RESEARCH ARTICLE

Efficiency measures the conversion of agonist binding energy into receptor conformational change

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Receptors alternate between resting↔active conformations that bind agonists with low↔high affinity. Here, we define a new agonist attribute, energy efficiency (η), as the fraction of ligand-binding energy converted into the mechanical work of the activation conformational change. η depends only on the resting/active agonist-binding energy ratio. In a plot of activation energy versus binding energy (an “efficiency” plot), the slope gives η and the y-intercept gives the receptor’s intrinsic activation energy (without agonists; ΔG0). We used single-channel electrophysiology to estimate η for eight different agonists and ΔG0 in human endplate acetylcholine receptors (AChRs). From published equilibrium constants, we also estimated η for agonists of KCa1.1 (BK channels) and muscarinic, γ-aminobutyric acid, glutamate, glycine, andaryl-hydrocarbon receptors, and ΔG0 for all of these except KCa1.1. Regarding AChRs, η is 48–56% for agonists related structurally to acetylcholine but is only ~39% for agonists related to epibatidine; ΔG0 is 8.4 kcal/mol in adult and 9.6 kcal/mol in fetal receptors. Efficiency plots for all of the above receptors are approximately linear, with η values between 12% and 57% and ΔG0 values between 2 and 12 kcal/mol. Efficiency appears to be a general attribute of agonist action at receptor binding sites that is useful for understanding binding mechanisms, categorizing agonists, and estimating concentration–response relationships.

Introduction

Nicotinic acetylcholine receptors (AChRs) from vertebrate skeletal muscle have two neurotransmitter-binding sites located in the extracellular domain, at α−δ and either α−ε (adult) or α−γ (fetal) subunit interfaces (Fig. 1a). At adult sites, 4 α−subunit aromatic amino acids combine to determine neurotransmitter-binding energy, and at the fetal site a tryptophan in the γ subunit also contributes (Cohen et al., 1991; Kearney et al., 1996; Zhong et al., 1998; Brejc et al., 2001; Nayak et al., 2014; Purohit et al., 2014). AChRs operate by a cyclic mechanism (Fig. 1b) in which the global, activation (“gating”) conformational change, R ↔ R*, occurs either with or without a bound agonist, and agonists bind weakly to R* (free-energy ΔGR* or strongly to R* (ΔG0). In mouse AChRs and for a series of acetylcholine (ACH)−like agonists, ΔGR* is a constant fraction of ΔG0* (Jadey and Auerbach, 2012). A fixed ΔGR*/ΔG0* ratio generates a linear correlation between the log of the receptor gating equilibrium constant and the agonist resting equilibrium dissociation constant (Auerbach, 2016). Recently, free-energy changes in each step of the activation cycle were measured experimentally for small, ACh-class agonists at individual mouse AChR-binding sites (Nayak and Auerbach, 2017). Despite a wide range in resting affinity, at all sites and for all tested agonists, ΔGR* was always approximately twice ΔG0. That is, at all three kinds of neurotransmitter−binding sites, the interaction energy of each ligand in the resting conformation was approximately half as strong as in the active conformation. Here, we show that the ΔGR*/ΔG0* ratio defines η, which is the energy-conversion efficiency, and that a fixed binding-energy ratio pertains to other classes of nicotinic receptor agonist and other receptors.

The new nicotinic agonists we investigated have an azabicycloheptane (Aza) group. Some of these occur naturally, such as anatoxin (from cyanobacteria) and epibatidine (Epi; a frog toxin), and other bridged, bicyclic compounds have been approved for treatment of neurodegenerative diseases (memantine, amantadine, and biperiden). We used single-channel kinetics to estimate binding energies of these and ACh-class agonists and compared energy efficiencies at individual α−ε, α−δ, and α−γ neurotransmitter-binding sites of human AChRs.

So far, a fixed binding-energy ratio has been observed only in endplate AChRs. To explore the generality of this result, we estimated from published values of binding and gating equilibrium constants agonist energy efficiencies at binding sites of BK channels (KCa1.1) and muscarinic, GABA_A, NMDA, glycine, and aryl-hydrocarbon receptors. We also estimated for the first

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time the intrinsic gating energy ($\Delta G_0$ in Fig. 1b) of adult- and fetal-type human AChRs and of these other receptors. $\Delta G_0$ not only determines the basal activity level but also contributes to the high-concentration asymptote and midpoint of the concentration–response curve (CRC). An increase or decrease in $\Delta G_0$ caused, for example, by a mutation or an allosteric modulator can alter the CRC and the physiological response enough to cause disease, often without a noticeable change in baseline activity (Zuo et al., 1997; Zhou et al., 1999; Lester and Karschin, 2000; Labarca et al., 2001; Hatton et al., 2003).

The results regarding energy efficiency indicate that (a) Epi-class nicotinic agonists are less efficient than ACh-class agonists, (b) the same agonist can have different efficiencies at different binding sites, and (c) many receptors have a fixed binding-energy ratio. The structural correlates of energy efficiency in AChRs are considered elsewhere (Tripathy et al., 2019).

Materials and methods

Electrophysiology

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s Minimal Essential Medium supplemented with 10% FBS and 1% penicillin–streptomycin, pH 7.4. AChRs were expressed in HEK293 cells by transient transfection (CaPO4 precipitation method) of mouse $\alpha,\beta,\delta,\varepsilon/\gamma$ subunits in a ratio of 2:1:1:1. Most electrophysiological experiments were started ~24 h after transfection. Single-channel currents were recorded in the cell-attached patch configuration (23°C). The bath solution was (in mM) 142 KCl, 5.4 NaCl, 1.8 CaCl2, 1.7 MgCl2, and 10 HEPES/KOH, pH 7.4. Because of the high extracellular [K$^+$], the cell membrane potential ($V_m$) was ~−0 mV. Unless noted otherwise, the pipette potential was +100 mV.

Patch pipettes were fabricated from borosilicate glass, coated with Sylgard (Dow Corning), and fire polished to a resistance of ~10 MΩ when filled with pipette solution (Dulbecco’s PBS; in mM): 137 NaCl, 0.9 CaCl2, 2.7 KCl, 1.5 KH2PO4, 0.5 MgCl2, and 8.1 Na2HPO4, pH 7.3/NaOH. Single-channel currents were recorded using a PC505 amplifier (Warner Instruments), low-pass filtered at 20 kHz, and digitized at a sampling frequency of 50 kHz, using a National Instruments data acquisition board (SCB-68). For unliganded-activation experiments, the pipette holder and pipettes were never exposed to agonists.

