplementary Fig. 13). The refinement of ATP-Sp-aS within the binding pocket lowers the B-factors obtained for the sulfur position to values more comparable to the overall molecule in all 16 ligands and was therefore included in the model. As in other AC structures, residues that anchor the adenine and phosphate tail of ATPaS in the binding pocket belong to different monomers of the dimeric CaAC. In the following, residues belonging to the nonmer that bind the phosphate tail are marked (*).

Similar to other type III cyclases, 7 conserved residues in CaAC provide ligand binding and are mainly involved in formation of cAMP and PPi from ATP (indicated in Supplementary Fig. 6). ATP cleavage and cyclization is considered to follow an intramolecular nucleophilic substitution (SN2), which is initiated through the attack of ribose-3′-OH oxygen at Pa, resulting in a negatively charged a-phosphate during the transition state.

The adenine base is held within the hydrophobic crev of the nucleotide binding pocket defined by F455, L504, L567, V568, V572, V496, I499 and I499*, while the mutated residues E497K and C566D anchor the base via hydrogen bonds (Fig. 7d, e). In particular, D566 together with the backbone oxygen of L567 function as hydrogen bond acceptors for the amino group of the adenine base while K497 serves as donor for the ring nitrogen N1. An additional water-mediated interaction ties purine N7 to the conserved N573 (a4) and G569 (a4). This observed donor/acceptor pattern would be unfavorable for a similarly positioned guanine. Although these key positions for base selectivity were identified 20 years ago, the CaAC structure explains the implications of E497K and C566D for the substrate specificity switch on a structural level. Different to most other adenyl cyclase structures, the ribosyl moiety (2′-endo conformation) is tilted perpendicular to the purine plane and orients towards the β2/3* loop with the 2′-OH in hydrogen bond distance to D501* and the backbone carbonyl of I499* (Fig. 7d, f). Two waters are seen that are expected to stabilize this binding to β2/3* by mediating further interactions to 2′-OH and 3′-OH. Different to other adenyl cyclases, the ribose ring oxygen of CaAC forms a weak hydrogen bond with S576 (a4, 3.5 Å) (Fig. 7e) and does not interact with the conserved and catalytically important N573 (a4).

In particular, the orientation of the conserved arginine (R577 in CaAC), located on a4 (Fig. 7d, f), towards Pa was claimed to be essential to stabilize the additional negative charge of the a-phosphate after the ribose-3′-OH attack. In CaAC this arginine (R577) (Fig. 7f) is found to be flexible and electron density for its side chain is not present in all protein biochemical analysis showed a similar inhibitory potential for both (Supplementary Fig. 13). The refinement of ATP-Sp-aS within the binding pocket lowers the B-factors obtained for the sulfur position to values more comparable to the overall molecule in all 16 ligands and was therefore included in the model. As in other AC structures, residues that anchor the adenine and phosphate tail of ATPaS in the binding pocket belong to different monomers of the dimeric CaAC. In the following, residues belonging to the nonmer that bind the phosphate tail are marked (*).
Fig. 7 Crystal structure of CaAC in complex with ATPαS. a, CaAC (E497K/C566D) homodimers form an intertwined helical superstructure within the crystal lattice (blue, red, gray indicate individual superhelices, yellow indicates 8 homodimers within a single asymmetric unit). b, Each homodimer consists of two antiparallel arranged monomers and harbors 2 active sites at the dimer interface, occupied by the ATP analog: ATP-SP-αS. Arrows indicate β4/5 loops, N-termini are labeled, chain A: blue/green, chain B: green/red. c, Comparison of the β4/5 loop orientation in CaAC. Overlay of the six monomers of CaAC found in the loop-proximal (green) position close to the catalytic core, or the loop-distal (orange) position. The protein backbone is visualized as a ribbon with selected residues and the base head group shown as sticks. d, CaAC active site; the adenine base and phosphate tail of ATP-SP-αS are anchored by different monomers. Residues of the phosphate binding monomer are marked (*). The previously mutated residues E497K (orange), C566D (red) form hydrogen bonds to the adenine base. The ribose is tilted perpendicular to the adenine plane and points towards β2*, β3*. The conserved arginine, R577 (α4) orients towards Pα. The ion B site is occupied by Ca2+, which is octahedrally coordinated among others by two conserved aspartates, D457* and D501*. The phosphate tail of ATP-SP-αS is anchored to the α1/β1-loop via polar interactions. mFo-DFc omit map for ATP-SP-αS contoured at 5σ, contour level is shown as gray mesh. e, Magnification of the adenine base binding residues. Neighboring residues of D566, situated on β5 and mutated in RhACs-6× are colored in red. f, Magnification of the residues, involved in nucleotide phosphate binding, coloring as in d. Gray dashed lines indicate hydrogen bonds or metal coordination between 2.3 Å and 3.6 Å in length.
subunits. In most cases the guanidine group points towards the Pa,β anhydride bond, a position suitable for stabilizing the transition state.

