SUPPLEMENTAL MATERIAL

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Phi and r values

We modify and extend phi (Φ) analysis of a linear Markov reaction chain with absorbing end states (C and O) separated by n short-lived, intermediate states (T1–n) that we define as the TSE (Zhou et al., 2005):

\[
\begin{array}{cccccc}
C & \xrightarrow{k_1} & T_1 & \xrightarrow{k_3} & T_2 & \ldots & T_n & \xrightarrow{k_{2n+1}} & O
\end{array}
\]

Assuming that the exit rate constants from the end states (k1 and k2n+2) are much smaller than all of the other rate constants, the mean first passage rate for a complete C→O transition \((k_f)\) is

\[
k_f = \frac{k_1}{1 + \sum_{i=1}^{n} \prod_{j=1}^{i} r_j}, \quad (S1)
\]

where \(r_j\) is the ratio of the exit rates (backward/forward) from \(T_j\). Defining the denominator as \(M\), the Φ values of the reaction chain (first derivative of the rate-equilibrium free energy relationships) are

\[
\Phi_q = \frac{\sum_{i=q}^{n} \prod_{j=1}^{i} r_j}{M}. \quad (S2)
\]

Note that this corrects Eq. 16 in Zhou et al. (2005).

There are five phi populations for diliganded AChR gating, so \(n = 4\) and

\[
\begin{align*}
\Phi_1 &= \frac{(r_1 + r_2 + r_1 r_2 + r_1 r_2 r_3)}{M} \\
\Phi_2 &= \frac{(r_1 r_2 + r_1 r_2 r_3 + r_1 r_2 r_3 r_4)}{M} \\
\Phi_3 &= \frac{(r_2 r_3 + r_2 r_3 r_4)}{M} \\
\Phi_4 &= \frac{(r_3 r_4)}{M} \\
\Phi_5 &= 0, \text{ by definition.}
\end{align*}
\]

An example WT energy landscape is the solid line and for a \(\Phi_2\) perturbation is the dashed line:

The \(r_j\) values can be calculated from the phi values by solving simultaneous equations represented by a matrix equation: \(AY = B\). \(A\) is an \(n\)-by-\(n\) square matrix and \(Y\) and \(B\) are column vectors of length \(n\):

\[
A_{ij} = \begin{cases} 
\Phi_i, & i < j \\
(1 - \Phi_i), & i \geq j 
\end{cases}
\]

\[
Y_i = \prod_{j=1}^{i} r_j
\]

\[
B_i = \Phi_i. \quad (S4)
\]

\(Y\), and hence \(r_j\) values, can be obtained by finding the matrix inverse of \(A\) (\(Y = A^{-1}B\)). For \(n = 4\), Eq. S4 is

\[
\begin{bmatrix}
(1 - \Phi_1) & (1 - \Phi_1) & (1 - \Phi_1) & (1 - \Phi_1) \\
-\Phi_2 & (1 - \Phi_2) & (1 - \Phi_2) & (1 - \Phi_2) \\
-\Phi_3 & -\Phi_3 & (1 - \Phi_3) & (1 - \Phi_3) \\
-\Phi_4 & -\Phi_4 & -\Phi_4 & (1 - \Phi_4)
\end{bmatrix}
\begin{bmatrix}
Y_1 \\
Y_2 \\
Y_3 \\
Y_4
\end{bmatrix}
= \begin{bmatrix}
\Phi_1 \\
\Phi_2 \\
\Phi_3 \\
\Phi_4
\end{bmatrix} \quad (S5)
\]

We write explicitly the set of four simultaneous equations:
\( (1 - \Phi_1) \cdot Y_1 + \Phi_1 \cdot Y_2 + (1 - \Phi_2) \cdot Y_2 + (1 - \Phi_3) \cdot Y_3 + (1 - \Phi_4) \cdot Y_4 = \Phi_1 \)

\(-\Phi_1 \cdot Y_1 + (1 - \Phi_2) \cdot Y_2 + (1 - \Phi_3) \cdot Y_3 + (1 - \Phi_4) \cdot Y_4 = \Phi_2 \)

