Supplementary Table 1 – MD simulation of membrane potential.

Orientation	Choline presence	Electric field [mV]	Occupancy*	Release time (ns)
Outward-facing	In solution [380mM]	0	12.46	n.a.**
Outward-facing	In solution [380mM]	0	1.3	n.a.
Outward-facing	In solution [380mM]	0	80.62	n.a.
Outward -facing	In cavity	0	19.4	348
Outward -facing	In cavity	0	99	n.r.***
Outward -facing	In cavity	0	98.4	n.r.
Inward-facing	In cavity	0	70.7	895
Inward-facing	In cavity	0	92.8	n.r.
Inward-facing	In cavity	0	100	n.r.
Outward-facing	In solution [380mM]	−200	50.55	n.a.
Outward-facing	In solution [380mM]	−200	73.43	n.a.
Outward-facing	In solution [380mM]	−200	0	n.a.
Outward -facing	In cavity	−200	98.38	n.r.
Outward -facing	In cavity	−200	99.59	n.r.
Outward -facing	In cavity	−200	99.29	n.r.
Inward-facing	In cavity	−200	100	n.r.
Inward-facing	In cavity	−200	64.4	662
Inward-facing	In cavity	−200	17.4	675

* Percentage of the trajectory in which at least one choline molecule is closer than 5 Å to the center of mass of TRP 102.
** n.a.: not applicable
***n.r.: no release observed

Supplementary Table 2 – Nucleotide sequences of human FLVCR variants used in this study.