For ligand-activation experiments, agonists were added to the pipette solution at the desired concentrations. The ACh-class agonists were the neurotransmitter ACh, carbamylcholine (CCh; Martin et al., 2017), tetramethylammonium (TMA), and choline (Cho), and the Epi-class agonists were the arrow toxin Epi, its synthetic analogue epiboxidine (Ebx), the very fast death factor anatoxin (Anx), and azabicyclo heptane (Aza). To estimate gating equilibrium constants, a saturating concentration of agonist ($≥10$ μM) was used. The channel activity (cluster PO; see below) increased as [Aza] diffused into the tip. We estimated the opening rate constant after ~120 s of diffusion time.

For experiments with α-conotoxin, cells were incubated in 100 nM α-conotoxin M1 (CTX M1), a specific blocker of the α-δ site (Bren and Sine, 2000) for 15 min before patching. The membrane potential was ~−100 mV when low [agonist] was used and +100 mV when high [agonist] was used.

Protein engineering

Mutations were incorporated into AChR subunits using the QuikChange site-directed mutagenesis kit (Agilent Technologies) and were verified by nucleotide sequencing. These “background” mutations were ~20 Å away from the agonist-binding sites, had no effect on agonist binding, and were added to facilitate the kinetic analyses (Jadey et al., 2011). We could not
resolve completely components of interval duration distributions having time constants briefer than \( \sim 100 \mu s \) or longer than \( \sim 200 \text{ ms} \) (see below). Hence, with WT AChRs, we could estimate accurately rate constants only over a narrow range of \( \sim 50 \text{ s}^{-1} \) to \( 10,000 \text{ s}^{-1} \). To extend this range almost indefinitely, we added mutations that only changed the unliganded gating equilibrium constant \( \Delta G_0 \) to known extents in order to place the interval durations into a readily measurable range. The mutations had no effect on binding to either the active or resting state. We multiplied the observed values by the fold changes caused by the mutations to obtain parameters for the WT condition.

The effect of each background mutation on unliganded gating was estimated by measuring its effect on gating with the weak partial agonist Cho and by assuming the change in open-channel probability \( P_O \) was entirely due to changes in unliganded gating (Fig. 4a).

To study AChRs having just one functional binding site, a disabling mutation (see below) was added to the \( \varepsilon, \gamma, \) or \( \delta \) subunit to effectively eliminate binding and activation at \( \alpha-\varepsilon, \alpha-\gamma, \) or \( \alpha-\delta \), respectively (Gupta et al., 2013). In mouse AChRs, this mutation reduces the coupling constant \( K_{UB}/K_{BR} \) for ACh from \( \sim 5,700 \) to \( \sim 12 \), to effectively eliminate activation from just the mutated site. We incorporated SP123R to make AChRs having only a functional \( \alpha-\gamma \) or \( \alpha-\varepsilon \) site, and \( \varepsilon \)P121R (adult type) or \( \gamma \)P121R (fetal type) to make AChRs having only a functional \( \alpha-\delta \) site. These mutations also change unliganded gating \( \Delta G_0 \) to an extent that was measured for each construct, in order to correct for the background. The results from the \( \delta \)P123R experiments were corroborated independently by using the \( \alpha-\delta \) site-specific inhibitor CTx MI.

To reduce the fast channel block by the agonist apparent at high concentrations, the membrane was depolarized to \(+100 \text{ mV} \) (pipette potential, \(-100 \text{ mV} \)). The effect of depolarization on unliganded gating of human AChRs was taken into account in the same way as with background mutations—namely, by correcting for the effect of voltage on the \( \Delta G_0 \). Fig. 4a (inset) shows that in adult-type human AChRs, there is an e-fold reduction in \( \Delta G_0 \) with a 66-mV depolarization. In mouse endplate AChRs, membrane potential does not influence agonist binding. All of the rate constants reported below have been corrected for the background perturbations (mutations and voltage) and pertain to WT AChRs at \(-100 \text{ mV} \).

Figure 2. **Energy measurements from electrophysiology.** The \( \alpha-\delta \) site of the adult-type human AChRs was studied in isolation after disabling the \( \alpha-\varepsilon \) site by adding the mutation \( \varepsilon \)P121R. (a) Gating with CCh. Top: Gating with CCh. [CCh] = 20 mM (to fully saturate the \( \alpha-\delta \) site) and \( V_m = +70 \text{ mV} \) (to reduce channel block by CCh). Openings (top) are clustered; intercluster gaps reflect desensitization and intracluster intervals mainly reflect \( 4 \text{R}^\text{E}_{2} \text{R}^* \) gating. Intracluster interval duration histograms (bottom) and an example cluster. (b) CCh binding. Association and dissociation rate constants were estimated by fitting across [CCh] (see Materials and methods). (c and d) Ebx gating and binding. Free energies were calculated from the equilibrium constants estimated from the forward/backward rate constant ratios.
Kinetic modeling

Kinetic analyses of single-channel currents were performed by using the QuB software suite (Nicolai and Sachs, 2013). Rate constants were obtained by analyzing clusters of single-channel activity (representing binding and gating) flanked by nonconducting intervals ≥20 ms (representing desensitization; see Fig. 2, top). The currents within clusters were idealized into noise-free intervals by using the segmental K-means algorithm after digitally filtering the data at 12 kHz (Qin, 2004). At the highest [agonist] (in mM: 10 Epi; 20 ACh, CCh, TMA, Ebx, and Anx; 50 Aza; and 100 Cho), the forward (channel-opening) rate constant ($f_1$; $n$, number of bound agonists) and backward (channel-closing) rate constant ($b_1$) were estimated from the idealized intracluster interval durations by fitting the data to a $C \leftrightarrow O \leftrightarrow D$ scheme, where $C$ is resting (closed channel and low affinity), $O$ is active (open channel and high affinity), and $D$ is a short-lived desensitized state (closed channel and high affinity) that was inside clusters (Salamone et al., 1999; Elenes and Auerbach, 2002). The rate constants of the model were optimized by using a maximum interval likelihood algorithm after imposing a dead time of 20–50 µs (Qin et al., 1997). The gating equilibrium constants were calculated from the ratios of the forward/backward rate constants, and the gating free energies in kilocalories per mole were calculated by taking the natural log and multiplying by $-0.59$ ($-RT$; $R$, universal gas constant and $T$, absolute temperature in K). The error limit on the energy values is ±0.6 kcal/mol (Gupta et al., 2017).

