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Title Page

Dose-response analysis when there is a correlation between affinity and efficacy

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Running title page

Affinity-efficacy correlation in AChRs

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Non-standard abbreviations:
 CRC, concentration-response curve
 AChR, acetylcholine receptor

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ABSTRACT

The shape of a concentration-response curve (CRC) is determined by underlying equilibrium constants for agonist binding and receptor conformational change. Typically, agonists are characterized by the empirical CRC parameters efficacy (the maximum response), EC_{50} (the concentration that produces a half-maximum response) and Hill coefficient (the maximum slope of the response). Ligands activate receptors because they bind with higher affinity to the active vs. resting conformation, and in skeletal muscle nicotinic acetylcholine receptors there is an exponential relationship between these two equilibrium dissociation constants. Consequently, knowledge of two receptor-specific, agonist-independent constants - the activation equilibrium constant without agonists (E_o) and the affinity-correlation exponent (M) - allows an entire CRC to be calculated from a measurement of either efficacy *or* affinity. Here, I describe methods for estimating CRCs of partial agonists in receptors that have a correlation between affinity and efficacy.

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INTRODUCTION

A simple reaction scheme describes the basic mechanism of agonist action: (L+R) ↔ LR ↔ LR* (Colquhoun, 1998; Del Castillo and Katz, 1957). L is the ligand, R is the resting (inactive) receptor and R* is the active receptor. The equilibrium dissociation constant for the first, ligand-binding step is K_d, which is "affinity". The equilibrium constant for the second, receptor-activation step is E_1 (the subscript indicates the number of bound ligands) which relates to the maximum response for that ligand, or "efficacy". The reaction can be extended to accommodate multiple agonist-binding steps. Together, the equilibrium constants K_d and E_n determine the response as a function of ligand concentration (Fig. 1).

Nicotinic endplate acetylcholine receptors (AChRs) mediate synaptic transmission between vertebrate nerve and skeletal muscle (Ashcroft, 2000; Changeux and Edelstein, 2005). The CRC methods described below are based on the experimental observation that in these receptors, affinity and efficacy are not independent (4). In endplate AChRs, if you know E_n then you also know K_d (and *vice versa*).

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MATERIALS AND METHODS

Simulations and analyses were done using QUB software (www.qub.buffalo.edu). The model was a linear scheme with 2 equivalent agonist binding steps (Fig. 1), with backward/forward rate constants for binding (10,000 s^{-1}/[L]·100 $(\mu Ms)^{-1}$; K_d=100 μM) and forward/backward rate constants for activation (1000 s^{-1}/2000s^{-1}; E_2=0.5). In the multi-receptor simulations the sampling interval was 10 kHz and the peak macroscopic response as a function of the agonist concentration was fitted by the Hill equation. In the single-receptor simulations the sampling interval was 100 kHz and the rate constants were estimated by fitting interval durations globally by the 2-site scheme (Purohit et al., 2015).

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RESULTS

Affinity-efficacy correlation. Fig. 1 shows by simulation 2 ways to estimate K_d and E_2. For the example partial agonist, the high-concentration asymptote of the macroscopic peak response was 0.34 (a full agonist would be ~1) and the EC_{50} was 173 μM. From the equations in Fig. 1 we calculate E_2=0.52 and K_d=102 μM. The rate constants obtained from fitting single-receptor interval durations are given in the legend, with the result K_d=99 μM and E_2=0.50. Both methods give the same equilibrium constant estimates and require the measurement of responses to mutliple [agonist].

Receptor desensitization can alter the shape of the CRC and the equilibrium constant estimates. When desensitization is as fast (or faster) than activation, peak macroscopic responses are truncated, especially at high [agonist] and possibly to different extents with different agonists. When desensitization and activation mix, the CRC parameters efficacy and EC_{50} do not provide accurate estimates of either E_n or K_d. In single-receptor responses desensitization typically appears as long-duration gaps (inactive periods) with lifetimes that do not change with [agonist](Sakmann et al., 1980). These, too, can foil rate and equilbrium constant estimation(Salamone et al., 1999). In the experimental results shown below, desensitization events were excluded from the analysis.

