Supplementary information

Specific residues in the cytoplasmic domain modulate photocurrent kinetics of channelrhodopsin from *Klebsormidium nitens*

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BR           1   -----------------------------------------------------QAQITGR   7
CrChR2       1   --MDYGGALSAVGRELLFVTNPVVVNG-SVLVPEDQCYCAGWIESRGT---NGAQTASNV   54
PsChR        1   ---------------MGFQLNPEYLNE-TILLDD----CTPIYLNVGP---LWEQKVARG   37
KnChR        1   MTVDAHSTVDA--------HSTVTDSH-SAGNGTESCYVADFLGMHHESHEGALYSVYKS   51
GtCCR4       1   ----------------------MTTSAPSLSDPNWQYGMGGWNNPRLPNFNL-HDPTVIG   37

BR           8   PEWIWLALGTALMGLGTLYFLVKGMGVSDPDAKKFYAITTLVP-------AIAFTMYLSM   60
CrChR2      55   LQWLAAG-----FSILLLMFYAYQTWKSTCGWEEIYVCAIEMV-------KVILEFFFEF   102
PsChR       38   TQWFGVI-----LSLAFLIYYIWITYKATCGWEELYVCTIEFC-------KIVIELYFEF   85
KnChR       52   LEWGCFL-----ISIGLFVFYLQQYRKKTAGWEVIYIAFIESF-------KYIFEIFWPH   99
GtCCR4      38   VDWLGFL---CLLGASLALMYKLMSFKGPDGDQEFFVGYREEKCLSIYVNLIAAITYWGR   94
                  :*         :.                .  : ::                    :

BR          61   LLGYGLTMVPFGGEQNPIYWARYADWLFTTPLLLLDLALLVDADQG---TILALVGADGI   117
CrChR2     103   ---KNPSMLYL-ATGHRVQWLRYAEWLLTCPVILIHLSNLTGLSNDYSRRTMGLLVSDIG   158
PsChR       86   ---SPPAMIYQ-TNGEVTPWLRYAEWLLTCPVILIHLSNITGLNDDYSGRTMSLITSDLG   141
KnChR      100   ---NNPAQLNIYGVNKSVPWVRYMEWMITCPVILMALSNISGEEGEYTHRSMQLLATDQG   156
GtCCR4      95   IC---AHFNNDMGLSLSVNYFKYLDYIFTCPILTLDLLWSLNLPYKITYSLFV-----GL   146
                                    : :* ::::* *:: : *    .         :

BR         118   MIGTGLVGALTKVYSYRFVWWAISTAAMLYILYVLFFGFTSKAESM--RP--EVASTFKV   173
CrChR2     159   TIVWGATSAM----ATGYVKVIFFCLGLCYGANTFFHAAKAYIEGYHTVPKGRCRQVVTG   214
PsChR      142   GICMAVTSAL----SKGWLKWLFFVIGCCYGASTFYHAALIYIESYYTMPHGVCKNMVLA   197
KnChR      157   AILCAITAAA----SEGAISAVFYAIGVCYGICTFYFCLQIYIEAYFTLPE-TCHSAVKW   211
GtCCR4     147   TIACGVFCNAF-EPPARYLWFMFGCFIFAFTWISIIRLVYARFQQFLNEDAKKIRAPLKL   205
                  *  .             :   :      :    :        :             .

BR         174   LRNVTVVLWSAYPVVWLIGSEGAGIVPLNIETLLFMVLDVSAKVGFGLILLRSRAIFGEA   233
CrChR2     215   MAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLMSKNCWGLLGHYLRVLIHEH   274
PsChR      198   MAAVFFTSWFMFPGLFLAGPEGTNALSWAGSTIGHTVADLLSKNAWGMIGHFLRLEIHKH   257
KnChR      212   MAVIFYAGWLCYPCFFLAGSEGWGNLSYEGSAIGHCIADLLSKNAWGVMHWWIRCQLEEY   271
GtCCR4     206   SLTLYFSIWCGYPALWLLTE--FGAISQLAAHVMTVIMDVAAKSVYGFALLKFQLGVDKR   263
                    :    *  :* .::      . :      :   : *: :*  :*.     :  . :

BR         234   EAP--------------EP----SAGDGAAATSD--------------------------   249
CrChR2     275   ILIHG--------D----------------------------------------------   280
PsChR      258   IIIHG--------DVRRPI----TVNT---------------LGREVTVSCFVDKE-EED   289
KnChR      272   KHTHN--------GQLPHY----SLETRAKMRAEAGHIIAGSLGSVLHVAGHQH------   313
GtCCR4     264   DVWLDELKSVRYRDVVPQIRPSKTRESR-MEYSEDGDFMRPSKGKRAEGDYMNPRWDHHD   322