The gating properties of unliganded AChRs are complex. There are multiple exponential components apparent in both the shut (nonconducting) and open (conducting) dwell-time distributions. Therefore, a simple shut→open kinetic scheme

### Table 1. Human AChR rate and equilibrium constants

| Site | Agonist | $f_1$(s$^{-1}$) | $b_1$(s$^{-1}$) | $E_1$ | $k_{on}$(M$^{-1}$s$^{-1}$) | $k_{off}$(s$^{-1}$) | $K_{dR}$ (µM) | $K_{dR^*}$ (nM) |
|------|---------|----------------|----------------|-------|--------------------------|-----------------|--------------|--------------|
| α–ε | ACh     | 55.8           | 6,771          | $8.2 \times 10^{-3}$ | $5.2 \times 10^{-1}$ | $3.662$       | 70.8         | 5.5          |
|      | CCh     | 32.0           | 7,884          | $4.05 \times 10^{-3}$ | $1.7 \times 10^{-1}$ | $2.236$       | 182          | 21           |
|      | TMA     | 20.1           | 10,615         | $1.9 \times 10^{-3}$ | $7.8 \times 10^{4}$  | $4.448$       | 573          | 195          |
|      | Cho     | 2.12           | 12,325         | $1.72 \times 10^{-4}$ | $2.04 \times 10^{6}$ | $5,884$       | 2,884        | 10,867       |
| α–δ | ACh     | 24.3           | 5,292          | $4.6 \times 10^{-3}$ | $3.6 \times 10^{-1}$ | $4.631$       | 130          | 18.1         |
|      | CCh     | 10.4           | 6,830          | $1.5 \times 10^{-3}$ | $8.1 \times 10^{4}$  | $3.345$       | 413          | 176          |
|      | TMA     | 7.1            | 8,540          | $8.3 \times 10^{-4}$ | $4.6 \times 10^{8}$  | $3,559$       | 773          | 587          |
|      | Cho     | 1.1            | 12,950         | $8.5 \times 10^{-5}$ | $1.6 \times 10^{6}$  | $7,601$       | 4,750        | 34,697       |
| α–γ | ACh     | 377            | 6,658          | $5.6 \times 10^{-5}$ | $2.9 \times 10^{6}$  | $4,020$       | 13.8         | 0.02         |
|      | CCh     | 65.4           | 8,167          | $8.0 \times 10^{-6}$ | $6.9 \times 10^{7}$  | $7,689$       | 934          | 6,053        |
|      | TMA     | 23.5           | 12,071         | $1.9 \times 10^{-3}$ | $2.7 \times 10^{7}$  | $8,696$       | 322          | 14.9         |
|      | Cho     | 5.5            | 13,598         | $4.1 \times 10^{-4}$ | $8.8 \times 10^{6}$  | $10,456$      | 1,188        | 230          |

The active-state equilibrium constant was calculated from the activation thermodynamic cycle (Fig. 1 b) assuming microscopic reversibility, $K_{dR^*} = (K_{dR}E_0/E_1)$, where $E_0$ is the unliganded gating equilibrium constant and is equal to $6.6 \times 10^{-7}$ (Δ$G_0$ = 8.4 kcal/mol) in adult-type and $8.6 \times 10^{-8}$ (Δ$G_0$ = 9.6 kcal/mol) in fetal-type AChRs. $f_1$ and $b_1$, monoligated forward and backward gating rate constants ($E_1 = f_1/b_1$), $k_{on}$ and $k_{off}$ agonist association and dissociation rate constants to a resting receptor ($K_{dR} = k_{off}/k_{on}$).

### Table 2. Human AChR gating and binding free energy changes

| Site | Agonist | Δ$G_1$ | Δ$G_R$ | Δ$G_{R^*}$ |
|------|---------|--------|--------|------------|
| α–ε | ACh     | 2.8    | -5.6   | -11.2      |
|      | CCh     | 3.3    | -5.1   | -10.4      |
|      | TMA     | 3.7    | -4.4   | -9.1       |
|      | Cho     | 5.1    | -3.4   | -6.7       |
| α–δ | ACh     | 3.2    | -5.3   | -10.5      |
|      | CCh     | 3.8    | -4.6   | -9.2       |
|      | TMA     | 4.2    | -4.2   | -8.5       |
|      | Cho     | 5.5    | -3.2   | -6.1       |
| α–γ | Epi     | 3.7    | -7.0   | -11.7      |
|      | Ebx     | 4.0    | -5.9   | -10.4      |
|      | Anx     | 4.8    | -5.0   | -8.7       |
|      | Aza     | 5.4    | -4.1   | -7.1       |
| α–γ | ACh     | 1.7    | -5.2   | -13.1      |
|      | CCh     | 2.9    | -5.4   | -12.1      |
|      | TMA     | 3.7    | -4.7   | -10.6      |
|      | Cho     | 4.6    | -4.1   | -9.0       |

All values are kilocalories per mole. Δ$G_1$, gating with one bound agonist; Δ$G_R$, binding to the resting conformation; Δ$G_{R^*}$, binding to the active conformation (Fig. 1 b).
is inadequate to describe unliganded gating activity. In mouse AChRs, unliganded gating schemes have three shut and two open states, irrespective of background mutations (Grosman and Auerbach, 2000; Gonzalez-Gutierrez and Grosman, 2010; Nayak and Auerbach, 2017). We did not carry out elaborate modeling of unliganded gating in human AChRs. Instead, we estimated the unliganded gating forward and backward rate constants, $f_0$ and $b_0$, from the inverse of time constant of the predominant components of the shut and open dwell-time distributions (Nayak et al., 2012). Hence, the occasional, unliganded long openings were excluded.