There are many additional obfuscating factors in dose-response analyses, including solution exchange times, receptor heterogeneity, modal activity, inhibition by the agonist (for example, channel-block), non-stationary receptor number, unequal binding sites, activation of partially-liganded receptors, sublevel responses of single receptors, kinetic complexity and rate/equilibrium constants that are too fast/large or slow/small to be measured accurately. In the preparation I consider below – single-channel currents from adult-type mouse skeletal muscle nicotinic AChRs expressed in HEK cells – these confounding issues were either not present or were incorporated into the analyses so that the activation equilibrium constants were estimated accurately.

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Fig. 2 shows K_d and E_2 values for 8 different agonists of endplate AChRs plotted on a log-log scale (Jadey and Auerbach, 2012; Jadey et al., 2011; Purohit et al., 2014). Remarkably, there is a linear relationship that holds over a ~25,000-fold range in affinity. Partial agonists that do not have a ring (carbamylcholine, tetramethylammonium, choline) or that have a ring (anabasine, nicotine, DMT and DMP) fall on the same line, as do those with a tertiary amino group (anabasine and nicotine) or a quaternary amino group (all of the others). The affinity-efficacy correlation in AChRs is robust. A correlation between affinity and efficacy has also been reported for partial agonists of $GABA_A$ (Jones and Westbrook, 1995) and NMDA receptors (Kalbaugh et al., 2004). The log of an equilibrium constant is proportional to the free energy difference between the end states, so the linear correlation in Fig. 2 indicates that in AChRs the energy changes in (L+R)↔LR ('binding') and LR↔LR* ('gating') are not independent but are related by a constant factor.

Cycle. Although a linear reaction scheme is an excellent approximation for most wild-type (WT) receptors, activation is more-completely described by a cyclic mechanism called MWC, after those who first proposed it for hemaglobin (Monod et al., 1965) and that was applied to AChRs shortly thereafter (Karlin, 1967) (Fig. 3). Three aspects differentiate linear and cyclic schemes. First, in a cycle a receptor can activate spontaneously in the absence of any ligands, but typically with a low probability. In adult AChRs the unliganded, R⇌R* activation equilibrium constant (the 'allosteric' constant) is $E_0 \approx 10^{-6}$ (Jackson, 1986; Nayak et al., 2012). Second, in a cycle agonists are seen to increase activity because they bind more tightly to the active vs. resting conformation of the receptor. The extra, favorable agonist-binding energy that is generated when the receptor switches *spontaneously* to the active conformation serves to increase the relative stability of R* and, hence, increase E_n over E_0 (Auerbach, 2015). I will use the symbols J_d and K_d for the higher (active) and lower (resting) affinities. For ACh and adult-type AChRs, $J_d \approx 35$ nM and $K_d \approx 150$ μM, to make

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$K_d/J_d \approx 4500$. This low/high affinity ratio is called the 'coupling' constant. For a given receptor, each agonist will have its own, characteristic coupling constant.

Agonist binding is sometimes described as 'triggering' the activation conformational change. I think that this ballistic metaphor is misleading, for two reasons. First, a diffusing ligand does not have sufficient momentum to activate mechanically. Rather, the passive, K_d-to-J_d increase in affinity provides chemical energy that serves to stabilize (relative to R) both the $R \rightleftharpoons R^*$ transition state (to increase the activation rate constant) and the R* state (to increase the probability of being active). Once a ligand has bound with low affinity it behaves similarly to the other, covalently-linked side chains, and changes position (energy) whenever called upon to do so within the global isomerization of the protein. Second, the AChR allosteric transition appears to occur by the same essential mechanism with or without agonists(Purohit and Auerbach, 2009). If the agonist is the 'trigger', what starts the process when no ligand is present? It appears that the AChR conformational change initiates, propagates and terminates spontaneously (by thermal forces alone), by mechanisms that do not require the presence of an agonist. There is evidence suggesting that the AChR channel-opening conformational change starts at a linker in the transmembrane domain rather than at an agonist-binding site(Purohit et al., 2013).

The third important property of a cycle is that without input of external energy, the product of the equilibrium constants of steps connecting any 2 states does not depend on the pathway. Equating the products for the clockwise and counter-clockwise routes between R and L_nR^* in Fig. 3,

$$E_n = E_0(K_d/J_d)^n. \qquad \text{Eq. 1}$$

Or, efficacy is related to the product of the (receptor-specific) allosteric constant E_0 and the (agonist-specific) coupling constant K_d/J_d at n equivalent agonist binding sites. In AChRs E_2^{ACh} is ~20, which is ~20 million (4500^2) times greater than E_0. The response without agonists (n=0) and at full

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saturation (n=2) is equal to $(1+1/E_n)^{-1}$. The minimum and maximum asymptotes of the adult mouse endplate AChR CRC are ~10^{-6} and ~0.96, not 0 and 1.