Variant	Nucleotide sequence
FLVCR1 (wild-type)	gacggatcgggagatctcccgatcccctatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatctgctccctgcttgtgtgttggaggtcg ctgagtagtgcgcgagcaaaatttaagctacaacaaggcaaggcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcgctgcttcgcgatgt acgggccagatatacgcgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataact tacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgt caatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcct ggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatg ggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgt aacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataagcagagctctccctatcagtgatagagatctccctatcagtgat agagatcgtcgacgagctcgtttagtgaaccgtcagatcgcctggagacgccatccacgctgttttgacctccatagaagacaccgggaccgatccagcctccgg actctagcgtttaaacttaagcttgccaccatggcgcggccagacgatgaggaggggcggcggtggcgcccggacacccgctcgcgaaaggatacctcccgtt gccgaggggcgcgcccgttgggaaggagagcgtggagctgcagaacgggcccaaagcgggcaccttcccggtgaatggggcccccgggacagcctcgctgc cgcctcgggagttctgggcgggcctcagactccactggccccagaagaggagacccaggcccggctgctgcctgcgggcgcgggagctgagaccccgggggcc gagagcagcccgctgccccttacggcgctctccccgcggcgcttcgtggtgctcctgatcttcagcctgtactcgctggtcaacgcctttcagtggatccagtacag catcattagcaacgtcttcgagggcttctacggtgtcaccttgctgcacatcgactggctgtccatggtgtacatgctggcctacgtgcccctcatcttcccggccac ctggctgctggacaccagaggcctgcggctcaccgccctgctgggctccggcctcaactgcctgggtgcctggatcaagtgcggcagtgtgcagcagcatctcttc tgggtcaccatgttgggccagtgcttgtgctcggtggcccaggtgttcatcctgggcttgcccctcccgcatcgcctcagtgtggtttgggcccaaagaggtgtccac agcttgtgccaccgccgtgctgggcaatcagcttggaactgcagttggcttttgctaccaccagttttagtacccaacacacagaatgacacaaatctcctggctt gtaatatcagcaccatgttttatggaacatcagctgttgccacacttttatttattttaacagcaattgccttcaaagaaaaacctcggtatccaccaagtcaggctc aagcagctcttcaagacagtccccctgaagagtactcctataagaaatcaataagaaacctgtttaaaaacattccttttgtccttctgttgatcacttatggtatca tgactggtgccttttattcagtctcaacgttattaaatcaaatgatattgacatattatgagggagaagaagtcaatgctggaaggattgggctaacgctagtagta gctggaatggtgggctctattctttgtggcttatggctggattatactaaaacatacaaacagactactctgatagtttatattttgtcttttattggaatggttatcttt actttcacattggaccttagatatattatcatcgtgtttgttactggaggggtgcttggcttcttcatgactggttacctccctttgggttttgaatttgctgttgaaatc acttaccctgaatctgaaggtacttcatctggtcttcttaatgcttctgcacagatatttggaattttgttcacattggctcaaggaaagctcacatcagactatggtc ctaaggcagggaacatttttctctgtgtctggatgtttataggcatcatattaacagcattaatcaagtctgatctgcgaagacacaacatataaatataggaattaca aatgttgatgttaaagctataccagctgacagtcccacagaccaagaaccaaaaacggttatgttgtccaagcagtcagaatcagcaattgattacaaggatga cgacgataagtgagcggccgctcgagtctagagggcccgtttaaacccgctgatcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccc gtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggg gtggggcaggacagcaaggggaggattgggaagacaatagcaggcatgctggggatgcggtgggctctatggcttctgaggcggaaagaaccagctggggc tctagggggtatccccacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccctagcgcccgct cctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggctcccctttagggttccgatttagtgctttacggcacctcgac cccaaaaaacttgattagggtgatggttcacgtacctagaagttcctattccgaagttcctattctctagaaagtataggaacttccttggccaaaaagcctgaact caccgcgacgtctgtcgagaagtttctgatcgaaaagttcgacagcgtctccgacctgatgcagctctcggagggcgaagaatctcgtgctttcagcttcgatgta ggagggcgtggatatgtcctgcgggtaaatagctgcgccgatggtttctacaaagatcgttatgtttatcggcactttgcatcggccgcgctcccgattccggaagt gcttgacattggggaattcagcgagagcctgacctattgcatctcccgccgtgcacagggtgtcacgttgcaagacctgcctgaaaccgaactgcccgctgttctg cagccggtcgcggaggccatggatgcgatcgctgcggccgatcttagccagacgagcgggttcggcccattcggaccgcaaggaatcggtcaatacactacatg gcgtgatttcatatgcgcgattgctgatccccatgtgtatcactggcaaactgtgatggacgacaccgtcagtgcgtccgtcgcgcaggctctcgatgagctgatgc tttgggccgaggactgccccgaagtccggcacctcgtgcacgcggatttcggctccaacaatgtcctgacggacaatggccgcataacagcggtcattgactgga gcgaggcgatgttcggggattcccaatacgaggtcgccaacatcttcttctggaggccgtggttggcttgtatggagcagcagacgcgctacttcgagcggaggc atccggagcttgcaggatcgccgcggctccgggcgtatatgctccgcattggtcttgaccaactctatcagagcttggttgacggcaatttcgatgatgcagcttgg gcgcagggtcgatgcgacgcaatcgtccgatccggagccgggactgtcgggcgtacacaaatcgcccgcagaagcgcggccgtctggaccgatggctgtgtag aagtactcgccgatagtggaaaccgacgccccagcactcgtccgagggcaaaggaatagcacgtactacgagatttcgattccaccgccgccttctatgaaagg ttgggcttcggaatcgttttccgggacgccggctggatgatcctccagcgcggggatctcatgctggagttcttcgcccaccccaacttgtttattgcagcttataat ggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctgta taccgtcgacctctagctagagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcata aagtgtaaagcctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatg aatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatca gctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaa ggccgcgttgctggcgtttttccataggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagat accaggcgtttcccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctca tagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaact atcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttct tgaagtggtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccgg caaacaaaccaccgctggtagcggtggttttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtct gacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaa tctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccc cgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaa ccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaagctagagtaagtagttcgccagtt aatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttaca tgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgcat aattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccg gcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttg agatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaa aaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttg aatgtatttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgccacctgacgtc
FLVCR2 (wild-type)	gacggatcgggagatctcccgatcccctatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagtatctgctccctgcttgtgtgttggaggtcg ctgagtagtgcgcgagcaaaatttaagctacaacaaggcaaggcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcgctgcttcgcgatgt acgggccagatatacgcgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataact tacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgt caatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcct

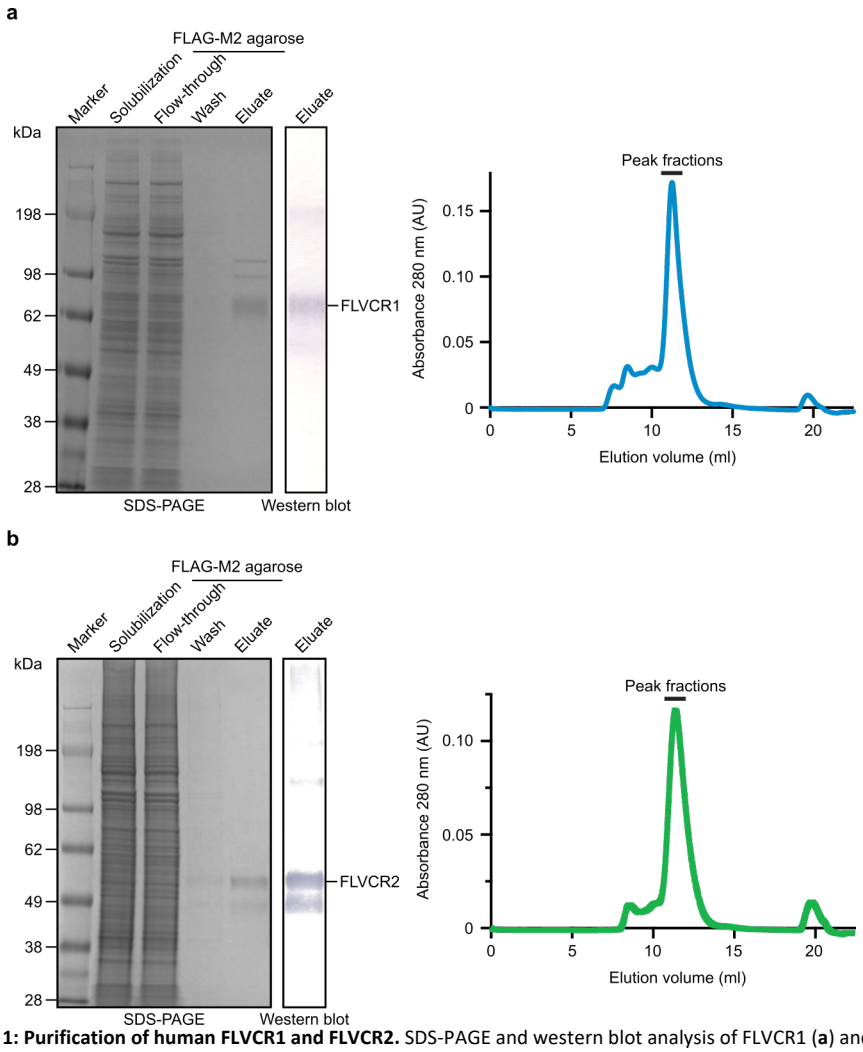
	ggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatg ggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgt aacaactccgcccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataagcagagctctccctatcagtgatagagatctccctatcagtgat agagatcgtcgacgagctcgtttagtgaaccgtcagatcgcctggagacgccatccacgctgttttgacctccatagaagacaccgggaccgatccagcctccgg actctagcgtttaaacttaagcttgccaccatggtgaatgaaggtcccaaccaggaagagagcgatgacacccctgtgccggagtccgcactccaagcggaccc cagcgtctcggtccatcccagcgtctcggtccatcccagcgtctccatcaaccccagcgtctctgtccaccccagcagttcggcccaccccagtgccttagcccaac ccagtggcttggctcaccccagtagctcgggccctgaggacctcagcgtgatcaaggtgagcaggcgccgttgggccgtggtcctggtgtttagctgctactccat gtgcaactcctttcagtggatccagtacggctccatcaataacatcttcatgcacttctacggtgtcagtgcctttgccattgactggctgtccatgtgctacatgctg acttacatccctctgctcctgccagtggcttggctgctggagaagttcggcctgcgcaccattgctctcactggctcggctctcaactgcctgggggcctgggtgaa gctgggcagcctgaagccgcatctctttccggtcaccgtggtgggccagctcatctgctctgtggcccaggttttcatcctgggcatgccctcccgcatcgcttccgt ctggttcggggctaatgaggtttcaacagcctgctccgtggctgtctttggcaatcagcttggaattgcgattgggttcttggtccctcctgttttggtacccaacatt gaagaccgggacgagcttgcctaccacatcagcatcatgttctatataataggaggtgtggccactctcctcctcatccttgtcatcattgtgttcaaggagaaacc taaatatccccccagcagggcccaatccctgagctatgccttgacctctcctgatgcctcatacttaggttccatcgcccggctcttcaaaaatctcaactttgtgct gcttgtcatcacctatggtctgaatgctggtgctttttatgccttgtccactcttctgaatcgcatggtgatctggcactacccgggggaagaagtgaatgctggaag aattggcctgacgatcgtcattgcaggaatgcttggggctgtgatctcaggaatctggctggataggtccaaaacctacaaagagacaaccctggtagtctatatc atgacactggtgggcatggtggtgtacacgtttaccttgaacctgggacacctgtgggtagtgttcatcactgctggcacaatgggcttctttatgactggctatctc ccactgggatttgagtttgctgtggagctcacgtacccagaatcagaaggcatctcctccggcctcctcaacatatctgcacaggtatttgggatcatctttaccatc tcccagggccagattattgacaactatggaaccaagcctgggaacatcttcctgtgtgtgttccttactcttggagcagccctcactgcattcattaaggcagatctc cggagacagaaagcaaacaaagaaactcttgagaacaaactccaagaggaggaggaggagagcaacaccagcaaagtgcccactgctgtgtcagaggatca tctcgattacaaggatgacgacgataagtgagcggccgctcgagtctagagggcccgtttaaacccgctgatcagcctcgactgtgccttctagttgccagccatc tgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcat tctattctgggggggtggggtggggcaggacagcaagggggaggattgggaagacaatagcaggcatgctggggatgcggtgggctctatggcttctgaggcgg aaagaaccagctggggctctagggggtatccccacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgcc agcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggctccctttagggttccgatttag tgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacgtacctagaagttcctattccgaagttcctattctctagaaagtataggaacttcctt ggccaaaaagcctgaactcaccgcgacgtctgtcgagaagtttctgatcgaaaagttcgacagcgtctccgacctgatgcagctctcggagggcgaagaatctcg