To estimate the single-site association and dissociation rate constants to resting AChRs ($k_{on}$ and $k_{off}$) we fitted globally intracluster interval durations across $\sim \mu M$ [agonist], using a bind-and-gate activation scheme (the clockwise activation pathway in Fig. 1b):

$$A + R \xrightleftharpoons{} A R \xrightleftharpoons{} A R^*,$$

where $R$ is a resting receptor, $R^*$ is an active receptor, and superscript $A$ is the agonist. The first step is binding to the resting state, and the second step is the global gating isomerization. The resting affinity ($K_{dR}$) was estimated as the ratio of the rate constants for the first step, $k_{off}/k_{on}$. $K_{dR}$ values were calculated from the cycle by assuming microscopic reversibility.

A free energy change ($\Delta G$) is proportional to the logarithm of the equilibrium constant ($K_{eq}$), $\Delta G = -RT\ln K_{eq}$, where $R$ is the gas constant and $T$ is the absolute temperature ($RT = 0.59$ at 23°C). In the cycle, $\Delta G_R$ and $\Delta G_R^*$ are the free-energy changes associated with low- and high-affinity binding to resting and active conformations (equilibrium dissociation constants $K_{dR}$ and $K_{dR^*}$).

**Statistical analyses of efficiency plots for nonnicotinic receptors**

In the analyses of published data from receptors other than endplate AChRs, we assumed equivalent and independent binding sites. In some reports, a gating equilibrium constant ($E$) was given, and in others, we calculated it from the maximum response ($P_{Dmax}$),

$$P_{Dmax} = \frac{1}{1 + \frac{1}{E}}.$$

To estimate more accurately the slopes and intercepts of the efficiency plots of nonnicotinic receptors, outliers were identified statistically by a forward search algorithm (Hadi and Simonoff, 1993; Atkinson, 1994). In brief, the method orders the points by their closeness to the fitted model (in this instance, see Eq. 3, in Results) starting with an initial set of fewer observations and extending the regression to a larger dataset, with outliers identified by estimating the residuals. The method is insensitive to the choice of initial subset so long as it is free of “unmasked” (obvious) outliers. We calculated the residuals for each dataset using Excel and plotted them versus the predicted y values from the fitted model to identify the outliers.

**Mutations**

As described above, in order to make the low-$P_D$ AChRs constructs more amenable to single-channel kinetic analysis, we added background mutations that made $\Delta G_0$ (and, hence, $\Delta G^*$) more favorable but did not influence binding (Jadey et al., 2011). For example, the monoliganded gating equilibrium constant with CCh ($E_1^{CCh}$) at $\alpha-\delta$ was measured using 20 mM CCh with the added background perturbations $\alpha P272A + \delta L265T$ (to make $\Delta G_0$ less positive), EPI12IR (to disable the $\alpha-\varepsilon$ binding site), and $V_m = +100$ mV (to reduce channel block by CCh). These four perturbations changed the unliganded gating equilibrium constant by 182-, 37-, 0.1416-, and 0.1-fold, respectively, and together increased the unliganded gating equilibrium constant ($E_0$) by $\sim 100$-fold. The observed $E_1^{CCh}$ was 0.15 ($f_1 = 89$ s$^{-1}$/b$_1 = 583$ s$^{-1}$), which was corrected to the WT condition by dividing by 100 ($1.5 \times 10^{-3}$). For weaker agonists, a larger boost in unliganded gating was required; for instance, $\alpha D97A + \alpha Y127F + \alpha S269I + \varepsilon P121R$, which, in combination, increase $E_0$ by 2,981-fold.
Chemicals

NaCl, KCl, CaCl₂, MgCl₂, HEPES, NaOH, KOH, KH₂PO₄, Na₂HPO₄, ACh chloride, CCh, TMA, Cho, and Ebx were purchased from Sigma. Epi (±) and anatoxin A fumarate were obtained from Tocris Biosciences. 7-Azabicyclo [2.2.1] heptane was purchased from AstaTech. CTX-MI was obtained from the Alomone Laboratories.

Results

Efficiency definition

Fig. 1b shows the activation cycle for a receptor having one functional binding site. Microscopic reversibility is satisfied (Nayak and Auerbach, 2017), so

$$\Delta G_1 = \Delta G_0 + \Delta G_R \left( \frac{1}{\kappa} - 1 \right).$$

Each side of Eq. 1 is the “coupling” constant energy that determines the extent to which one bound agonist molecule increases activity above the baseline level.

The energy conversion efficiency ($\eta$) of a machine is the useful output energy divided by the total input energy (Schroeder, 1999). In a receptor, the useful output energy is that for activation above the baseline that from Eq. 1 is equal to the active-resting difference in binding free energy, $\Delta G_R^\ast - \Delta G_R$. The total input energy is the maximum from the ligand, $\Delta G_R^\ast$. Hence, agonist energy efficiency at a given binding site is

$$\eta = 1 - \Delta G_R / \Delta G_R^\ast.$$

An energy efficiency can be calculated for any agonist at any binding site of any receptor (that operates by a cyclic mechanism) from the resting/active binding energy ratio, that is equal to the ratio of the logarithms of the equilibrium dissociation constants ($\log K_{dR} / \log K_{dR^*}$).

In endplate AChRs and for a series of ACh-class agonists, experiments show that the binding-energy ratio is a constant (Jadey and Auerbach, 2012),

$$\kappa = \Delta G_R / \Delta G_R^\ast.$$

Rearranging Eq. 1 and substituting,

$$\Delta G_1 = \Delta G_0 + \Delta G_R \left( \frac{1}{\kappa} - 1 \right),$$

and from Eq. 2,

$$\Delta G_1 = \Delta G_0 + \Delta G_R \left( \eta / (1 - \eta) \right).$$

Figure 4. Intrinsic gating of human AChRs. (a) Left: Mutations far from the binding sites produce similar changes in the diliganded gating energy with Cho ($\Delta \Delta G_{2\text{Cho}}$) in mouse and human AChRs (slope, 1.0 ± 0.1; $R^2 = 0.95$). Each symbol is a different mutation. Right: In adult-type human AChRs, $\Delta \Delta G_{2\text{Cho}}$ is caused exclusively by a change in the unliganded gating energy ($\Delta \Delta G_{0\text{obs}}$; slope = 1.0 ± 0.1; $R^2 = 0.91$; dashed lines, 95% confidence limits). The y intercept (no change in $\Delta \Delta G_{2\text{Cho}}$) is $\Delta G_0$ in the WT. Inset: Voltage dependence of $E_0$ in adult-type human AChRs. (b) Example unliganded single-channel current clusters from mutations added to four different background constructs. The clusters (top to bottom) and the backgrounds (left to right) are arranged with increasing open-channel probability (excluding long openings).
Eq. 3 describes an “efficiency” plot, which is a plot of $\Delta G_1$ versus $\Delta G_R$ ($\log E_1$ versus $\log K_{dR}$) for a series of agonists. If the energy efficiency is the same for all of the agonists, then the points will fall on a straight line with slope $\eta/(1 - \eta)$ and y intercept $\Delta G_0$. An average $\eta$ value is estimated from the slope,