All of the constants in Eq. 1 have been measured independently for AChRs, with much effort. E_o was estimated by extrapolating the effects of dozens of mutations on constitutive activity back to the zero-effect intercept (Nayak et al., 2012) or from the E_2/E_1 ratio (Jha and Auerbach, 2010). K_d was measured by using standard CRC methods and multiple [L] (Jadey and Auerbach, 2012; Jadey et al., 2011). J_d was estimated either by closing the cycle (microscopic reversibility) or by CRC methods using receptors that were active constitutively because of mutations (Grosman and Auerbach, 2001; Purohit and Auerbach, 2013). In WT AChRs the CRC equations for the simple linear reaction scheme are approximately correct because the unliganded, R* state is hardly visited. The unliganded activation pathway ($R \rightleftharpoons R^*$) is rarely taken because the allosteric constant is small, and dissociation from L_nR^* is infrequent because the active-state affinity is high because of a slow dissociation rate constant(Grosman and Auerbach, 2001) However, both of these uncommon routes have been revealed experimentally. For AChRs, the cyclic mechanism has been confirmed (Auerbach, 2012).

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M. The affinity-efficacy correlation in Fig. 2 arises because the low- and high-affinity binding constants (K_d and J_d) are correlated exponentially (Jadey and Auerbach, 2012):

$$J_d = K_d^M. \qquad \text{Eq. 2}$$

Because of this relationship, the coupling constant in Eq. 1 changes from (K_d/J_d) to $K_d^{(1-M)}$. M has been estimated for different agonists of AChRs, and for all of these the *extra* energy of binding to the active receptor happens to be almost equal to the energy of binding to the resting receptor (M≈1.92). M has been measured both for agonists (Jadey and Auerbach, 2012) and for dozens of mutations of aromatic binding site residues (Purohit et al., 2014), and is approximately the same. Like E_o, M appears to be a fundamental constant of the receptor. Two non-aromatic residues at the binding site that do not

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contact the agonist have different M-values (1.1 for αG153 and 3.3 for εP121). This suggests that the characteristic value of M=1.9 pertains only to the agonist and to structural elements that interact with the ligand.

The low-high affinity correlation appears to be an intrinsic part of how the AChR operates. *A priori*, this makes sense. Establishing the low affinity complex (K_d) involves diffusion *and* a local conformational change at the binding site (Jadey and Auerbach, 2012). If this structural change continues along the same, local reaction co-ordinate, to further increase the stability of the agonist molecule (J_d), then a correlation between the affinities will prevail. This is illustrated as an energy landscape in Fig. 4, that happens to look like the letter M. For each agonist, log J_d is larger by a constant factor than log K_d, with the factor in AChRs being M=1.92. (In a previous report the factor was called κ=1/M (Purohit et al., 2014)). In AChRs, establishment of the low-affinity complex is a stopover about halfway (energetically) between the apo and high-affinity conformations of the agonist binding site. It is possible that a correlation between low- and high-affinity binding is a common property of receptors.

We can now modify Eq. 1 by incorporating Eq. 2:

$$E_n = E_o(K_d)^{n(1-M)} \qquad \text{Eq. 3}$$

$$\log(E_n) = \log(E_o) + n(1-M)\log(K_d)$$

It is relatively easy to measure K_d and E_n by a standard CRC. However, it took many years and hundreds of mutations to estimate the receptor-specific, agonist-independent constants E_o and M. By using Eq. 3, these can be estimated readily from the affinity-efficacy log-log correlation plot for a WT receptor, as follows.