tgctttcagcttcgatgtaggagggcgtggatatgtcctgcgggtaaatagctgcgccgatggtttctacaaagatcgttatgtttatcggcactttgcatcggccgc gctcccgattccggaagtgcttgacattggggaattcagcgagagcctgacctattgcatctcccgccgtgcacagggtgtcacgttgcaagacctgcctgaaacc gaactgcccgctgttctgcagccggtcgcggaggccatggatgcgatcgctgcggccgatcttagccagacgagcgggttcggcccattcggaccgcaaggaatc ggtcaatacactacatggcgtgatttcatatgcgcgattgctgatccccatgtgtatcactggcaaactgtgatggacgacaccgtcagtgcgtccgtcgcgcagg ctctcgatgagctgatgctttgggccgaggactgccccgaagtccggcacctcgtgcacgcggatttcggctccaacaatgtcctgacggacaatggccgcataa cagcggtcattgactggagcgaggcgatgttcggggattcccaatacgaggtcgccaacatcttcttctggaggccgtggttggcttgtatggagcagcagacgc gctacttcgagcggaggcatccggagcttgcaggatcgccgcggctccgggcgtatatgctccgcattggtcttgaccaactctatcagagcttggttgacggcaa tttcgatgatgcagcttgggcgcagggtcgatgcgacgcaatcgtccgatccggagccgggactgtcgggcgtacacaaatcgcccgcagaagcgcggccgtct ggaccgatggctgtgtagaagtactcgccgatagtggaaaccgacgccccagcactcgtccgagggcaaaggaatagcacgtactacgagatttcgattccacc gccgccttctatgaaaggttgggcttcggaatcgttttccgggacgccggctggatgatcctccagcgcggggatctcatgctggagttcttcgcccaccccaactt gtttattgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaat gtatcttatcatgtctgtataccgtcgacctctagctagagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaaca tacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtc gtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggc tgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaagg ccaggaaccgtaaaaaggccgcgttgctggcgttttccataggctccgcccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccg acaggactataaagataccaggcgtttcccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaa agcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctg cgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtagg cggtgctacagagttcttgaagtggtggcctaactacggctacactagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagtt ggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatccttttg atcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaggatcttcacctagatccttttaaattaaaa atgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatcc atagttgcctgactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccag atttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaagctagagt aagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacg atcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatgg ttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcgac cgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaa ggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacagga aggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcat gagcggatacatatttgaatgtatttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgccacctgacgtc

Supplementary Table 3 – Amino acid sequences of human FLVCR variants used in this study.

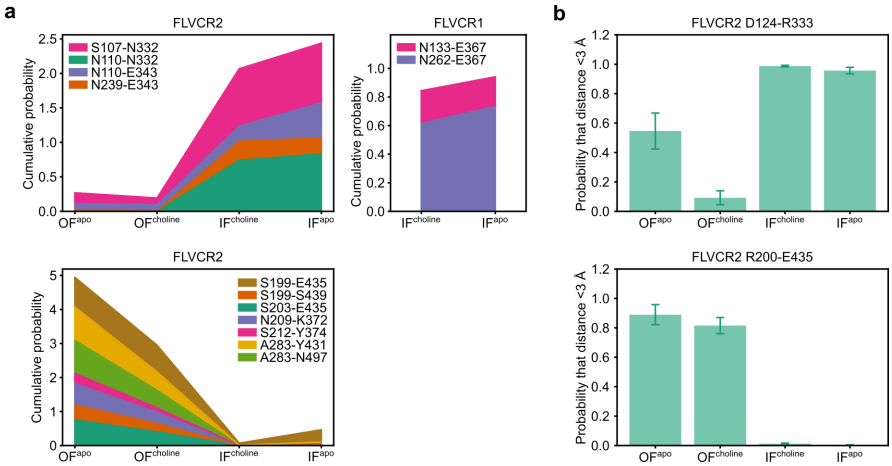
Variant	Amino acid sequence
FLVCR1a (wild-type)	MARPDDEEGAAVAPGHPLAKGYLPLPRGAPVGKESVELQNGPKAGTFPVNGAPRDSLAAASGVLGGPQTPLAPEEETQARLLP AGAGAETPGAESSPLPLTALSPRRFVVLLIFSLYSLVNAFQWIQYSIISNVFEGFYGVTLLHIDWLSMVYMLAYVPLIFPATWLLDTR GLRLTALLGSGLNCLGAWIKCGSVQQHLFWVTMLGQCLCSVAQVFILGLPSRIASVWFGPKEVSTACATAVLGNQLGTAVGFLL PPVLVPNTQNDTNLLACNISTMFYGTSAVATLLFILTAIAFKEKPRYPPSQAQAALQDSPPEEYSYKKSIRNLFKNIPFVLLLITYGIM TGAFYSVSTLLNQMILTYYEGEEVNAGRIGLTLVVAGMVGSILCGLWLDYTKTYKQTTLIVYILSFIGMVIFTFTLDLRYIIIVFVTGG VLGFFMTGYLPLGFEFAVEITYPESEGTSSGLLNASAQIFGILFTLAQGKLTSDYGPKAGNIFLCVWMFIGIILTALIKSDLRRHNINI GITNVDVKAIPADSPTDQEPKTVMLSKQSESAIDYKDDDDK
FLVCFR2 (wild-type)	MVNEGPNQEESDDTPVPESALQADPSVSVHPSVSVHPSVSINPSVSVHPSSSAHPSALAQPSGLAHPSSSGPEDLSVIKVSRRR WAVVLVFSCYSMCNSFQWIQYGSINNIFMHFYGVSAFAIDWLSMCYMLTYIPLLLPVAWLLEKFGLRTIALTGSALNCLGAWVK LGSLKPHLFPVTVVGQLICSVAQVFILGMPSRIASVWFGANEVSTACSVAVFGNQLGIAIGFLVPPVLVPNIEDRDELAYHISIMFYI IGGVATLLLILVIIVFKEKPKYPPSRAQSLSYALTSPDASYLGSIARLFKNLNFVLLVITYGLNAGAFYALSTLLNRMVIWHYPGEEVN AGRIGLTIVIAGMLGAVISGIWLDRSKTYKETTLVVYIMTLVGMVVYTFTLNLGHLWVVFITAGTMGFFMTGYLPLGFEFAVELTY PESEGISSGLLNISAQVFGIIFTISQGQIIDNYGTKPGNIFLCVFLTLGAALTAFIKADLRRQKANKETLENKLQEEEEESNTSKVPTAV SEDHLDYKDDDDK