$$
\eta = \frac{\text{slope}}{1 + \text{slope}}.
$$

Human endplate AChRs

To study one human endplate AChR neurotransmitter–binding site at a time, a mutation (or toxin) was added to disable the companion site, and background mutations were added to make $\Delta G_0$ more favorable so that a single agonist molecule would produce an easily measured response. The background mutations only decreased $\Delta G_0$ and had no effect on either $\Delta G_R$ or $\Delta G_{R*}$. The decrease in $\Delta G_0$ resulted in an equivalent decrease in $\Delta G_1$ (Eq. 3) and, hence, an increased level of activity that allowed rate constants to be estimated from single-channel interval durations at different agonist concentrations (Fig. 2). Rate constant ratios for binding and gating are equilibrium constants (Table 1), the logs of which are proportional to $\Delta G_R$ and $\Delta G_1$ (Table 2).

Fig. 3 b shows efficiency plots for ACh- and Epi-class agonists at the $\alpha$–$\delta$ binding site. Within each agonist family, there is a range of $\Delta G_R$ and $\Delta G_1$ values, but because the points fall on the same line we conclude that all four ligands within each class have approximately the same energy efficiency. From the slopes of the linear fits (Eq. 4), we estimate that $\eta_{\text{ACh-class}} = 0.53 \pm 0.04$ and $\eta_{\text{Epi-class}} = 0.39 \pm 0.05$ (mean $\pm$ SD). At the $\alpha$–$\delta$ binding site (that is common to adult and fetal AChRs), ACh-class agonists are $\sim 35\%$ more efficient than Epi-class agonists at converting agonist-binding energy into kinetic energy for gating. The average of the y intercepts, +8.5 kcal/mol, estimates $\Delta G_0$ in adult-type human AChRs (at $-100 \text{ mV}$) and is the same value as in adult-type mouse AChRs.

We repeated these experiments with ACh-class agonists and AChRs having only a functional $\alpha$–$\epsilon$ or $\alpha$–$\gamma$ binding site (Fig. 3 c).

### Table 3. Effect of mutations on $\Delta G_{2\text{Cho}}$ in human AChRs

| Mutation | $f_1(s^{-1})$ | $b_2(s^{-1})$ | $E_{2\text{Cho}}$ | $E_2^{\text{mut/Cho}}/E_2^{\text{WT}}$ | $\Delta G_{2\text{Cho}}$ |
|----------|--------------|---------------|-----------------|-------------------------------------|---------------------|
| $-\text{E}45R$ | 76           | 2,252         | 0.034           | 1                                   | 0.0                 |
| $\alpha96V$ | 4,002        | 2,095         | 1.91            | 56                                  | -2.4                |
| $\alpha979A$ | 3,380        | 885           | 3.82            | 112                                 | -2.8                |
| $\alpha127F$ | 6,624        | 1,533         | 4.3             | 126                                 | -2.8                |
| $\alpha5266E$ | 3,471        | 1,001         | 3.47            | 102                                 | -2.7                |
| $\alpha5266I$ | 30           | 3,023         | 0.01            | 0.30                                | 0.7                 |
| $\alpha5269I$ | 1,832        | 706           | 2.6             | 77                                  | -2.6                |
| $\alphaP272A$ | 1,458        | 236           | 6.2             | 182                                 | -3.1                |
| $\alphaC1418W$ | 731           | 184           | 3.98            | 116                                 | -2.8                |
| $\betaL262T$ | 844           | 675           | 1.25            | 36                                  | -2.1                |
| $\betaV266A$ | 424           | 28            | 15.1            | 445                                 | -3.6                |
| $\betaT456I$ | 101           | 826           | 0.14            | 3.6                                 | -0.8                |
| $\betaT456F$ | 314           | 342           | 0.92            | 27                                  | -1.9                |
| $\deltaI43Q$ | 200           | 1,893         | 0.105           | 3.1                                 | -0.7                |
| $\deltaI43H$ | 20.5          | 5,067         | 0.004           | 0.12                                | 1.3                 |
| $\deltaL265T$ | 190           | 148           | 1.48            | 37                                  | -2.1                |
| $\deltaL265S$ | 172           | 9.2           | 18.7            | 550                                 | -3.7                |
| $\epsilonL261S$ | 1,956         | 134           | 14.6            | 429                                 | -3.6                |
| $\epsilonL269F$ | 831           | 197           | 4.2             | 124                                 | -2.8                |

E$_2 = f_1/b_2$, $\Delta G_{2\text{Cho}}$, gating free energy change with two bound Cho molecules; $f_1$ and $b_2$, diliganded forward and backward gating rate constants.