\(-\Phi_1 \cdot Y_1 + (1 - \Phi_2) \cdot Y_2 + (1 - \Phi_3) \cdot Y_3 + (1 - \Phi_4) \cdot Y_4 = \Phi_3 \)

\(-\Phi_1 \cdot Y_1 - \Phi_4 \cdot Y_2 - \Phi_4 \cdot Y_3 + (1 - \Phi_4) \cdot Y_4 = \Phi_4 \)

Solving these equations for \( Y_i \)

\[ Y_1 = r_1; \quad Y_2 = r_1 r_2; \quad Y_3 = r_1 r_2 r_3; \quad Y_4 = r_1 r_2 r_3 r_4. \]

The experimental diliganded AChR gating phi values are \( \Phi_1 = 0.95, \Phi_2 = 0.79, \Phi_3 = 0.58, \) and \( \Phi_4 = 0.33 (\Phi_5 = 0.06; \text{Fig. 6 A}). \) Solving for \( \Phi \) (MATLAB R2014b; The MathWorks Inc.) and using the experimental \( k_f \) value of 2 ms\(^{-1}\) when the overall C-to-O equilibrium constant is equal to 1, the solution is \( r_1 = 3.20, r_2 = 1.32, r_3 = 1.19, \) and \( r_4 = 1.32. \) These values yield \( M = 19.96 \) and Scheme 3 (rate constants, ms\(^{-1}\)).

From the hypothetical energy landscape for unliganded gating (Fig. 6 E), we calculate \( r \) values of \( r_1 = 3,200, r_2 = 1.32, r_3 = 1.19, \) and \( r_4 = 1.32, \) to yield Scheme 5. From these \( r \) values, we calculate (Eq. S3) unliganded phi values of \( \Phi_1 = 0.99, \Phi_3 = 0.83, \Phi_3 = 0.61, \) and \( \Phi_4 = 0.35. \)

**Committer**

The position in the reaction chain where there is an equal probability of entering rapidly either absorbing end state is called the committer or separatrix (\( \dagger \)). The position of \( \dagger \) in the TSE was calculated (by optimization) from the A matrix (Qin et al., 1996) as the position in the Markov chain in which the probabilities of reaching either absorbing state were equal after time \( t \) (long compared with sojourns in the TSE and short compared sojourns in the end states; by Chris Nicolai; http://www.qub.buffalo.edu/online/commitor.html). A \( \dagger \) position between T states is the relative probability of either flanking state. In Scheme 3 and with all \( k \) values = 300 ms\(^{-1}\), the \( \dagger \) probabilities were 80\% in \( T_3 \) and 20\% in \( T_4 \).

**Transmission coefficient**

In Eyring theory, a TS is the point intersection of end-state parabolic wells, and a transmission coefficient (\( \kappa \)) corrects the rate constant for TS re-crossings. In Kramers theory, the separating barrier is a parabola and \( \kappa \) is proportional to the product of a diffusion constant and the barrier frequency (width) under the condition of moderate to high friction (Billing and Mikkelsen, 1996). Here, \( r \) values (phi values) set the overall barrier shape and \( \kappa \) is the fraction of exits from an end state that reach the committer \( \dagger \), which by definition is crossed with a 50\% probability.

With absorbing end states, the number of exits from C that result in one complete passage to O is \( M \). The fraction of visits to \( \dagger \) is, then, 2/\( M \):  

\[ \kappa_{C \rightarrow O} = 2 \cdot \left( 1 + \sum_{j=1}^{\infty} \prod_{i=1}^{j} r_j \right)^{-1} \]

and  

\[ \kappa_{O \rightarrow C} = 2 \cdot \left( 1 + \sum_{j=1}^{\infty} \prod_{i=1}^{j} r_j \right)^{-1}. \]

Note that these correct Eqs. 8 and 9 in Zhou et al. (2005). From the \( r \) values for diliganded gating Scheme 3, \( \kappa_{C \rightarrow O} = 0.10 \) and \( \kappa_{O \rightarrow C} = 0.66, \) and for unliganded gating Scheme 5, \( \kappa_{C \rightarrow O} = 1.1E-4 \) and \( \kappa_{O \rightarrow C} = 0.7. \) With regard to the mean number of the exit attempts required for a full crossing (\( \sim 2/\kappa \)), these four values correspond to approximately 20, 3, 18,000, and 3, respectively.