Supplementary Table 4 – Cryo-EM and model data statistics.

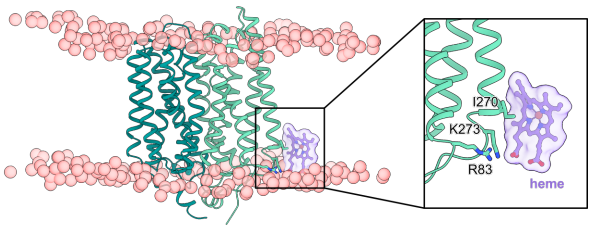
	FLVCR1-IF^{apo} (EMD-18334; PDB 8QCS)	FLVCR1-IF^{choline} (EMD-18335; PDB 8QCT)	FLVCR2-IF^{apo} (EMD-18336; PDB 8QCX)	FLVCR2-OF^{apo} (EMD-18337; PDB 8QCY)	FLVCR2-OF^{heme} (EMD-18338; PDB 8QCZ)	FLVCR2-IF^{choline} (EMD-18337; PDB 8QD0)
Data collection and processing						
Magnification	215,000	215,000	215,000	215,000	105,000	215,000
Voltage (kV)	300	300	300	300	300	300
Electron dose (e^{-}/Å^2)	55	55	80	80	80	57
Defocus range (μm)	−1.1 to −2.1	−1.1 to −2.1	−1.1 to −2.1	−1.1 to −2.1	−1.1 to −2.1	−1.1 to −2.1
Pixel size (Å)	0.573	0.573	0.573	0.573	0.837	0.573
Symmetry imposed	*C*1	*C*1	*C*1	*C*1	*C*1	*C*1
Initial particle images (no.)	3,247,307	3,251,081	3,497,517	3,497,517	5,419,952	4,790,154
Final particle images (no.)	56,664	25,873	50,167	49,532	51,266	51,511
Map resolution (Å)	2.9	2.6	3.1	2.9	3.1	2.8
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.4–6.5	2.6–4.9	2.8–5.5	2.7–3.8	2.7–5.8	2.6–3.8
Refinement						
Initial model used	AlphaFold model (AF-Q9Y5Y0-F1)		AlphaFold model (AF-Q9UPI3-F1)			
Model resolution (Å)	3.4	3.1	3.6	3.4	3.6	3.3
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5
Model resolution range (Å)						
Map sharpening *B* factor (Å^2)	−80	−30	−120	−100	−60	−30
Model composition						
Non-hydrogen atoms	3,255	3,273	3283	3,273	3,316	3,290
Protein residues	417	418	422	421	421	422
Ligands	NAG: 1	NAG: 1 CHT: 1	–	–	HEM: 1	CHT: 1
Average *B* factors (Å^2)						
Protein	87.45	40.28	76.93	82.55	109.15	63.63
Ligand	144.11	68.46	–	–	183.4	31.72
R.m.s. deviations						
Bond lengths (Å)	0.003	0.003	0.003	0.004	0.004	0.003
Bond angles (°)	0.570	0.512	0.512	0.592	0.631	0.641
Validation						
MolProbity score	1.50	1.40	1.20	1.31	1.43	1.31
Clashscore	6.82	7.22	4.20	5.71	7.24	5.68
Poor rotamers (%)	2.5	3.1	0.6	0.0	1.4	2.8
Ramachandran plot						
Favored (%)	97.3	98.1	99.0	98.6	98.1	99.0
Allowed (%)	2.7	1.9	1.0	1.4	1.9	1.0
Disallowed (%)	0.0	0.0	0.0	0.0	0.0	0.0