**Discussion**

In this study, we characterized the rhodopsin-guanylyl cyclase CaRhGC from the fungus *Catenaria anguillulae*, which belongs to a recently discovered class of enzyme rhodopsins (cyclase opsin). CaRhGC produces cGMP upon illumination with green light in oocytes and rat hippocampal neurons. As the guanilyl cyclase domain (aa 443–626) itself is active without the rhodopsin, we conclude that in full-length CaRhGC (and BeRhGC) the rhodopsin domain acts as a clamp, which keeps the cyclase inactive in darkness, and illumination releases the clamp allowing the cyclase to produce cGMP. Interestingly, the light-activated rhodopsin additionally influences the cyclase dynamics, as the RhGCs are 3–6× more active than the isolated cyclase domains (Fig. 4; Supplementary Figs. 4–5, Table 1). Possibly the activated rhodopsin stabilizes the dimeric arrangement of the enzyme or light causes other conformational changes that promote catalysis. A full understanding of the light-induced intramolecular signal transduction will require solving the structures of full-length dark-adapted and light-activated rhodopsin cyclases.

Spectroscopic analysis of the CaRh domain indicated a higher photo-stability compared to that of BeRh, which is beneficial for optogenetic approaches or biophysical studies where extended illumination protocols are required. Different to most microbial rhodopsins the prolonged M state is formed rather late in CaRh. But, in histidine kinase rhodopsin 1 (HKR1) of *Chlamydomonas* the M state is formed also rather late within 27 ms after excitation33. We expect that the M state (probably a late M) is the cGMP producing signaling state of CaRhGC, since pronounced structural changes occur during the M1 to M2 transition in other microbial rhodopsins as bacteriorhodopsin34. In addition, the median onset of photocurrents was around 25 ms in neurons expressing any of the *Catenaria* cyclases, setting an upper limit for formation of the active state. Thus, the M state or an earlier state (i.e., L2) must be the active state.

Compared to BeRhGC, the prolonged M state and associated active state of CaRhGC increases the number of molecules in the photocyte at non-saturating light intensities, which accelerates the accumulation of cGMP. This would explain (a) the 5× faster onset of the CaRhGC photocurrents and (b) the increased enzymatic activity of CaRhGC compared to BeRhGC (Table 1). The *V*max of isolated CaGC was also higher than BeGC, contributing to the improved performance of full-length CaRhGC.

Introducing the point mutations E497K/C566D into the two CaRhGCs successfully converted the rhodopsin-guanylyl cyclases into rhodopsin-adenyl cyclases. Unfortunately, BeRhAC had dark activity, as recently reported14 and the construct was poorly expressed and/or membrane-targeted. Additionally, the N-terminal YFP may stabilize the dark state of the full-length protein. The reason for the increased dark activity of RhGC with C-terminal YFP remains unclear but is not caused by the proteolytic separation of the constitutively active GC domain as shown by immunodetection (Supplementary Fig. 14). Even attaching smaller mycHis tags to the C terminus had a negative impact (Supplementary Table 3).

Several other optogenetic tools exist for raising cAMP. In comparison to the soluble bPAC or euPAC, YFP-CaRhAC allows faster control of intracellular cAMP (ms vs seconds time-scale) and being a transmembrane protein, more closely mimics the endogenous transmembrane adenylyl cyclases35. Additionally, it may prove easier to target CaRhAC (and wt RhGCs) to specific cellular compartments, which will enable studying subcellular effects of cAMP (and cGMP)36. While bPAC and CaRhAC are single proteins, the G protein-coupled rhodopsins OptoXRs and JellyOps rely on activation of endogenous G proteins and endogenous transmembrane ACs to raise cAMP17,38. We expect that single component cyclases will less likely activate additional cNMP independent signaling pathways and that they will function in a wider variety of cell types.

