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Supplemental Information

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Supporting Information included:

- Figure S1
- Figure S2
- Figure S3

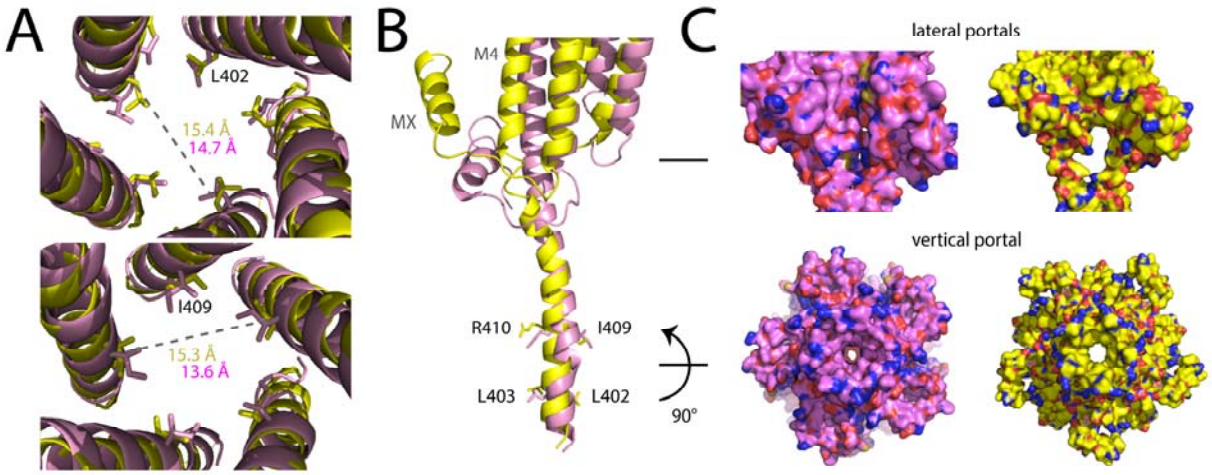

Figure S1. Structural alignment of 5-HT$_{3A}$ Apo (PDB: 6BE1, pink) and State 2 (PDB: 6DG8, yellow) and detailed view of possible ion exit pathways. (A) View down the pore of the ICD of 5-HT$_{3A}$. Side chains visible for L402/L403 (top panel) and I409/R410 (bottom panel) as well as the pore diameter for the different structures. **(B)** Alignment of a single subunit of 5-HT$_{3A}$, cartoon representation, emphasizing the differences between the open and closed conducting states. **(C)** Surface representation of 5-HT$_{3A}$ in different conformational states, showing a detailed view of the plausible ion exit pathways. (top panel) Detailed view of the lateral portals between two adjacent MA-helices around the R0' (R436), viewed parallel to the membrane. State 2, which is potentially conducting (PDB: 6DG8), shows a larger lateral opening as compared to the apo structure (PDB: 6BE1). (bottom panel). Receptors viewed from the intracellular side, comparing the diameter of the vertical pore in the different structures, again with state 2 having a slightly larger aperture as compare to the apo state.