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      290   EDERISTKTYANRASFMKMRNDMEQRGIQTRKS-LE----MLAPPPALNDGSIV-LA---   340
KnChR      314   --------------HHFHFDEDSGMQGAQIQGQPLAQANERANLKASLLNGNAVNYNQAV   359
GtCCR4     323   DGRRLPDS--------REMDEQVHEKDQEIS-STMKQIADLNKQLSAMQESEAV------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      341   VADPMTLTFFTQQLSQ-LDATIRATPAMGQGQLEQVLEKGGFDGVLVSPEYIQQVGLVQ-   398
KnChR      360   QAQPQAFQIQSGEDGYVISMNQGESSASGSRRTTQNIEGP--KKDPVASAFSKALGSMKS   417
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      399   RLKDKYHMPVYAFGWGKSSPWRSVIEGSGVDGWLEGPYFGSTFDTDALSDAIAEMQRIKT   458
KnChR      418   KVTEHFTKDLPPS-ARPDSPSRRGSARGGADAPTAQPQQSMKIDLRD----LANNPQLQQ   472
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      459   SYSMVVNGVGMNGAGMN----GVGMN-----GMGMDGVGMNGAGMNGVGMNGMGGYGSVG   509
KnChR      473   QLSMMLKGQMAGGYALKTDLKDLANNPQLQQQLSMMLKGQMGAGNSNTN------QGSNN   526
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      510   NNMHSMPMMSQ---QAVM-MPQSA--PQMMGGMSQTQQPAMMGGMQGASPHYSVGNLQNM   563
KnChR      527   GSPRARPVSEELAQPARLQKAQSARATSNLDDEIEEQAPAQMSRRPSA----RVPRAT--   580
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      564   EGQQAVGSPQVLASSWQQSALHGGMGQQQQYGQVQQMPMMVGMQTPAS---PGGVQTPPH   620
KnChR      581   ---AAADEEQAPARTLSRKISRGDGGEPVSEERLQKRPSYRAAKAAAEAADEDMTDAPAP   637
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      621   TMAGQPQMSPQQLQQQLYFMQQLQQRQLQQ------------------------------   650
KnChR      638   ALARRPSARAPAADADE-ETAPVRTASLRRAGSKSPGNSLPSSPSRRVSRANSLRAPVSA   696
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      651   ----QQYQGGTGQR----------------------------------------------   660
KnChR      697   SVLLCDADGDMGDFFLDQFAAVPNSKCSVKVVATPDELIDALATGRAYDFVMVPKGVITS   756
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ------------------------------------------------------------   249
CrChR2     281   ------------------------------------------------------------   280
PsChR      661   ------------------------------------------------------------   660
KnChR      757   DPEISEEIRAHAQTPLVAFGPALEGMAVAEVKAHMFELDVDDYIGLPKRGQEYPVEDLEA   816
GtCCR4     368   ------------------------------------------------------------   367

BR         250   ---------------                                               249
CrChR2     281   ---------------                                               280
PsChR      661   ---------------                                               660
KnChR      817   LVWKYKKVADDSNKD                                               831
GtCCR4     368   ---------------                                               367
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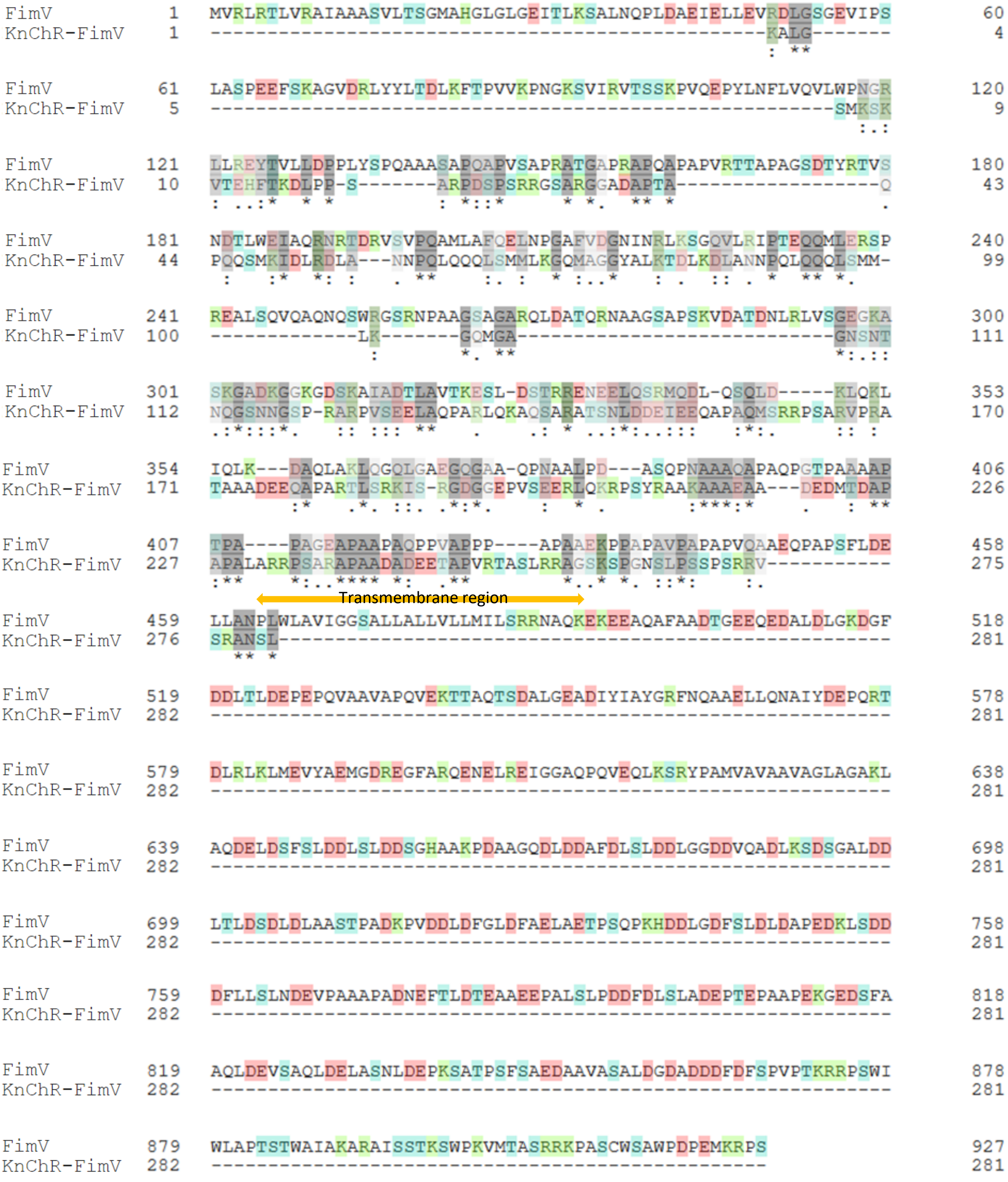
Supplementary Figure 1. Full amino acid alignments of bacteriorhodopsin (BR), CrChR2, KnChR and GtCCR4
The transmembrane region was shown based on the membrane topology of CrChR2. Putative FimV domain in KnChR was indicated.