In nucleotidylic cyclases, a head-to-tail arrangement of two cyclase subunits is required for catalysis. Surprisingly, the isolated GCs crystalized as monomers or unusual head-to-head dimers32. In contrast, CaAC crystallized as an antiparallel homodimer, which indicates a classical type III cyclase reaction mechanism. According to current knowledge (mostly derived from ACs), the reaction is initiated by substrate-binding to an open conformation of the protein mainly through interactions between the triphosphate and metal ion B19. The transient binding of the second, catalytic metal ion A facilitates the deprotonation of the ribose-3′-OH needed for its nucleophilic attack at Pa. It is assumed, that the base discriminating interactions and substrate specificity is achieved during and not upon substrate-binding. During cyclization the nucleotide must adopt an inline orientation of the attacking ribose-3′-O and the ‘to be broken’ Pa-O bond. This leads to a pentavalent transition state, in which the α-phosphate harbors an additional negative charge stabilized by ion A and the conserved arginine R577 in CaAC. Proper placement of this arginine is thought to be facilitated by protein closure based on the approach of the β7/8 loop and helix α1 towards the dimer center. We believe that CaAC adopts a closed conformation for two reasons: the CaAC helix α1 position resembles α1 in other closed-state AC structures as for example tmAC (1CJK)31 (Supplementary Fig. 12c) and the hydrogen bond pattern of β7/8 to the neighboring helix α3 and α1* match that of closed transmembrane AC. It remains to be answered, if this closure results (a) from a ligand-induced fit and shows the enzyme before catalysis29 or, (b) represents the product/enzyme complex30.

The CaAC binding site is highly similar to other ligand-bound ACs with either both metal sites occupied or a single ion B. Ion A, believed to bind transiently during catalysis, is only present in closed-state structures of tmAC and cyanobacterial AC, in which the non-productive nucleotide conformation stabilizes the metal interaction. Different to most other AC structures, the ribose interacts with the β2* /3* loop (via D501*). Interestingly, this placement brings the 3′-OH close to the position of the ion A-site deduced from the structure of tmAC (Supplementary Fig. 12d). Indeed, density functional calculations (DFT)39 of the active site based on tmAC suggest that the sugar approaches Ion A to allow deprotonation of the attacking 3′-OH. Thus, the CaAC ribose position may be of catalytic relevance.

Electron density for a single conformer, assigned as ATP-Sp-S was found in the CaAC crystal. Since ATP-Sp-S (but not ATP-Rp-S) is catalyzed by other ACs30, finding this epimer in an AC crystal structure is unexpected. In other ACs, stereospecific inhibition by ATP-Rp-S is attributed to a deviation from the inline arrangement of the ribose-3′-O and the ‘to be broken’ Pa-O bond, which prevents catalysis. In CaAC the epimer ATP-Sp-S is an equally potent inhibitor (Supplementary Fig. 13) and the ribose-3′-OH and Pa-O are not arranged inline, similar to other inhibitor bound AC structures. The contribution of the sulfur to the nucleotide conformation remains uncertain.

An open question is how the rhodopsin photon absorption results in cyclase activity. Similar to other rhodopsins, we assume...
a light-induced conformational change, which is transmitted via the coiled-coil domain to the cyclase. Recently the structures of two bacterial light-activated type III adenyl cyclases (OaPAC and bPAC) were solved and the studies addressed how light-induced conformational changes within the BLUF domain (sensors of blue light using flavin adenine dinucleotide) are propagated via the coiled-coil domain to regulate the AC activity. In contrast, Lindner et al. proposed a light-induced conformational change within the BLUF structures. Ohki et al. suggested that intramolecular signal transmission is propagated via the coiled-coil domain to regulate the AC activity. Although we assume by analogy a central role for a light-induced conformational change, which is transmitted via the coiled-coil domain to regulate the AC activity. According to the crystallographic model, this flexible loop directly precedes β4/5, which forms the main protein contacts to the nucleotide base. Thus, movement of the β4/5 loop is expected to directly affect base binding and consequently substrate turnover. Indeed, the two orientations of this loop present in the CaAC structure show slightly different hydrogen bonding to DS66 due to a shift of H564 (Fig. 7c), nevertheless base coordination is unchanged. Additional mutations near position 566 in RhACs-6x decrease both dark- and light-induced enzymatic activity, indicating the importance of this region for enzyme activity. Although our study provides structural insights into the recently discovered class of enzyme rhodopsins, the structure of the full-length protein will be crucial for a detailed understanding of the intramolecular phototransduction mechanism.