The results were $\eta_{\text{ACh-class}} = 0.50 \pm 0.08$ and $0.56 \pm 0.02$. ACh-class agonists have approximately the same energy efficiency at the two adult sites ($\alpha$–$\delta$ and $\alpha$–$\epsilon$) but, perhaps, a slightly greater efficiency at the fetal $\alpha$–$\gamma$ site. It appears that the same ligand can

### Table 4. Mutant AChR construct unliganded gating rates, equilibrium constants and free energies

| Construct | $f_0(s^{-1})$ | $b_0(s^{-1})$ | $E_0^{\text{mut/Cho}}$ | $E_0^{\text{WT}}$ | $\Delta G_0^{\text{Obs}}$ | $\Delta G_{2\text{Cho}}^{\text{mut/Cho}}$ |
|-----------|---------------|---------------|------------------------|------------------|--------------------------|--------------------------------------|
| aN217KβL262TδL265T | 22.5 (6) | 786 (192) | 0.028 (0.01) | 2.1 (0.18) | 3.9 $\times 10^4$ | -6.25 |
| aD979AβL262TδL265T | 19 (2) | 260 (17) | 0.073 (0.009) | 1.5 (0.07) | 1.7 $\times 10^5$ | -7.08 |
| aL279WβL262TδL265T | 56 (4) | 633 (54) | 0.088 (0.01) | 1.4 (0.067) | 1.7 $\times 10^5$ | -7.1 |
| aC1418WβL262TδL265T | 85 (7) | 879 (92) | 0.095 (0.013) | 1.4 (0.08) | 1.6 $\times 10^5$ | -7.1 |
| aP272AβL262TδL265T | 225 (58) | 889 (78) | 0.25 (0.06) | 0.8 (0.12) | 2.4 $\times 10^5$ | -7.3 |
| aN217KβL262TδL265TεS450W | 35 (4.3) | 126 (13) | 0.27 (0.04) | 0.8 (0.09) | 2.9 $\times 10^5$ | -7.43 |
| aY127FβL262TδL265TεS450W | 433 (14) | 752 (35) | 0.58 (0.02) | 0.3 (0.02) | 9.3 $\times 10^5$ | -8.1 |
| aC1418WβV266AδL265S | 5285 (236) | 120 (14) | 44.1 (5.1) | -2.2 (0.07) | 2.9 $\times 10^5$ | -10.1 |
| βT456IδL265SεL261S | 269 (34) | 825 (93) | 0.32 (0.06) | 0.7 (0.11) | 8.4 $\times 10^5$ | -8.0 |
| aA96VβV266AδL265S | 735 (55) | 160 (17) | 4.6 (0.22) | -0.9 (0.07) | 2.7 $\times 10^5$ | -10.0 |
| aA96VβV266AεL261S | 6,925 (655) | 477 (126) | 14.6 (4.0) | -1.6 (0.16) | 2.1 $\times 10^5$ | -9.9 |

Free energies are in kilocalories per mole. $E_2 = f_1/b_2$, $\Delta G_{2\text{Cho}}$, gating free energy change with two bound Cho molecules; $f_0$ and $b_0$, unliganded forward and backward gating rate constants (±SEM, n patches); $\Delta G_0$, change in gating free energy with two bound Cho molecules.
have different efficiencies at different binding sites. As expected, the y intercept of the α−ε plot gives the same ΔG₀ as in the α−δ plot, but that from the α−γ plot estimates the intrinsic gating energy of fetal-type human AChRs to be +9.6 kcal/mol, again similar to the mouse fetal-type AChR value.

It is of considerable importance to know the intrinsic gating energy of a receptor, so we applied two additional methods to measure it more accurately in adult-type human AChRs. Many mutations away from the binding sites have the same effect on gating with two bound Cho molecules (ΔG₂Cho) in human and mouse AChRs (Fig. 4 a, left). We assumed that, as in mouse, the observed changes relative to the WT (ΔΔG₂Cho; Table 3) were caused exclusively by equivalent changes in intrinsic gating (ΔΔG₀). We measured ΔG₀ for human AChR mutants (Table 4) and plotted the values against the corresponding values of ΔΔG₂Cho (Fig. 4 a, right). The slope of the fitted straight line was 1.0 ± 0.1, validating the assumption. The y intercept of the plot in Fig. 4 b provides a second estimate of ΔG₀, +8.4 ± 0.8 kcal/mol.

A third method of estimating ΔG₀ does not require extrapolation or mutations (Jha and Auerbach, 2010). When the binding sites operate independently (see below), the difference between gating energies with two versus one bound agonist is the same as the difference between one versus none,

\[ ΔG₂ - ΔG₁ = ΔG₁ - ΔG₀, \]

where ΔG₁ is the average of the two, single-site gating energies. We measured ΔG₂ and calculated ΔG₁ from the single-site ΔG₁ values. The calculated average ΔG₀ for the four agonists at adult-type binding sites was +8.3 kcal/mol.

All three methods of estimating ΔG₀ produced the same result. We estimate that the human AChR intrinsic gating energies are 8.4 kcal/mol in adult-type and 9.6 kcal/mol in fetal-type AChRs, which correspond to unliganded gating equilibrium constants.
Table 5. Energy efficiencies (η, for the native agonist) and intrinsic gating energies (ΔG₀)

| Receptor | η % | ΔG₀ (kcal/mol) |
|----------|-----|----------------|
| Endplate AChR | | |
| Human | | |
| a−ε | 47 | 8.4 |
| a−δ | 51 | |
| a−γ | 56 | 9.6 (w/a−δ) |
| Mouse | | |
| a−ε | 55 | 8.4 |
| a−δ | 58 | |
| a−γ | 59 | 9.8 (w/a−δ) |
| Human a1β2y2S GABA A receptor (constitutive PO values) | 39 | 1.9 |
| Human NR1A/NR2A NMDA receptor (unliganded Gly site, Glu site saturated) | 49 | 3.6 |
| Human NR1A/NR2A NMDA receptor (unliganded Gly site, Glu site saturated) | 57 | 9.3 |
| Human M3 muscarinic receptor | 52 | 5.4 |
| Human α1 GlyR | 59 | 4.5 |
| Fish aryl-hydrocarbon nuclear receptor | 50 | 12.3 |

ΔG₀ values for endplate AChRs are for adult (α−ε and α−δ) or fetal types (α−γ).

(Constitutive PO values) of 6.6 × 10⁻⁷ in adult-type and 8.6 × 10⁻⁸ in fetal-type AChRs.

To learn if the two WT binding sites interact with each other with regard to receptor activation, we compared the two-site gating energies with the sums of one-site gating energies. The two were the same in both adult- and fetal-type human AChRs, for all agonists. As in mouse AChRs (Nayak and Auerbach, 2017), the human AChR-binding sites operate independently with regard to activation by agonists.