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FimV             1    MVRLRTLVRAIAAASVLTSGMAHGLGLGEITLKSALNQPLDAEIELLEVRDLGSGEVIPS    60
KnChR-FimV       1    -------------------------------------------------KALG-------    4
                                                                       : **

FimV            61    LASPEEFSKAGVDRLYYLTDLKFTPVVKPNGKSVIRVTSSKPVQEPYLNFLVQVLWPNGR    120
KnChR-FimV       5    -------------------------------------------------------SMKSK    9
                                                                               :.:

FimV           121    LLREYTVLLDPPLYSPQAAASAPQAPVSAPRATGAPRAPQAPAPVRTTAPAGSDTYRTVS    180
KnChR-FimV      10    VTEHFTKDLPP-S-------ARPDSPSRRGSARGGADAPTA------------------Q    43
                      : ..:*  * *         : *::*     * *.  ** *                  .

FimV           181    NDTLWEIAQRNRTDRVSVPQAMLAFQELNPGAFVDGNINRLKSGQVLRIPTEQQMLERSP    240
KnChR-FimV      44    PQQSMKIDLRDLA---NNPQLQQQLSMMLKGQMAGGYALKTDLKDLANNPQLQQQLSMM-    99
                       :   :*  *: :   . **    :. :  * :..*   : .  :: . *  ** *.

FimV           241    REALSQVQAQNQSWRGSRNPAAGSAGARQLDATQRNAAGSAPSKVDATDNLRLVSGEGKA    300
KnChR-FimV     100    -------------LK-------GQMGA----------------------------GNSNT    111
                                   :       *. **                            *:.::

FimV           301    SKGADKGGKGDSKAIADTLAVTKESL-DSTRRENEELQSRMQDL-QSQLD-----KLQKL    353
KnChR-FimV     112    NQGSNNGSP-RARPVSEELAQPARLQKAQSARATSNLDDEIEEQAPAQMSRRPSARVPRA    170
                      .:*:::*.   :: ::: **   .    .: * ..:*:..:::   :*:.     :: :

FimV           354    IQLK---DAQLAKLQGQLGAEGQGAA-QPNAALPD---ASQPNAAAQAPAQPGTPAAAAP    406
KnChR-FimV     171    TAAADEEQAPARTLSRKIS-RGDGGEPVSEERLQKRPSYRAAKAAAEAA---DEDMTDAP    226
                             :*   .*. ::. .*:*.    :  * .       :***:*    .   : **

FimV           407    TPA----PAGEAPAAPAQPPVAPPP----APAAEKPPAPAVPAPAPVQAAEQPAPSFLDE    458
KnChR-FimV     227    APALARRPSARAPAADADEETAPVRTASLRRAGSKSPGNSLPSSPSRRV-----------    275
                      :**    *:..**** *:  .**        *..* *. ::*:    :.
                                 Transmembrane region
FimV           459    LLANPLWLAVIGGSALLALLVLLMILSRRNAQKEKEEAQAFAADTGEEQEDALDLGKDGF    518
KnChR-FimV     276    SRANSL------------------------------------------------------    281
                        ** *

FimV           519    DDLTLDEPEPQVAAVAPQVEKTTAQTSDALGEADIYIAYGRFNQAAELLQNAIYDEPQRT    578
KnChR-FimV     282    ------------------------------------------------------------    281

FimV           579    DLRLKLMEVYAEMGDREGFARQENELREIGGAQPQVEQLKSRYPAMVAVAAVAGLAGAKL    638
KnChR-FimV     282    ------------------------------------------------------------    281

FimV           639    AQDELDSFSLDDLSLDDSGHAAKPDAAGQDLDDAFDLSLDDLGGDDVQADLKSDSGALDD    698
KnChR-FimV     282    ------------------------------------------------------------    281

FimV           699    LTLDSDLDLAASTPADKPVDDLDFGLDFAELAETPSQPKHDDLGDFSLDLDAPEDKLSDD    758
KnChR-FimV     282    ------------------------------------------------------------    281

FimV           759    DFLLSLNDEVPAAAPADNEFTLDTEAAEEPALSLPDDFDLSLADEPTEPAAPEKGEDSFA    818
KnChR-FimV     282    ------------------------------------------------------------    281

FimV           819    AQLDEVSAQLDELASNLDEPKSATPSFSAEDAAVASALDGDADDDFDFSPVPTKRRPSWI    878
KnChR-FimV     282    ------------------------------------------------------------    281