**Methods**

**Animal experiments.** All animal experiments were approved by the local authorities.

Berlin: All experiments were conducted in accordance with the guideline given by the Landratsamt für Gesundheit und Soziales Berlin and were approved by this authority.

Würzburg: Xenopus laevis surgery for oocytes in Würzburg was under License #70/14 from Landratsamt Würzburg Veterinaeramt.

Hamburg: Rats were housed and bred at the University Medical Center Hamburg. Animal care and use procedures were conducted according to protocols approved by the Animal Research Ethics Board (AREB) and the Institutional Animal Care and Use committee of the City of Hamburg.

**Molecular biology.** Both rhodopsin-guanylyl cyclase sequence (1–626 residues) from Catenaria anguillulae (Ca) (gb: M9399579) and Blastocladiella emersonii (Be) (gb: ACO77007.1) were ordered from GenScript and the RhAC variants (with 2–6 amino acids mutated) were generated with the help of a quickchange PCR reaction. An overview of the used constructs is found in Supplementary Table 4.

For electrophysiological measurements in Xenopus oocytes, RhGCs and RhACs (Be/ Ca) were subcloned via BamHI and HindIII into pGEM (Promega), (#2, Supplementary Table 4).

For Elisa assays, the N-terminally YFP-tagged versions were cloned from pGEM-YFP, CaRhAC in oocyte recordings into pAAAV-Syn-Rub2-2G-2A-GCAMP6s-WPRE-pA (Addgene plasmid # 50942) via SacI/ HindIII and PstI/HindIII, respectively (primers: 5’-CGAGGCTCATGTCCTGATGAGAAGAGTAAAGA-3’, 5’-TAGTGGAACACGATAC-3’), (#14, Supplementary Table 4).

The untagged variants (CaRhGC and CaRhAC in text) were generated from the pAAV-Syn-Rub2-G2A-CaRhGC/CaRhAC variants into pAAV-Syn-lb-BeRhGC-2A-Dimer by PCR and restriction with EcoRI and Acc65I (primers: 5’-TCGAAATCGGAATATGCTTCGATAGG-3’, 5’-CGCTTCACAGAGAACTGGTATTATAC-3’), (#10-11, Supplementary Table 4).

**YFP-tagged CaRhAC/CaRhGC (mouse codons) were cloned from pGEM-YFP, CaRhAC in oocyte recordings into pAAAV-Syn-YFP-CaRhAC via EcoRI and Acc65I (primers: 5’-TGGTTGAACACGATAC-3’, 5’-CAGTGGAGCTATCAACGGAG-3’), (#12-13, Supplementary Table 4).

Restriction enzymes and Plu DNA Polymerase were acquired from Thermo Fisher Scientific, primers were made at Eurofins, oligonucleotides were synthesized at Talent MN Center’s NucleoSpin® Gel and PCR clean up/NucleoBond® PC 100 kits were used, as well as Thermo Fisher Bioscience’s GeneJET Plasmid Miniprep Kit and Agilent’s QuickChange Site-Directed.

**Split YFP assay in Xenopus oocytes.** The first 425 aa of CaRhGC which covers the opsin and a part of the predicted coiled-coil sequence, was amplified by PCR with primer pair CaRhGC Kp5F (5’-GGGATCCGATATGCATTACG-3’) and CaRhGC 425Xh3R (5’-CGCCATGATATGCATTACG-3’). The PCR product was cloned into the pEG-FYPC vector via EcoRI and Acc65I (primers: 5’-TGGTTGAACACGATAC-3’, 5’-CAGTGGAGCTATCAACGGAG-3’), (#12-13, Supplementary Table 4).