With ACh, the assumption that the exit rate constant from C is \( \ll \) than the other rate constants is not valid. Specifically, the exit rate from C (\( k_1 \)) is likely to be similar to the rate for exiting the TSE (\( k_{TSE} \)). Accordingly,

\[ \kappa_{C \rightarrow O} = 2 k_1 \left( \frac{1}{k_1} + \frac{1}{k_{TSE}} \right). \]

The mean number of the exit attempts required for a full C\( \rightarrow \)O crossing is the inverse of half this value.

In WT AChRs with two bound ACh, \( k_1 = 50 \text{ ms}^{-1} (-100 \text{ mV, 23°C}). \) However, \( k_1 \) is not known, and \( k_{TSE} \) depends on the absolute values of the rate constants in Scheme 3. In Fig. 6 E, we have assumed \( k_1 = 1,000 \text{ ms}^{-1}, \) which is close to maximal (Chakrapani and Auerbach, 2005). With \( k_3, k_5, k_7, \) and \( k_9 \) in Scheme 3 all equal to 300 ms\(^{-1}\), \( k_{TSE} \approx 180 \text{ ms}^{-1} \) (the inverse of the longest TSE time constant, 1/5.6 \( \mu \text{s}; \) Fig. 6 B), and we calculate using Eq. S7 that \( \kappa_{C \rightarrow O} = 0.66. \) With these assumptions regarding \( k_1 \) and \( k_{TSE}, \) the mean number of exit attempts from C before achieving a full C\( \rightarrow \)O crossing is \( \kappa_{C \rightarrow O}/2 \) or \( \sim 3. \) The O\( \rightarrow \)T 4 rate constant is slow with or without ACh, so the mean number of exit attempts from O before achieving a full O\( \rightarrow \)C crossing is in both cases also \( \sim 3. \)
Figure S1. **Cyclic activation model.** C, closed-channel conformation; O, open-channel conformation; superscript A, agonist; \( K_d \), low-affinity equilibrium dissociation constant; \( J_d \), high-affinity equilibrium dissociation constant; \( E_0 \), unliganded gating equilibrium constant; \( E_2 \), gating equilibrium constant with two bound agonists. Without external energy, the product of equilibrium constants connecting any two states is independent of the pathway (Hess’s law). Considering \( C \) and \( 2AO \): \( \frac{1}{K_d^2} \cdot (E_2) = (E_0) \cdot \frac{1}{J_d^2} \) or, \( E_2/E_0 = (K_d/J_d)^2 \).

Figure S2. **Energy landscapes corresponding to Scheme 3 with \( k = 500 \text{ ms}^{-1} \), except for the intermediate state(s) marked by an open circle (Table S3).** \( \tau \) is the brief shut interval flip/primed lifetime measured after filtering and fitting by C-C'-O (Fig. 6C). Lifetime (\( \mu s \)) of each TSE intermediate state is shown below each well.
Video 1. **A model of AChR gating.** Part 1 (~2 min): Description of the model detailing the structural components, energy landscape, sequence of rearrangements, and corresponding kinetic scheme. Part 2 (~3 min): Simulations of the model at three different time scales. (top left) Cartoon structure showing components that switch between locally off (black) and on (red) conformations; (top right) gating energy landscape; (bottom) simulated single-channel current. (i) 100 kHz (10 µs/sample). Only sojourns in C and O are clearly resolved. (ii) 2 MHz (500 ns/sample). Sojourns in the T states are also resolved. Notice that C→T structural transitions occur in long-duration shut intervals without a change in current and that brief closures in the current trace (flip/primed events) mainly reflect gate bubble formation. (iii) 50 MHz (20 ns/sample) using two different kinetic models (see Fig. S2).