FimV           879    WLAPTSTWAIAKARAISSTKSWPKVMTASRRKPASCWSAWPDPEMKRPS    927
KnChR-FimV     282    -------------------------------------------------    281
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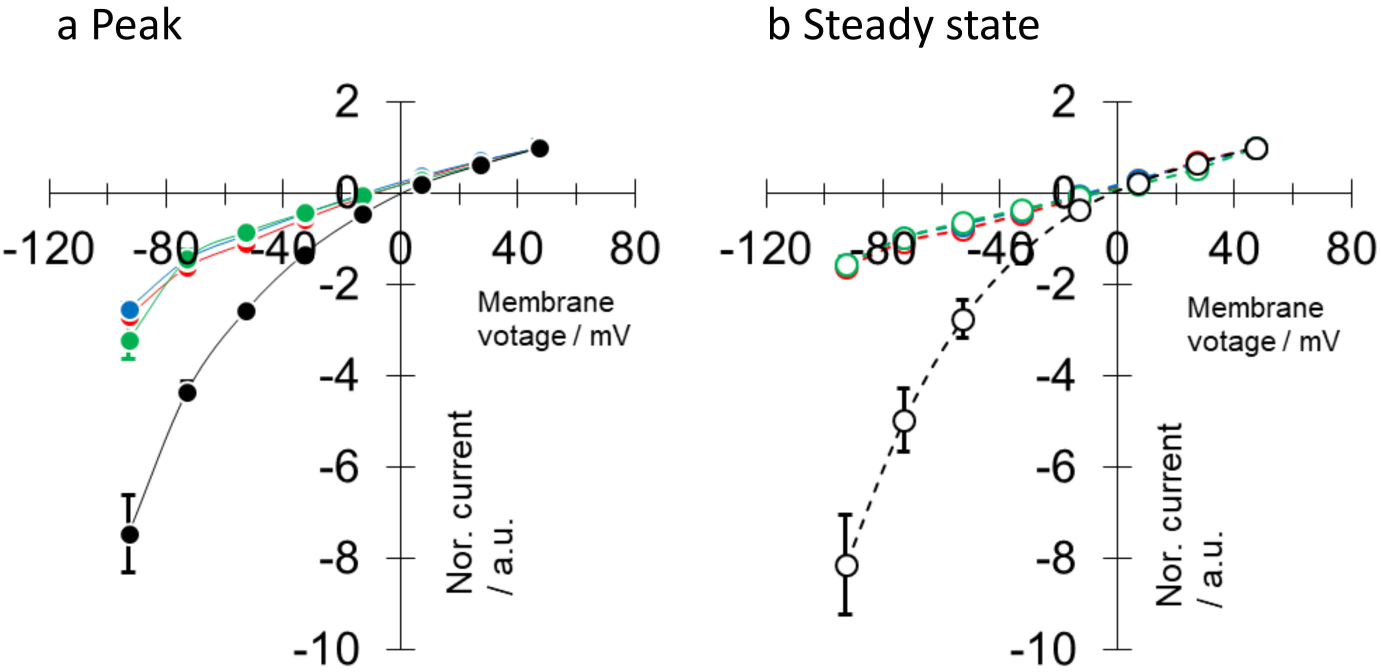
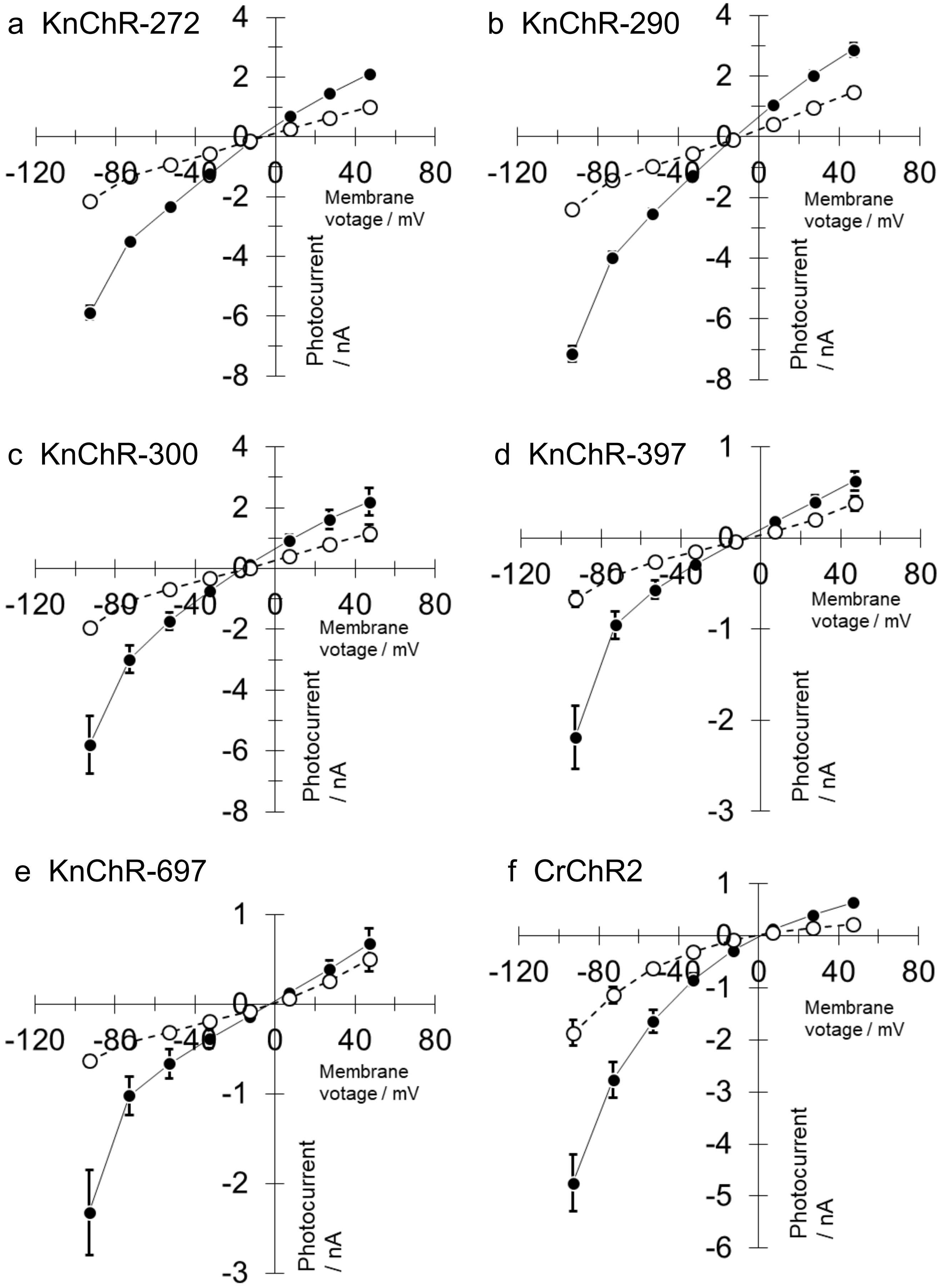
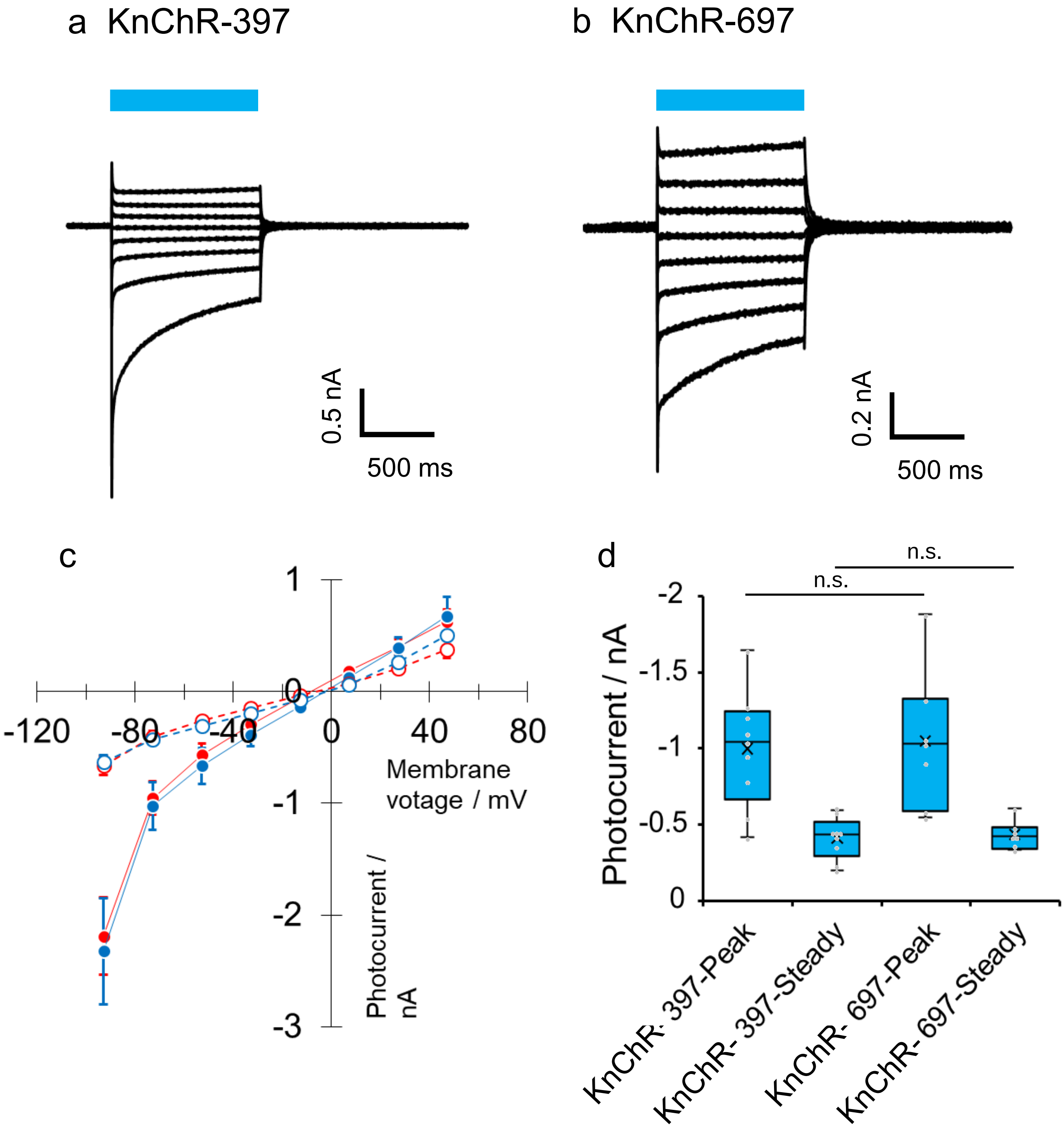
Supplementary Figure 2. Full amino acid alignments of FimV from *Pseudomonas aeruginosa* and KnChR Transmembrane region is indicated.