**TEVC in oocytes.** Coding RNA (cRNA) for two electrode voltage clamp (TEVC) measurements was synthesized from linearized DNA (Nhel) according to the manufacturer’s instructions (mMESSAGE mMACHINE, 100 ng/pl, Invitrogen). cRNA of CaRhGC (2.5 ng), together with 5 ng of the cGMP-sensitive CNGA2 channel (rat olfactory neurons (gb: 6978671, NP_037060.1)) or 5 ng of the double mutated (C460W, E583M) version of the CNGA2 channel, sensitive to cAMP, was injected in Xenopus oocytes. After incubation for 3–5 days in Ringer solution (96 mM NaCl, 5 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES and 50 µg/ml gentamycin, pH 7.4) containing 1 µM all-trans retinal. Fluorescence pictures were taken 3 days after injection with confocal microscope (Leica DM6000).

**YFP-tagged CaRhAC/CaRhGC (mouse codons) were cloned from pGEM-YFP, CaRhAC in oocyte recordings via EcoRI and Acc65I (primers: 5’-TGGTTGAACACGATAC-3’, 5’-CAGTGGAGCTATCAACGGAG-3’), (#12-13, Supplementary Table 4).

**cAMP and cGMP ELISA assay from whole oocyte lysates.** Oocytes injected with 30 ng cRNA of CaRhGC/BeRhGC/CaRhAC/BeRhAC variants were incubated in 18 °C for 3 days in Ringer solution supplemented with 1 µM all-trans retinal. Oocytes were either kept in the dark or illuminated for 1–4 min with green light (532 nm, 0.3 mW mm⁻²). Five oocytes injected with the same construct were pooled and homogenized by pipetting in Sample Diluent (containing 0.1 N HCl and pH indicator of the solus). To remove cell debris samples were centrifuged at 12,000 r.p.m. for 6 min at room temperature. cAMP/cGMP within the oocyte was quantified with a Deltec X High Sensitivity Direct Cyclic AMP/GMP Chemiluminescent Immunoassay Kit (Epicentre). Statistical significance between mean cAMP/cGMP concentrations light vs dark were tested with an ANOVA and post-hoc tests between the conditions. All dark were performed using a one-way ANOVA followed by Tukey’s multiple comparison tests.
Xenopus oocyte membrane extraction and in vitro reaction. Oocytes were frozen in liquid N2 after expressing different constructs (30 ng cRNA, CaRhGCs, were provided by a tunable Rainbow OPO/Nd:YAG laser system. Laser energy was

NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-04428-w | www.nature.com/naturecommunications

...in reservoir solution (0.2 M potassium thiocyanate, 1.0 mM sodium cacodylate pH 6.5, 25% w/v PEG 2000 MME) and equilibrated against 70 µM of reservoir solution (Clear strategy I, Molecular Dimensions). For data collection crystals were soaked in reservoir solution, supplemented with 7.5 mM Ca2+, 7.5 mM Mg2+ and 10 mM HEPES on the spectrometer for a linear absorbance measurement at 540 nm. 

Enzymatic activity assays and pH dependence. Activity assays (triplicates) at 22 °C were carried out in 50 mM HEPES, 100 mM NaCl, pH 7.5 (final volume 100 µl) with varying concentrations of NTP (GTP/ATP) and Mg2+ (0.25–12 mM). The assay was started by addition of the respective purified enzyme (0.05 nmol full-length CaRhGC, 0.1 nmol BeRhGC, 2.3 nmol CaAC(E497K/C566D), 1.7 nmol CaAC) and 0.08 nmol BeRhGC (E497K/C566D). Four time points within the linear increase of cNMP were used to determine the initial velocity at a certain substrate concentration: for full-length CaRhGC (30, 60, 120, 180, 240 s), for truncated cyclases CaRhGC, BeRhGC, CaAC: 30, 1 min, 1.5 min, 2 min. The full-length protein was illuminated with green light (522 nm, 0.010 mW mm−2) and the supernatant was filtered through a 0.2 µm chromaf filter (Macherey-Nagel) and 25 µl was applied on a C18 Reversed Phase High Pressure Liquid Chromatography Column (Supelco 100A, 1 mm, 15 cm × 4.6 mm, Sigma Aldrich), equilibrated to 100 mM K2HPO4/KH2PO4, 4 mM tetra-butylammonium iodide, pH 5.9, 10 % methanol at a flow rate of 1.2 ml/min−1. Analyte elution was recorded via absorbance at 260 nm (retention time for cGMP ~7 min, for cAMP ~16 min). cNMP was quantified by peak area in Origin 8.5.3 (Originlab) and compared to peak values of cNMP standards (Sigma Aldrich) of known concentration. For each substrate concentration, cNMP concentrations were blotted against the time and the initial velocities were retrieved with the help of a linear fit. For the full-length constructs, the initial velocities of the dark samples were subtracted from the initial velocities of the illuminated samples. For the truncated substrate and pH dependence, purified full-length CaRhGC/BeRhGC (E497K/C566D) or truncated cyclase CaRhGC/BeRhGC/CaAC (E497K/C566D) protein (4 µl, 55.36 µg) was incubated for 5 min with 0.1 mM ATP, 1.4 mM ATP-Rp-[13C6]-S/ATP-Sp-[12C3]-S/A hydrolysates (Sigma Aldrich) and 800 µM ATP analogs (900 µM). The pH dependence was determined using Mg2+ concentrations as described above. 