Other receptors

Next, we investigated energy efficiency in other receptors. In terms of equilibrium constants, Eq. 2 is

\[
\eta = 1 - \log(K_{DR})/\log(K_{DR^*}),
\]

where K_{DR} is the equilibrium dissociation constant of the active conformation and K_{DR^*} is the equilibrium dissociation constant of the resting conformation (Fig. 1 b). For example, K_{DR} and K_{DR^*} for ACh measured at the mouse AChR α−ε site are 12 nM and 153 µM (Nayak and Auerbach, 2017), from which we calculate η_{ACh} = 52%.

We used Eq. 5 to estimate the efficiency of the agonist Ca²⁺ at binding sites of K_{Ca,1.1} (BK; a potassium-selective ion channel) using published values of the equilibrium dissociation constants (Sweet and Cox, 2008). At Ca-bowl sites, K_{DR} = 3.1 mM and K_{DR^*}=0.9 µM, from which we calculate η_{Ca} = 9%. At RCK1 sites, K_{DR} = 15.8 mM and K_{DR^*} = 2.1 µM, from which we calculate η_{Ca} = 13%.

So far, binding equilibrium constants have been published only for Ca²⁺, so we could not make an efficiency plot and ascertain if other agonists of K_{Ca,1.1} have the same energy efficiency.

Affinities and efficacies for agonist series have been reported for several other receptors, including M3 muscarinic (Sykes et al., 2009), GABA A (Mortensen et al., 2004), glycine (Lewis et al., 2003), NMDA (Priestley and Kemp, 1994; Priestley et al., 1995), and aryl-hydrocarbon (Hestermann et al., 2000). From these, we could calculate gating and binding energies and construct efficiency plots to estimate η and ΔG₀ (Fig. 5). In all of these receptors except M3, a positive correlation between binding and gating energies is apparent. We considered that the scatter in these plots was caused, in part, by including agonists that belong to different energy efficiency classes. For example, combining all of the points for ACh- and Epi-class agonists at the human AChR α−δ site (Fig. 2 a) would obscure the linear relationship between gating and binding energies apparent for each agonist family.

To improve the accuracy of the slope and intercept estimates for the non-nicotinic receptors, we used an unbiased, statistical method to identify outliers (see Materials and methods). After their removal, the activation versus binding free energies all fell on the same line, including for M3. This result suggests that in these receptors and for these agonists there is a constant energy efficiency and, hence, a fixed binding-energy ratio. In Fig. 5, the η values estimated from the slopes are in the range of 39–59% and the ΔG₀ values estimated from the y intercepts are in the range of 1.9–12 kcal/mol (Table 5).

In some cases, the “outlier” ligands had structures that differed from the main group. For example, in GABA A receptors, the outliers were the only agonists with a sulfur atom, and in M3 muscarinic receptors, the outliers were large and with rings. This result supports the hypothesis that combining data from agonists belonging to different efficiency classes creates scatter in the efficiency plots. However, for other receptors, the basis for the scatter was less clear and possibly can be attributed to experimental errors.

Rate-equilibrium free energy relationships (REFERs)

Our fundamental measurements were rate constants, so we were also able to probe the transition states of binding and gating in human AChR activation. Fig. 6 shows REFERs for binding and gating in human AChRs activated by different agonists. The REF ER slope (φ) gives the relative extent to which the agonist dependence of the equilibrium constant is determined by changes in the forward versus backward rate constant on a scale from 1 to 0. For ACh-class agonists, the single-site φ-value for both binding and gating is ~0.83, indicating that differences between the agonists are caused mainly by differences in the forward processes, namely agonist association and channel opening. The binding and gating φ-values were similar at α−δ, α−ε and α−γ sites. For Epi-class agonists at α−δ, the binding φ-value was smaller (0.70) and the gating φ-value larger (0.93) than for ACh-class agonists.

That is, with Epi compared with ACh, the transition state for binding is earlier (when achieved, the ligand is more “free-like” in energy) and that for gating is later (the ligand is a more “open-like” in energy).
Efficacy is $\Delta G_R - \Delta G_R^*$, and efficiency is $1 - \Delta G_R / \Delta G_R^*$.

At all sites, agonists differ mainly with regard to association rate constant $k_{on}$ (M$^{-1}$s$^{-1}$), association rate constant; $K_dR$, equilibrium dissociation constant. At all sites, agonists differ mainly with regard to the forward versus backward rate constant.

Energy conversion efficiency ($\eta$) is the fraction of the stimulus energy transformed into the mechanical work of a global conformational change. In energy terms, affinity is $\Delta G_R$ or $\Delta G_R^*$, relative energy transformed into the mechanical work of a global conformational change. Ca$^{2+}$ at KCa1.1-binding site is substantially less efficient, for unknown reasons. It is possible that the low per-site efficiency is compensated by the large number of binding sites ($n = 8$).

The spread in receptor $\Delta G_0$ values is substantial. The estimate for GABA$A$ receptors suggests a relatively high level of constitutive activity ($P_o \sim 4 \times 10^{-2}$), consistent with literature reports (Wagner et al., 2005; Shin et al., 2017). M3 muscarinic, glycine, and NMDA receptors appear to be less active in the absence of agonists ($\sim 10^{-4}$). Interestingly, the intercepts of the efficiency plots for the glycine versus glutamate agonist series suggests that NMDA receptors have an even lower level of constitutive activity in the absence of the coagonist glycine compared with the neurotransmitter glutamate. Adult-type neuromuscular synapses (mouse and human) and KCa1.1 channels have about the same probability of being active constitutively ($10^{-7}$). Of the receptors we examined, the fetal endplate and aryl-hydrocarbon receptors have the most positive $\Delta G_0$ and, hence, the smallest estimated level of constitutive activity ($\sim 10^{-8}$). Even in this small sample, there is a wide range in constitutive $P_o$.

In mouse AChRs, only a few amino acids at the neurotransmitter binding site determine the agonist-binding energies, whereas a large number of amino acids throughout the protein determine $\Delta G_0$ (Corringer et al., 2000; Sine, 2012; Auerbach, 2013; Purohit et al., 2013). The physiological reasons for the wide variation in the level of constitutive activity are not known ($\sim 15$-fold smaller in fetal versus adult endplate AChRs and $\sim 70$-fold larger in GABA$A$ versus glycine receptors). However, the wide range in $\Delta G_0$ values and the participation of many side chains suggest that the level of intrinsic activity is fine tuned by natural selection. We note that the lower intrinsic activity of fetal versus adult endplate receptors pertains to both mouse and human AChRs.