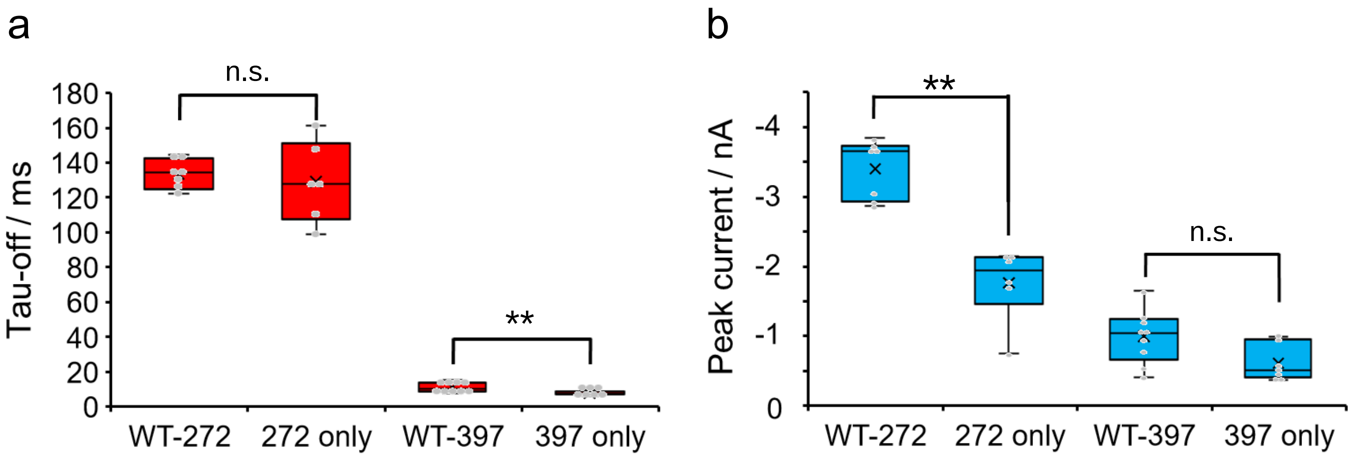
Supplementary Figure 3. I-V relationship of KnChR and CrChR2.
Photocurrent amplitude form each variant and CrChR2 is normalized (at +50 mV as 1.0). Filled (a) and empty (b) symbols indicate peak (I_p) and steady-current (I_s), respectively. Red: KnChR-272 (*N*=7), Blue: KnChR-290 (*N*=7), Green: KnChR-397 (*N*=9), Black: CrChR2 (*N*=6). Standard solutions were used (See *Materials and Methods*). Data were presented as the mean ± SEM.