### Table S1. Backgrounds for MCA analysis

| Mutation(s) | Agonist | Mutant pair |
|-------------|---------|-------------|
| αA96V       | 100 mM ACh | αS268P→αY196P |
| αC418W      | 100 mM ACh | aP265A→aG147A, aP265A→aW149A, aI260P→aG147A, aI260P→aW149P |
| αC418Y      | 100 mM ACh | aP265A→αY198A |
| αC418W+αI43Q| 100 mM ACh | aS268A→aP121A, aS268A→aP121S, aS268A→aP123A |
| βT456F      | 100 mM ACh | aP265A→αY190F, aI260P→αP123G, aI260P→αP121G, aI260P→αP121G, aI260P→αP121G |
| βT456F+α43H | 100 mM ACh | aP265A→αY193A, aP265A→αP121G, aI260P→αP121G, aI260P→αP121G |
| βT456F+α43H | 100 mM ACh | aP265A→αP112A |
| βP229A+α43Q | 100 mM ACh | aS268P→αP121G, aP265G→αP121G |
| δH43Q       | 20 mM Cho | aS268A→αT133A, aS268A→αY93A, aS268A→αY93A, aT267A→αK219A |
| δH43I       | 100 mM ACh | aE262G→aV193A, aE262G→aW149A, aE262G→aW149A, aE262G→aW149A, aE262G→aY193A, aE262G→aY193A |
| δH43Q+εL260Q| 100 mM ACh | aP265A→αG147A |
| εL260F      | 100 mM ACh | aS268A→αY196P |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |
| δA96Y+εL266A+δP123R | 100 mM ACh | γP121A→αY93A |

### Table S2. Backgrounds for unliganded REFERs (Fig. 5)

| Mutation | Background(s) |
|----------|---------------|
| αA260M   | α(D97A+Y127F+S269I+W149F) |
| S        | α(D97A+Y127F+S269I+W149F)+αS269C |
| A        | α(D97A+Y127F+S269I+W149F+C418W) |
| αA262G/L | α(D97A+Y127F+S269I+W149F) |
| αA262G/L | α(D97A+Y127F+S269I+W149F) |
| αA262G/L | α(D97A+Y127F+S269I+W149F+C418W) |
| G        | α(D97A+Y127F+S269I) |
| D        | α(D97A+Y127F) |
| L        | α(D97A+Y127F)+αS269C |

### Table S3. Simulation results using Scheme 3 and different k values

| k (ms⁻¹) | τ⁻|| (µs) | τ⁻|| (µs) | k⁻| (ms⁻¹) | k⁺| (ms⁻¹) |
|----------|---------|---------|---------|---------|------|---------|------|---------|
| 100      | 15.8    | 15.2    | 35      | 30     |
| 200      | 8.4     | 9.3     | 51      | 55     |
| 300      | 5.6     | 6.4     | 61      | 82     |
| 400      | 4.3     | 5.8     | 61      | 107    |
| 500      | 3.5     | 5.0     | 58      | 137    |
| 600      | 2.9     | 4.6     | 49      | 161    |

τ⁻|| is the slowest component of the simulated TSE shut distribution (Fig. 6 B), τ⁻|| is the fitted shut component in facsimile patch experiments (Fig. 6 C), k⁻| and k⁺| are the fitted exit rate constants from C, backward and forward. In experiments with human adult AChRs expressed in cells, τ⁻|| ~9 µs, k⁻| ~19 ms⁻¹, k⁺| ~86 ms⁻¹.
Table S4. Effects of stabilizing individual TSE states

| n  | λ  | τ  | k₉ | k₈ | ΔG | kᵦᵣᵩ |
|----|----|----|----|----|----|-------|
| 1  | 5.2 | 5.6 | 46 | 128 | 1  | 55    |
| 2  | 6.3 | 7.7 | 37 | 88  | 0.74 | 150   |
| 3  | 6.1 | 7.3 | 53 | 80  | 0.5 | 200   |
| 4  | 5.9 | 7.0 | 79 | 62  | 0.7 | 150   |
| 1:4| 5.3 | 6.7 | 45 | 99  | 0.54 | 200   |

λ, τ, kᵢ, and kᵦ as in Table S3. ΔG, the free energy by which state(s) n was stabilized. kᵦᵩᵩ, forward exit rate constant from stabilized state(s). Corresponding energy landscapes are shown in Fig. S1.

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