To assess the inhibitory potential of ATP analogs, purified CaAC/E497K/ C566D (70 µg) was incubated with 1.4 mM ATP-Rp-aS/ATP-Sp-aS (Biolog) or APCPP (Jena Bioscience) in the presence of 0.5 mM ATP/Mn2+ (Sigma Aldrich) (100 mM NaCl, 50 mM HEPES, pH ~7.5) for 10 min. cAMP amounts were quantified as described before.

Crytallography and structure solution of cyclases. The cyclase domain CaAC (E497K/C566D) was co-crystallized with ATPαS (Jena Bioscience) at 20.0 °C. 2 µl of CaAC/E497K/C566D solution (15.5 mg ml−1) was applied to a 20 µl Tris/HCl pH 8, 50 mM NaCl, 7.5 mM MgCl2 buffer (7.5 mM MgCl2, 10 mM ATPαS) was mixed with 0.5 µl of reservoir solution (0.2 mM potassium thiocyanate, 1.0 mM sodium cacodylate pH 6.5, 25% w/v PEG 2000 MME) and equilibrated against 70 µl of reservoir solution (Clear strategy I, Molecular Dimensions). For data collection crystals were soaked in reservoir solution, supplemented with 7.5 mM Ca2+, 7.5 mM Mg2+ and 10 mM HEPES on the spectrometer for a linear absorbance measurement at 540 nm. Transition spectroscopy was performed on an LKS.60 flash photolysis system (Applied Photophysics Ltd.) at 22 °C. Excitation pulses of 10 ns (at 332 nm) were provided by a tunable Rainbow OPO/InDyAG laser system. Laser energy was adjusted to the sample (spectral resolution of 1.6 nm). Light absorption spectra were measured with a Perkin-Elmer UV-Vis spectrophotometer (The MathWorks).
HIPPOCAMPSAL SLICE CULTURES AND TRANSFECTION. Hippocampal slice cultures were prepared from P7-14 Wistar rat (Janvier) and maintained in slice culture medium without antibiotics as described51. After 10 days to 3 weeks in vitro, hippocampal neurons were transfected by single-cell electroporation52. For the initial experiments with BeRhGC, CaRhGC, BeRhAC and CaRhAC, the person performing the transfection was performed with MATLAB, whereas graphs and curve-fitting were generated with GraphPad Prism 6.0. For the dose-response analysis, the slope was calculated between the 30 and 50% value of the peak response. Time to onset was defined as the intersection of the slope of the initial segment of the response and the start point of the illumination. The time of decay to half response was identified as the time after the stimulus when the response decayed to half of the value before the stimulus was off (sustained response). Error bars represent median and interquartile range if not otherwise stated.

**Data analysis and statistics neuronal electrophysiology.** Analysis of the currents was performed with MATLAB, whereas graphs and curve-fitting were generated with GraphPad Prism 6.0. For the dose-response analysis, the slope was calculated between the 30 and 50% value of the peak response. Time to onset was defined as the intersection of the slope of the initial segment of the response and the start point of the illumination. The time of decay to half response was identified as the time after the stimulus when the response decayed to half of the value before the stimulus was off (sustained response). Error bars represent median and interquartile range if not otherwise stated.

**Data availability.** Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request. The sequence of BeRhGC is available under gb: AI970797.1 (gb:KF313861 or gb:KF309806) (humanized codon-usage)), the sequence of CaRhGC is available under gb: MF393579. The atomic coordinates and diffraction data of the adenylyl cyclase in complex with ATP-Sp-A3 are deposited at the Protein Data Bank, www.pdb.org: PDB ID code SOYH. Plasmids will be available on Addgene.