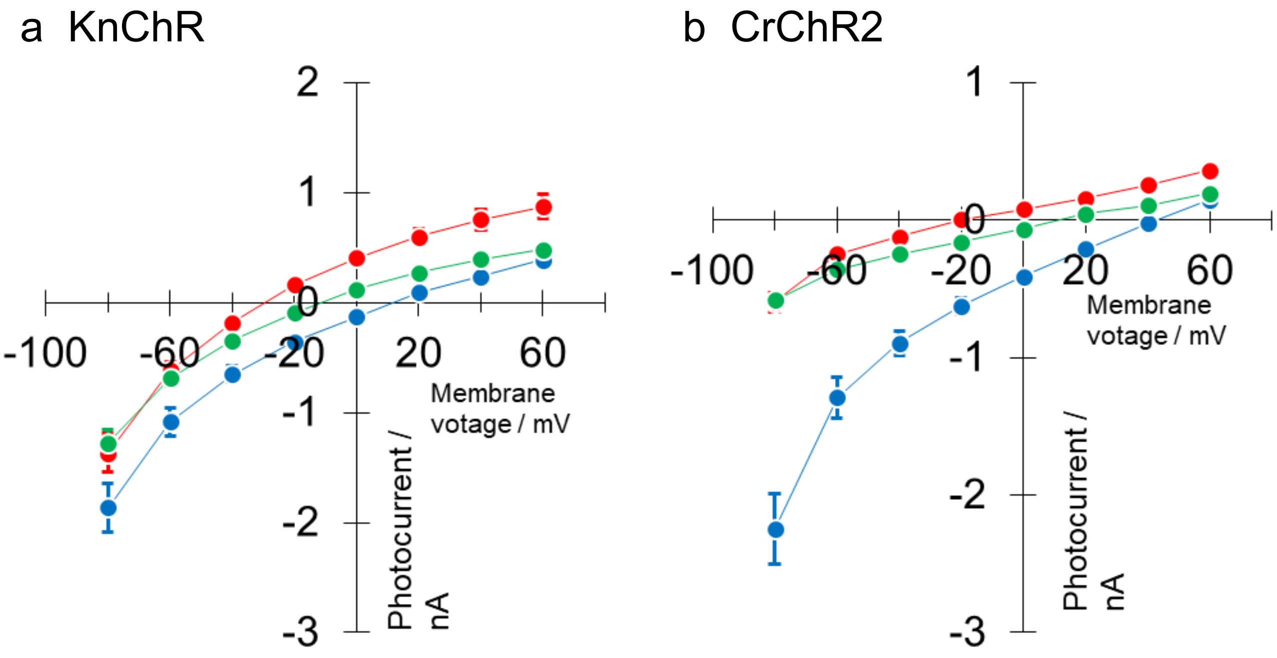
Supplementary Figure 4. I-V relationship of C-terminus length variants of KnChR and CrChR2. Amino acid length of KnChR variants is indicated on each panel (**a-e**). **f.** CrChR2. Filled circle, a peak component of the photocurrent (I_p). Empty circle, a steady-state component of the photocurrent (I_s). Standard solutions were used (See *Materials and Methods*). Data were presented as the mean ± SEM with $N = 6$ (panel c and f), 7 (panels a, b and e), 9 (panel d).



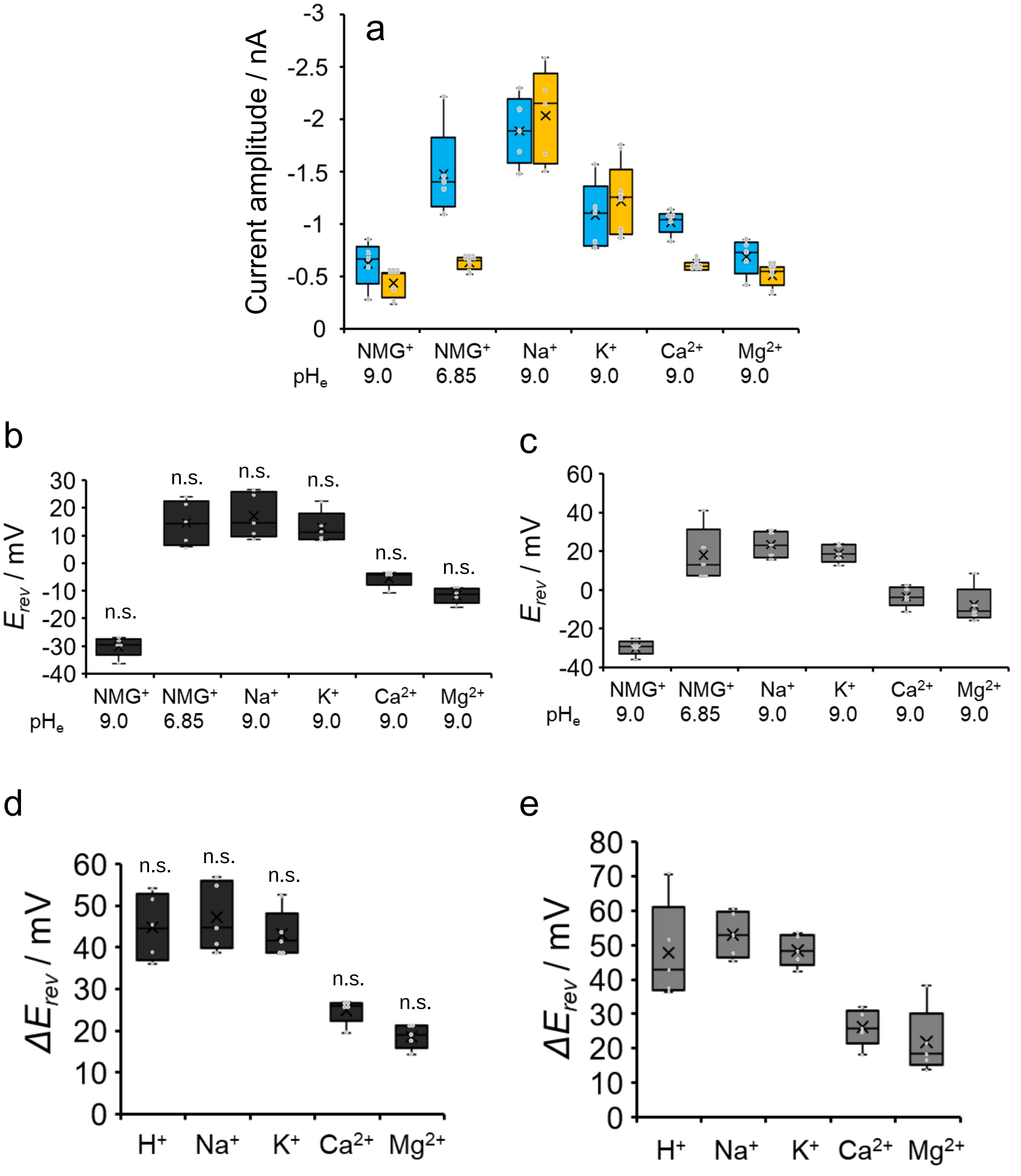
Supplementary Figure 5. Comparison of the length variants KnChR-397 and KnChR-697. Typical photocurrent traces of KnChR-397 amino acids (**a**) and KnChR-697 amino acids (**b**). Blue bars indicate light application (480 nm, 12.3 mW/mm^2). Membrane voltage was clamped from at -90 to +50 mV by 20 mV steps. Standard solutions were used (See *Materials and Methods*). **c**, I-V relationship of KnChR-397 (red) and KnChR-697 (blue). Peak component (filled circle) and steady-state component (empty circle) of the photocurrent were depicted. **d**, Photocurrent amplitudes at -70 mV were shown. Data were presented as the mean ± SEM with N=9 (KnChR-397), and 7 (KnChR-697).