Implications of $\eta$

In this section, we discuss the value of knowing energy efficiency. First, $\eta$ informs of the binding mechanism. The main activation
pathway connecting R with $^4\text{R}^*$ (Fig. 1b) involves the formation of a low-affinity complex followed by a switch (within the gating isomerization) to a high-affinity complex: $\text{A+R} \leftrightarrow \text{AR} \leftrightarrow \text{AR}^*$. The corresponding ligand-dependent free energy changes in this two-step sequence are $\Delta G_R$ and $(\Delta G_{R^*} - \Delta G_R)$. A linear efficiency plot indicates that $\Delta G_R/\Delta G_{R^*}$ is the same for all agonists, or that $\Delta G_R$ is a constant fraction of $\Delta G_{R^*}$ for all agonists in the family. Hence, a shared efficiency implies that the energy changes in the two steps in the above reaction sequence are correlated linearly.

Several lines of evidence suggest that in endplate and other receptors, both steps involve local rearrangements of the binding sites. In mouse AChRs (Nayak and Auerbach, 2017) and all of the receptors shown in Fig. 5, the resting association rate constant ($k_{\text{on}}$) is slower than diffusion (Grewer, 1999; Lewis et al., 2003; Dravid et al., 2008; Sykes et al., 2009; Mortensen et al., 2010). This suggests that the formation of the low-affinity complex is not by diffusion alone. Also, $k_{\text{on}}$ can be highly temperature dependent in AChRs (Gupta and Auerbach, 2011) and independent of the agonist’s diffusion constant in nicotinic and GABA$_\gamma$ receptors (Zhang et al., 1995; Jones et al., 2001; Jadey and Auerbach, 2012). These results suggest that A+R$^2\text{R}^2\text{R}$ involves a local rearrangement of the binding site (“catch”). Certainly, the subsequent $^4\text{R}^2\text{R}^2\text{R}^*$, affinity-changing step that triggers the global isomerization (“hold”) involves structural changes at the binding sites.

The linear efficiency plots suggest that in AChRs and the receptors shown in Fig. 5, the energy change associated with low-affinity binding ($\Delta G_R$) is correlated linearly with the energy change in the switch to high affinity ($\Delta G_{R^*} - \Delta G_R$, which in an efficiency plot is the agonist-dependent part of the y axis). This correlation between catch and hold energies, however, does not necessarily imply a correlation in the catch and hold structural changes. It is possible that in some receptors, distinct ligand–protein interactions govern the energy changes in each step of the reaction sequence.

Second, $\eta$ can be used to categorize agonists. Defining an agonist family by members that have the same energy efficiency (fall on the same straight line in an efficiency plot) is a new way to classify ligands. In AChRs, it appears that the relative movement of the ligand toward the center of the binding pocket is greater for ACh-class versus Epi-class agonists (Tripathy et al., 2019). We speculate that the classification of agonists by efficiency will become increasingly useful as we learn more about the structural basis of low- versus high-affinity binding in other receptors.

Third, $\eta$ simplifies CRC analysis. There are four free energies in the activation cycle, but one is constrained by microscopic reversibility and $\Delta G_0$ is agonist independent, leaving just two to be measured for each ligand. If the agonist’s efficiency is known, then only one energy value needs to be measured in order to construct a full CRC. An experimental measurement of either the resting affinity or gating equilibrium constant is sufficient (Auerbach, 2016). Once the receptor and agonist family have been calibrated ($\Delta G_0$ and $\eta$ have been measured), an entire CRC, including absolute efficacy and EC$_{50}$, can be calculated from just one affinity estimate, either for a resting or active site.

**Human versus mouse AChRs**

Our study of human AChRs involved a comprehensive analysis of binding and gating rate and equilibrium constants for eight different agonists at three kinds of binding sites (Fig. 7). Some values for adult-type human AChRs were reported previously based on kinetic modeling of single-channel currents from receptors having two functional binding sites (Wang et al., 1997; Mukhtasimova et al., 2016). These previous reports suggested that $\alpha$–$\delta$ and $\alpha$–$\varepsilon$ have distinctly different affinities for ACh, CCh, Epi, and Cho, whereas our results show unambiguously that these affinities are almost the same at the two human adult neurotransmitter-binding sites (within a factor of ∼2, or ∼0.5 kcal/mol; Tables 1 and 2). As pointed out elsewhere (Salamone et al., 1999), this discrepancy can be traced to a modeling error in the previous experiments. In AChRs, there is an approximately millisecond shut interval component apparent at all agonist concentrations that may reflect sojourns in a short-lived desensitized state (Elenes and Auerbach, 2002).

If, as in the previous analyses, this state is not included in the modeling scheme, then the equilibrium dissociation constant of one binding step will be underestimated, leading to the incorrect conclusion that the two sites have different affinities. Our results using individual binding sites show definitively...
that the adult sites of human AChRs have approximately the same affinities for the tested agonists and, furthermore, operate independently.

Binding and gating constants of human endplate AChRs are almost the same as those in mouse endplate AChRs, for both fetal and adult types. For a complete list of the results for mouse AChRs, see Nayak and Auerbach (2017). Receptor ΔG0 values, too, are nearly identical. In both species, agonists at the fetal α−γ site have higher affinities, relative efficacies, and energy efficiencies than those at either adult site. The only significant difference between human and mouse AChRs we have detected so far is that binding and gating φ-values for ACh-class agonists are lower in human AChRs (~0.8 versus ~0.9; Fig. 6), but for unknown reasons. We also observed that there is greater kinetic heterogeneity in human versus mouse AChRs that may be caused by amino acid differences in the δ subunit in the region that flanks a conserved glycine in loop E (Vij et al., 2018).

Mouse and human AChRs share ~90% sequence identity. There are n = 10 (α−γ) or n = 21 (α−δ or α−ε) amino acid mismatches between human and mouse AChRs within 20 Å of the aromatic cluster of the binding site. The similarity in function between species suggests that these mismatches (in combination) have little effect on binding, efficacy, energy efficiency, or intrinsic gating. The conservation of the fetal versus adult ΔG0 difference between species suggests that the specific values are optimal, but different, at developing versus mature neuromuscular synapses.

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