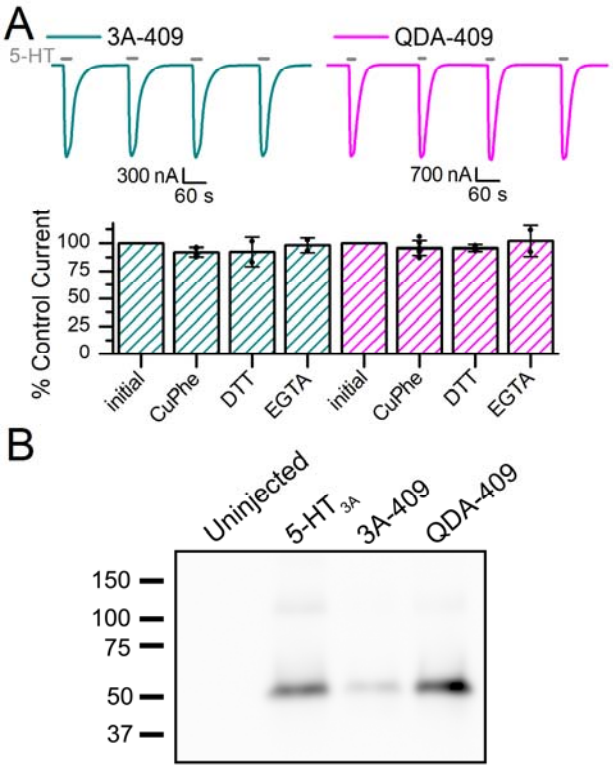

Figure S2. **Evaluation of single Cys at I409 in response to 5-HT, Cu:Phe, DTT, and EGTA. (A)** (top panel) Sample traces of 5-HT_{3A}-I409C and QDA-I409C in response to 3 μM 5-HT, showing stable current amplitudes with each agonist application. (bottom panel) Quantification of current amplitudes after 5-HT_{3A}-I409C and QDA-I409C were exposed to Cu:Phe (100:200 μM), 10 mM DTT, and 1mM EGTA. The first inward current recorded in response to 3 μM 5-HT is the reference current amplitude (100% of current). Experimental set-up was the same as in Fig. 3A. Data is shown as mean±S.D. Individual data points are shown as circles overlaid on top of the bar graph (n≥2). Statistical significance was determined with one-way ANOVA, Dunnett's multiple comparisons test between the initial inward current and each set of conditions. **(B)** Western blot of single Cys and uninjected oocytes and 5-HT_{3A} as controls. Experimental set-up was the same as in Fig. 2. Expressing *X. laevis* oocytes were exposed to 2 min of 100:200 μM Cu:Phe. SDS-PAGE fractions were run without reducing agent.

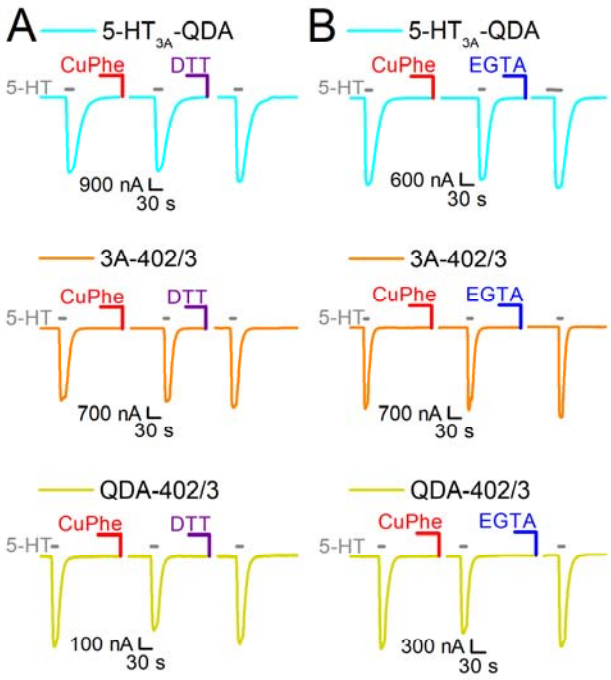

Figure S3. **No Effect of Cu:Phe, DTT, and EGTA on QDA and L402C/L403C Cys pairs. (A)** Sample traces of QDA (cyan), 5-HT_{3A}-L402C/L403C (orange), and QDA-L402C/L403C (yellow). The inward currents were evoked with 3 μM 5-HT (grey bars). The initial inward current represents the reference current amplitude (100% of current). Following the 5-HT application(s), the oocytes were exposed to 2 min of Cu:Phe (100:200 μM) and then a 6 min wash with OR-2 (not pictured) before another application of 5-HT (second inward current depicted). 5-HT was applied until a stable current response was achieved (stable response to 5-HT is the inward current before DTT, labeled "5-HT" in Fig. 4 for I409C/R410C mutants). Lastly, 10 mM DTT was applied to the oocytes for 2 min, followed by a 6 min OR-2 wash (not pictured) and 5-HT (last inward current pictured). **(B)** Sample traces of QDA (cyan), 5-HT_{3A}-L402C/L403C (orange), and QDA-L402C/L403C (yellow). Same experimental set-up as in **(A)** but during the last step 1 mM EGTA was applied instead of DTT.