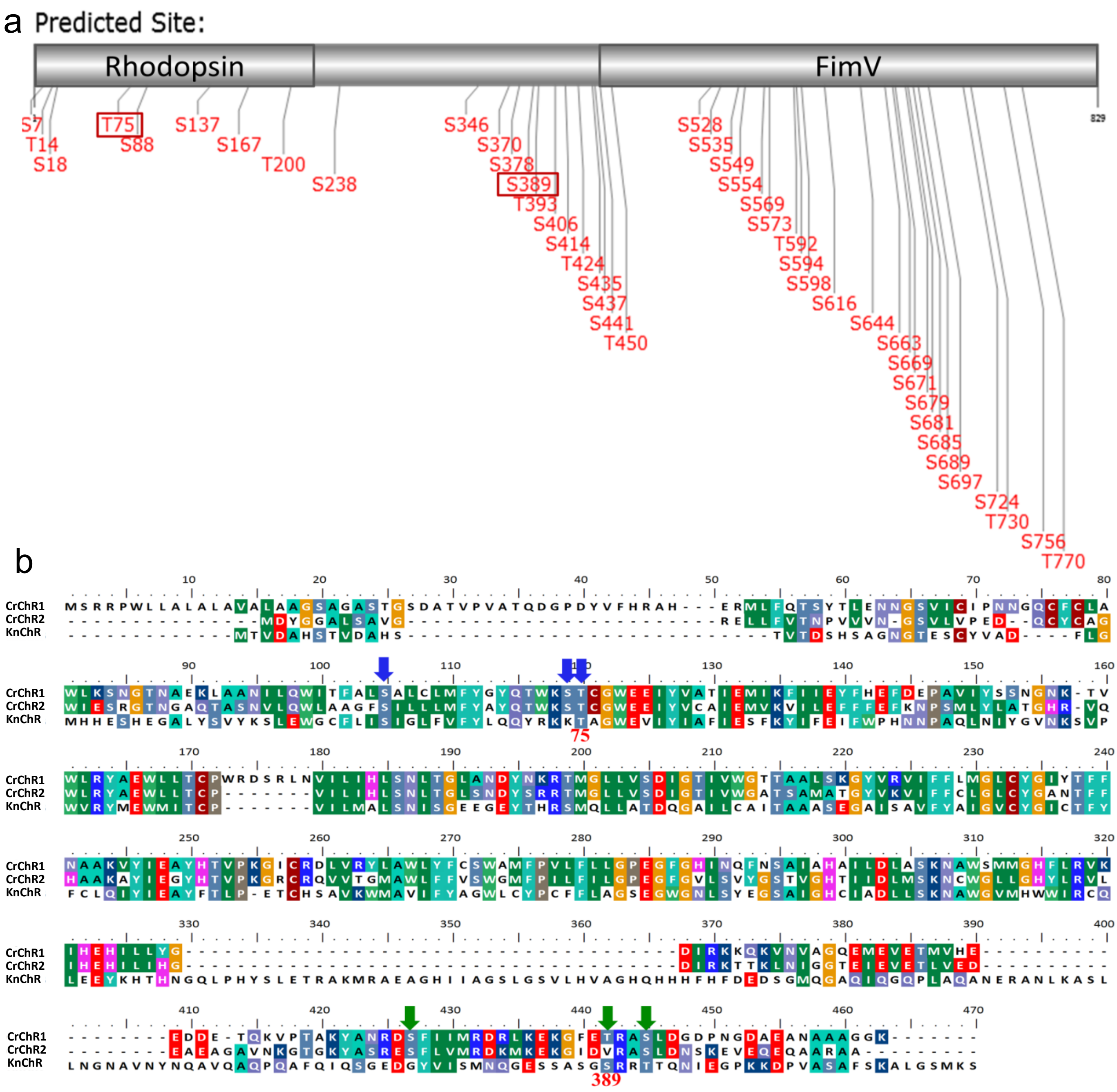
Supplementary Figure 6. eYFP does not alter the channel kinetics of KnChR. Photocurrent amplitudes at -70 mV were analyzed. Time constants (**a**) and peak current amplitude (**b**) of KnChR-272 amino acids and -397 amino acids variants with or without eYFP. Standard solutions were used. Data were presented as the mean ± SEM. *N* =7 (WT-272), 6 (272 only), 9 (WT-397), and 7 (397 only).