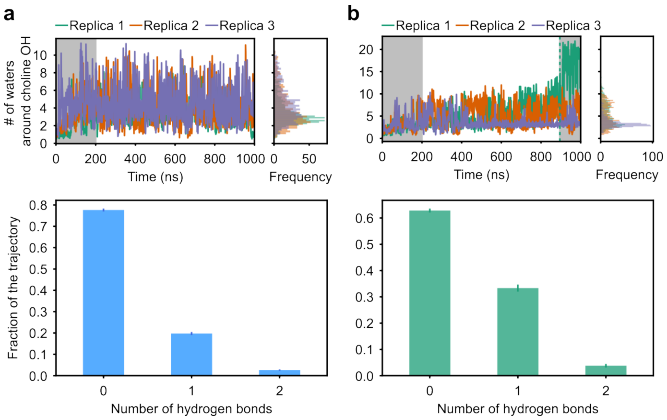
Supplementary Fig. 1: Purification of human FLVCR1 and FLVCR2. SDS-PAGE and western blot analysis of FLVCR1 (**a**) and FLVCR2 (**b**) affinity purification (left) and subsequent size-exclusion chromatography (SEC; right).



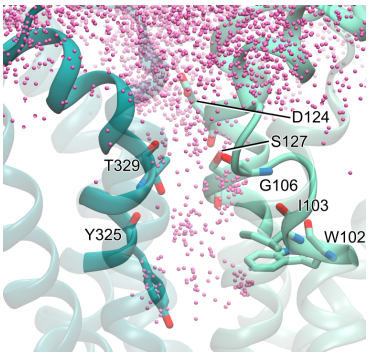
Supplementary Fig. 2: Persistence of critical interactions for stabilization of FLVCR1 and FLVCR2 in MD simulations. a, Cumulative probability of inter-domain hydrogen bond formation at the external gate (top) and the internal gate (bottom) with or without choline in the binding cavity. For each pair of residues, the probability was calculated as the fraction of the trajectory in which the distance between the residues remained below 3 Å. **b,** Stability of salt bridges within the external gate (top) and internal gate (bottom) of FLVCR2 in MD simulations. The displayed data represents the mean values obtained from three independent replicas, with error bars representing standard errors of the mean (s.e.m.).