Received: 18 October 2017 Accepted: 26 April 2018 Published online: 24 May 2018

**References.**

1. Pilis, S. J. & Granner, D. K. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annu. Rev. Physiol. 54*, 885–909 (1992).

2. Pilz, R. B. & Castelo, D. E. Regulation of gene expression by cyclic GMP. *Circ. Res. 93*, 104–106 (2003).

3. Beavo, J. A. & Brunton, L. L. Cyclic nucleotide research—still expanding after half a century. *Nat. Rev. Mol. Cell Biol. 3*, 710–718 (2002).

4. Rainskjaer, K., Madriaju, A. & Montminy, M. In *Handbook of Experimental Pharmacology* Vol 233 (ed. Barrett, J. E.) 29–49 (Springer, Cham, Switzerland, 2013).

5. Chen, S. J. et al. Cyclic nucleotide compartmentalization: contributions of phosphodiesterases and ATP-binding cassette transporters. *Annu. Rev. Pharmacol. Toxicol. 53*, 231–253 (2013).

6. Sterl, M. et al. Light modulation of cellular cAMP by a small bacterial photoactivated adenylyl cyclase, bPAC, of the soil bacterium *Beggiaota*. *J. Biol. Chem. 286*, 1181–1188 (2011).

7. Jansen, V. et al. Controlling fertilization and CAM signaling in sperm by optogenetics. *eLife 4*, e05161 (2015).

8. Steuer Costa, W., Yu, S., Liewald, J. F. & Gottschalk, A. Fast cAMP modulation by a cGMP-controlled cAMP phosphodiesterase. *Curr. Biol. 27*, 495–507 (2017).

9. Iseki, M. et al. A blue-light-activated adenylyl cyclase mediates light avoidance in *Euglena gracilis*. *Nature 415*, 1047–1051 (2002).

10. Ryu, M.-H., Moskvin, O. V., Siliberg-Liberles, J. & Gomelsky, M. Natural and engineered photoactivated nucleotidyl cyclases for optogenetic applications. *J. Biol. Chem. 285*, 41501–41508 (2010).

11. Avelar, G. M. et al. A rhodopsin-guanylyl cyclase gene fusion functions in light-coupled action in a fungus. *Cell 124*, 1234–1246 (2011).

12. Gao, S. et al. Optogenetic manipulation of cGMP in cells and animals by the tightly regulated guanylyl-cyclase opsin CyChOp. *Nat. Commun. 6*, 8046 (2015).

13. Scheib, U. et al. The rhodopsin-guanylyl cyclase of the aquatic fungus *Blastocladiella emersonii* enables fast optical control of cGMP signaling. *Sci. Rep. 6*, 19612 (2016).

14. Trieu, M. M. et al. Expression, purification, and spectral tuning of RhoGC, a retinylidene/guanylyl cyclase fusion protein and optogenetic tools from the aquatic fungus *Blastocladiella emersonii*. *J. Biol. Chem. 292*, 10379–10389 (2017).

15. Penzkofer, A., Scheib, U., Hegemann, P. & Stehfest, K. Absorption and emission spectroscopic investigation of thermal dynamics and photodynamics of the rhodopsin domain of the rhodopsin–guanylyl cyclase from the aquatic fungus *Blastocladiella emersonii*. *BAOJ Phys. 2*, 006 (2016).

16. Rich, T. C., Tse, T. E., Rohan, J. G., Schaack, J. & Karpen, J. W. In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. *J. Gen. Physiol. 118*, 63–78 (2001).

17. Penzkofer, A., Scheib, U., Stehfest, K. & Hegemann, P. Absorption and emission spectroscopic investigation of thermal dynamics and photodynamics of the rhodopsin domain of the rhodopsin-guanylyl cyclase from the nematophagous fungus *Catenaria anguillulae*. *Int. J. Mol. Sci. 18*, 2099 (2017).

18. Linder, J. U. Substrate selection by class III adenyl cyclases and guanylyl cyclases. *J. Bioenerg. Biomembr. 57*, 797–803 (2005).

