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SUPPLEMENTARY INFORMATION

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Structural mechanisms of selectivity and gating in anion channelrhodopsins

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Supplementary Discussion

Distinct photocycles in iC++ and *Gt*ACR1

Both iC++ and *Gt*ACR1 work as light-gated anion channels, but their photocycles are remarkably different. While *Gt*ACR1 has 5 intermediate states (K, L, M, N, and O), iC++ has only three photo-distinguishable intermediate states (K, M and O), and the lifetime of the K intermediate is unusually long. The K-intermediate state is generally characterized by the 13-*cis* retinal twisted around the C14-C15 bond. The twisted retinal becomes planar during the transition form K- to L- intermediate state, and the Schiff base proton is transferred from the Schiff base nitrogen to the proton acceptor during the transition from L- to M- intermediate state. Since iC++ has an unusually long K-intermediate state and does not exhibit the L-intermediate, some of the mutations introduced to engineer iC++ (i.e. T98S, E129Q, E162S, and/or N297Q) may change the local environment around the Schiff base and inhibit the efficient relaxation of the twist around the C14-C15 bond of 13-*cis* retinal. The proton would be directly transferred from the Schiff base nitrogen of twisted retinal to the proton acceptor (probably Asp292), and thereby iC++ could skip the L-intermediate state.

Ion selectivity in ACRs

This study supports a framework in which both nACR and dACR follow the overall surface-electrostatic model for pore selectivity, in which the contribution of each individual amino acid position to anion selectivity can vary between ACRs. In iC++, amino acids at the CCS (Q129 and Q297) position are particularly important for ion selectivity, and as shown in Fig. 3e, the Q129E/Q297E double mutation depolarizes V_{rev} by ~40 mV. An additional 8 mutations (T98S, E122N, E140S, V156R, E162S, V281R, T285N, and E312S) mainly introduced inside the ion-conducting pathway work cooperatively, creating an electropositive surface suitable for anion selectivity, and thus further hyperpolarize V_{rev} by 20 mV. This pattern also holds for another dACR (iChloC, in which the E-to-R mutation to E90 (Glu-129 in iC++) decreases V_{rev} from ~+10 to ~-40 mV[4]). An additional four mutations, E83Q, E101S, D156N, and T159C (E122Q, E140S, D195N, and T198C in iC++ numbering, respectively), of which the first two are introduced inside the ion-conducting pathway, further hyperpolarize V_{rev} to ~-65 mV[5]. The mutational tests for positions inside the nACR ion-conducting pathway tend to show more

variable contribution to anion selectivity; while Gln-46, another residue comprising the CCS in *Gt*ACR1, does contribute to anion selectivity (lowering V_{rev} by ~15 mV), the E-to-Q mutation of Glu-68 (Glu-129 in iC++) does not significantly change V_{rev} (Fig. 3e; this mutation however would be predicted to give rise to a more mild electrostatic effect than the E-to-R mutation in iChloc noted above), and positively-charged residues positioned at the nACR ion-conducting pathway vestibules appear important for tuning the electropositive surface suitable for anion conduction. Therefore, although both dACR and nACR follow the pore surface-electrostatic model, thus far the tested residues contributing most to anion selectivity in dACRs have been found positioned at the CCS or inside the ion-conducting pathway, and the tested residues found to contribute more to anion selectivity in nACRs have been positioned near the vestibules of the ion-conducting pathway. Structures for additional ChRs will be informative regarding possible evolutionary contributions to this pattern and to chloride/cation selectivity in general, and already our demonstrated ability to interconvert ChR ion selectivity (CCR→ACR and now ACR→CCR), guided by our crystal structures and structure-informed electrostatic model, may help in the screening of ChR sequences for informative new variants in nature.

Relationship between distribution of charged residues and photocurrent amplitude

Both iC++ and *Gt*ACR1 have several positively charged residues that govern exclusive anion selectivity, but their distributions are completely different, consistent with the overall surface-electrostatic model and not with a model involving singular high-affinity binding sites. These different distributions (in the setting of similar ion selectivity) not only support the selectivity model but also may be relevant to explaining differences in photocurrent amplitude, a distinct ChR property in which iC++ and GtACR1 differ markedly. Except for the Schiff base lysine, iC++ has four positively charged residues along the pore (Lys-132, Arg-156, Arg-159, and Arg-281), but only one of these is conserved in *Gt*ACR1, and Lys-132, Arg-156, and Arg-281 are replaced by Thr-71, Pro-91, and Glu-223, respectively. Instead, *Gt*ACR1 has 12 positively charged residues at and near the pore vestibules, which create an electropositive surface suitable for anion selectivity. Because of its lysine and arginine residues inside the ion-conducting pathway, the surface of pore in iC++ is predicted to be more “sticky” for substrate anions. As proposed for potassium and calcium channels previously[48,49], ions tightly binding to the inner surface of the pore will exhibit lower mobility. Therefore the lower conductance of iC++ relative

to *Gt*ACR1 may be understood from the structures and a sticky-pore model, and it will be of interest to see if this pattern can be extended to CCRs.