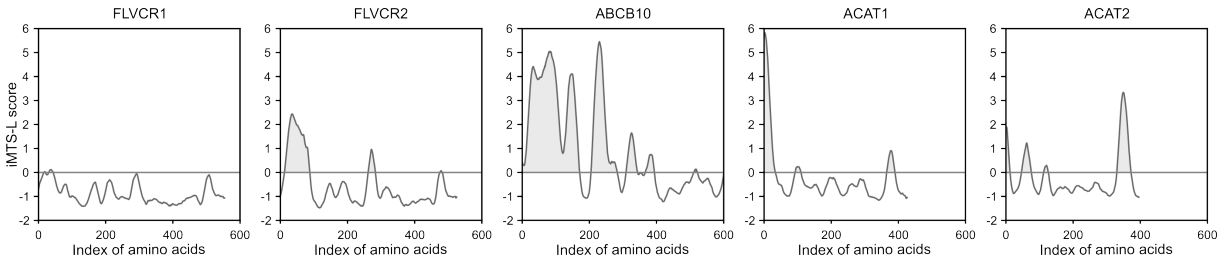
Supplementary Fig. 3: Snapshot of new, stable interactions formed between heme and FLVCR2 within the lipid bilayer in MD simulations. The heme molecule is shown in ball-and-stick representation and the phosphates of POPE/POPG are shown as red spheres. The right panel shows the close-up view of the heme molecule and the interacting residues of FLVCR2.



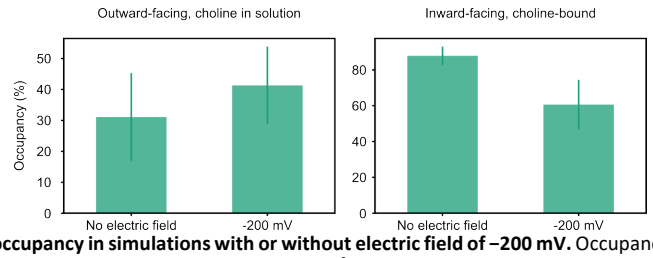
Supplementary Fig. 4: Hydration of choline in MD simulations. Interactions between choline and water in the binding site of inward-facing FLVCR1 (**a**) and FLVCR2 (**b**). The upper panels indicate the number of water molecules in the proximity of the hydroxyl group of choline with a cutoff of 5 Å in the $O^{choline}$-O^{water} distance. The first 200 ns of simulations were considered as equilibration and thus not included in the histograms. The last 105 ns of replica 1 in the FLVCR2 simulation were discarded as well due to choline release. The lower panels represent the hydrogen bond formation between waters and the hydroxyl group of choline in the simulations with a distance and angle cut-off of 3 Å and 20 °, respectively. Error bars represent s.e.m.



Supplementary Fig. 5: Choline entry from solution into the outward-facing cavity of FLVCR2 in MD simulations. Residues that interact with choline frequently are shown as sticks. Pink dots represent choline positions in all frames of one 1-μs trajectory.



Supplementary Fig. 6: Propensity of mitochondrial matrix targeting sequences in FLVCR1, FLVCR2 and well-characterized human mitochondrial proteins. The propensity scores of internal matrix targeting signal-like sequences (iMTS-Ls) for FLVCR1, FLVCR2, and other known mitochondrial proteins, including ATP Binding Cassette Subfamily B Member 10 (ABCB10), Acetyl-CoA acetyltransferase 1 (ACAT1) and Acetyl-CoA acetyltransferase 2 (ACAT2), were computed with iMLP (https://csb-imlp.bio.rptu.de). Curve areas with propensity score >0 are coloured grey.



Supplementary Fig. 7: Choline occupancy in simulations with or without electric field of −200 mV. Occupancy was defined as the percentage of the trajectory in which at least one choline molecule is closer than 5 Å to the centre of mass of W102. The average values of three replicas are shown. Error bars represent standard errors of the mean (s.e.m.).