Supplementary Figure 7. I-V relationship of KnChR (a) and CrChR2 (b) in various bath solutions. Red: NMG pH9.0, Blue: K$^+$ pH9.0, Green: Mg^{2+} pH9.0. See Supplementary Table 4 for more details of solutions. Data were presented as the mean ± SEM. N=5 (all the conditions for KnChR). N=8 (CrChR2 in NMG pH 9.0), 5 (CrChR2 in K$^+$ pH 9.0), and 6 (CrChR2 in Mg^{2+} pH 9.0).



Supplementary Figure 8. Ion selectivity of KnChR. Peak and steady-state component of photocurrents were analyzed. **a**, Photocurrent amplitude at -60 mV under various ionic conditions. blue, peak component. orange, steady-state component. Reversal potential (E_{rev}) of peak component (**b**) and steady-state component (**c**). Shift of E_{rev}, ΔE_{rev} analyzed from peak component (**d**) and from steady-state component (**e**). The Mann-Whitney *U* test was performed between b and c, and d and e. No significant difference was observed between b and c, and d and e respectively. Data were presented as the mean ± SEM with $N = 5$.



Supplementary Figure 9. Prediction of phosphorylation site in KnChR (**a**) Prediction of kinase-specific phosphorylation site by GPS 5.0 database. T75 and S389 highlighted in red box correspond to Ser102 and Thr373 in CrChR1 phosphorylation site proposed by Böhm et al. (2019) and Wagner et al. (2008), respectively. (**b**) Proposed phosphorylation sites in CrChR1; Böhm et al. (2019) suggested multiple phosphorylation sites, one of them (Ser102) being in the first transmembrane domain (TM) and two (Ser 116 and Thr117) in the intracellular vestibule between (TM) domains marked by a blue arrow. One of them (Thr117) was conserved in KnChR (T75 marked by a red number below the sequence). Wagner et al. (2008) proposed three phosphorylation sites (Ser358, Thr373 and Ser376) in CrChR1 and one in CrChR2 (Ser358) marked by a green arrow. Out of three phosphorylation sites, one is common among CrChR1 and CrChR2 (RD/ESpF) but replaced by Gly in KnChR. Ser389 corresponding to Thr373 is marked by a red number (389) below the sequence.

Supplementary Table 1 Solutions for the ion selectivity measurement

		NaCl	KCl	$CaCl_2$	$MgCl_2$	NMG	EGTA	CHES	MES
Pipette	NMG pH9.0	1	1	2	2	140	10	10	-
Bath	NMG pH9.0	1	1	2	2	140	-	10	-
	NMG pH6.85	1	1	2	2	140	-	-	10
	Na^+ pH9.0	140	1	2	2	-	-	10	-
	K^+ pH9.0	1	140	2	2	-	-	10	-
	Ca^{2+} pH9.0	1	1	70	2	-	-	10	-
	Mg^{2+} pH9.0	1	1	2	70	-	-	10	-

Supplementary Table 2 List of primers used for insertions

Gene	Sense primers	Anti-sense primers
KnChR_cMyc	TCAGAAGAGGATCTGGGGGATCCAAAGAGC	GATGAGTTTTTGTTCGCTGGCGCTCACAGG
KnChR_272_stop	GTTGGAAGAATACAAGTAAGGGGATCCAAAGAGC	GCTCTTTGGATCCCCTTACTTGTATTCTTCCAAC
KnChR_397_stop	CAACACAGAACATCGAGTAAGGGGATCCAAAGAGC	GCTCTTTGGATCCCCTTACTCGATGTTCTGTGTTG

Supplementary Table 3 List of primers used for subcloning into an eYFP-3.0 vector

Gene	Sense primers	Anti-sense primers
KnChR_272 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCCTTGTATTCTTCCAACTGGC
KnChR_280 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCTGGCAACTGTCCGTTATGAG
KnChR_290 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCCATTTTGGCTCTTGTCTCAAG
KnChR_300 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCGCCAGCAATAATATGCCCAG
KnChR_310 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCGCCAGCCACGTGCAAGACGC
KnChR_317 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCGTGAAAATGATGATGCTGATG
KnChR_321 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCATCCTCATCAAAGTGAAAATG
KnChR_397 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCCTCGATGTTCTGTGTTGTCC
KnChR_697 aa	TTCGAATTCGCCACCGCTAGCATTATGACAGTGGATG	GCTCTTTGGATCCCCGCTGGCGCTCACAGGGGCCC
eYFP 3.0 vector	GGGGATCCAAAGAGCAGGAT	GGTGGCGAATTCGAAGCTTG

Supplementary Table 4 List of primers used for site-directed mutagenesis

Mutations	Sense primers	Anti-sense primers
KnChR_397_E285A	GCCACACTATAGCCTTGCGACAAGAGCCAAAATGA	TCATTTTGGCTCTTGTCGCAAGGCTATAGTGTGGC
KnChR_397_R287A	GTTGCCACACTATAGCCTTGAGACAGCAGCCAAAATGAGG	CCTCATTTTGGCTGCTGTCTCAAGGCTATAGTGTGGCAAC
KnChR_397_K289A	GCCTTGAGACAAGAGCCGCAATGAGGGCTGAAGCTG	CAGCTTCAGCCCTCATTGCGGCTCTTGTCTCAAGGC
KnChR_397_R291A	GAGACAAGAGCCAAAATGGCGGCTGAAGCTGGGCATA	TATGCCCAGCTTCAGCCGCCATTTTGGCTCTTGTCTC
KnChR_397_E293A	AGCCAAAATGAGGGCTGCAGCTGGGCATATTATTG	CAATAATATGCCCAGCTGCAGCCCTCATTTTGGCT