19. Steegborn, C. Structure, mechanism, and regulation of soluble adenyl cyclases—similarities and differences to transmembrane adenyl cyclases. *Biochim. Biophys. Acta 1842*, 2535–2547 (2014).

20. Sunahara, K. R. et al. Exchange of substrate and inhibitor specificities between adenyl and guanylyl cyclases. *J. Biol. Chem. 278*, 16322–16338 (1998).

21. Santoro, B. et al. Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. *J. Neurosci. 20*, 5264–5275 (2000).

22. Kumar, R. P. et al. Structure and monomer/dimer equilibrium for the guanylyl cyclase domain of the optogenetics protein RhoGC. *J. Biol. Chem. 292*, 21578–21589 (2017).

23. Sinha, S. C. & Sprang, S. R. Structures, mechanism, regulation and evolution of class III nucleotidyl cyclases. *Rev. Physiol. Biochem. Pharmacol. 157*, 105–140 (2006).

24. Rauch, A., Leipelt, M., Russwurm, M. & Steegborn, C. Crystal structure of the guanylyl cyclase CyChC, *Nat. Acad. Sci. USA 105*, 15720–15725 (2008).

25. Winger, J. A., Derbyshire, E. R., Lamers, M. H., Marletta, M. A. & Kuriyan, J. The crystal structure of the catalytic domain of a eukaryotic guanylate cyclase. *BMC Struct. Biol. 8*, 42 (2008).

26. Allerton, C. K., von Delft, F. & Gileadi, O. Crystal structures of the catalytic domains of eukaryotic guanylate cyclases. *Biochim. Biophys. Acta 1633*, 1621–1628 (1993).

27. Zhang, G., Liu, Y., Ruoho, A. E. & Hurley, J. H. Structure of the adenylyl cyclase catalytic core. *Nature 386*, 247–253 (1997).

28. Gerli, J. A., Coderre, J. A. & Wodin, M. S. Mechanism of the adenylyl cyclase reaction. Stereochemistry of the reaction catalyzed by the enzyme from *Brevibacterium lactofaciens*. *J. Biol. Chem. 255*, 331–334 (1980).

29. Chemer, J. J., Sunahara, R. K., Gilman, A. G. & Sprang, S. R. Crystal structure of the catalytic domains of adenyl cyclase in complex with GTPgS. *Science 278*, 1907–1916 (1997).
Acknowledgements

We wish to thank Mala Reh, Christina Schnick, Melanie Meiworm, Jan-Phillip Kehl, Iris Ohmert, Sabine Graf, and the Beamline staff for excellent technical assistance; Thomas Oertner for support and discussions; and Robert Lindner (Heidelberg) for constructive criticism on the manuscript. C.E.G. received funding from the Landesforschungsförderung Hamburg and the DFG (FOR2419, SPP1665). O.M.C. was supported by a DAAD scholarship. G.N. was supported by grants from the DFG (SFB 1047/A03, TRR 166/A03). G.N. and P.H. acknowledge support from the Louis-Jeantet-Foundation. P.H. received funding from the DFG (SFB1078) and from ERC (MERA). P. H. is Hertie Professor for neuroscience, supported by the Hertie Foundation.

Author contributions

U.S.: photocurrents in oocytes, Elisas for BeRhGC/AC CarRhGC/AC mutations, protein expression, kinetic studies, crystallization of GC/ACs of Be and Ca, data collection at the synchrotron, analysis of the structure, and writing of the manuscript. M.B.: established crystallization, support of crystallization, data collection and resolving, and analysis of the structure, and contribution to writing of structural results and discussion. O.M.C.: photocurrents and 2-photon microscopy in neurons data collection and analysis, and participated in writing the ms. S.Y. and S.G.: cAMP and cGMP measurements of cyclase activity in whole oocytes and in oocyte membranes (in vitro assays), split GFP, mutations to AC and to reduce dark activity, kinetic studies. S.M.: support of kinetic studies. K.S.: protein expression and photocycle kinetics, and writing contribution to the spectroscopic part. G.N.: coordinator of mutagenesis and cAMP/cGMP measurements in oocyte membranes. C.E.G.: supervision of all neuronal experiments, project coordination with P.H., and substantial contribution to manuscript writing. P.H.: Project coordinator, supervision of protein expression, crystallization and biochemistry, writing of the manuscript with U.S. and C.E.G. with support from several others authors who read and approved the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-04428-w.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.