The x-intercept of the plot in Fig. 2 is log(E_o). The fitted value of the intercept is -5.64±0.48, which gives E_o in the range 0.7-7.6 x 10^{-7}. The value of the allosteric constant estimated by the arduous extrapolation method was 7.4 x 10^{-7}, which is within this range (Nayak et al., 2012). In

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AChRs there are two binding sites (n=2), so the slope of the plot is 2(1-M). The fitted value of the slope is -1.90±0.16, so we calculate M=1.95±0.08, the same as the 1.92 value for agonists obtained by the more-difficult procedure. Simply by plotting K_d and E_2 against each other on a log-log scale, it is possible to estimate the allosteric constant E_0 and the low-high affinity correlation factor M. These two constants are the same for all of the agonists shown in Fig. 2.

Certainly, all ligands cannot have the same M. An antagonist is a ligand that binds but does not activate. For these, M~1, which means the same affinity for the resting and active state. An inverse agonist has an M<1. The molecular mechanism for having a particular M-value is not known. It is possible that for certain receptors and ligand families M will be constant but different than 1.9, or there could be a distribution of M values rather than a single, clear correlation.

Protocols. For physiologists, measuring the efficacy of a partial agonist is easier than measuring affinity because all that is needed is an estimate of the maximum response at a high [L]. The following whole-cell protocol can be used to estimate affinity from efficacy, given the correlation.

1) Apply a high concentration of a full agonist to estimate the maximum response. To minimize the effects of desensitization, the pulse onset should be as fast as possible. Try to minimize the effects of the other pitfalls in CRC construction, listed above.

2) In the same cell, measure EC_{50} and unnormalized efficacy values (as a fraction of the maximum response) for several partial agonists. From these calculate the activation constants using appropriate equations for the receptor in question. It is possible to fit a plot of log (EC_{50}) *vs* log (efficacy) directly to some non-linear function, but transforming these values to equilibrium constants and fitting by a straight line is perhaps more convenient.

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3) Plot the K_d and E_n estimates obtained by using this standard, multiple [L] approach on a log-log scale. A fit to a straight line will yield estimates of the fundamental constants E_0 (the intercept) and M (the slope).

4) Once the receptor has been so-calibrated, measure the efficacy (relative to the full agonist) of a novel ligand. This can be done by exposing the receptor to a very high [L]. Calculate E_n.

5) Use Eq. 3 to calculate K_d, EC_{50} (or whatever percentage) and the entire CRC, using the equations shown in Fig. 1.

For example, using the adult AChR values for the allosteric constant (E_0=7.4x10^{-7}), the J_d-K_d correlation (M=1.92) and Eq. 3 we calculate $K_d = \sqrt[1.9]{(7.4\text{x}10^{-7}/E_2)}$. Substituting the experimental estimate for ACh efficacy (0.96, or E_2≈20) we calculate K_d^{ACh}≈123 µM, which is close to the value obtained by standard CRC analysis. Using this value of K_d^{ACh} we now calculate EC_{50}^{ACh}=33 µM (Fig. 1), which again is close to the value measured by CRC analysis of whole-cell currents. It is possible to approximate K_d from an E_2 value measured at a single, saturating [ACh].

For both whole cell and single-receptor responses the typical, measureable efficacy range is ~0.05-0.90, which corresponds to $0.05<E_n<20$. Below or above this range the responses saturate and become difficult to quantify accurately. For some agonists it may be necessary to engineer E_0 (and, hence, E_n; Eq. 1) by adding a background muation(s) that does not affect K_d, so that the emergent E_n is in the suitable range (Jadey et al., 2011). The effect of the background on efficacy must be taken into account before making the log-log plot.

It is also possible to reverse the procedure, because knowledge of affinity can be used to estimate efficacy. Often, affinity is measured using a biochemical method, for example isothermal calorimetry (ITC). For partial agonists, these measurements are only 'effective' (not purely K_d) because the low and high affinities of resting, active and other conformations are jumbled, with fractional contributions weighted by the interconversion equilibrium constant(s). If M is common for all partial

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agonists of a receptor and E_o is known, then it is possible to estimate efficacy from just a single measurement of K_d, as follows.

1) Make a receptor mutation(s) that does not influence K_d or M, but that greatly reduces E_o. This will increase the stability of the resting state and reduce the mixing of affinities caused by agonist activation. In AChRs, almost all mutations away from the agonist binding sites only affect the allosteric constant (Purohit et al., 2013), and one(s) that makes it substantially smaller than the WT is what is needed here.

2) Use ITC (or another method) to characterize this receptor's resting affinity for a weak partial agonist (K_d), which is no longer 'effective' because the modified receptor has a low probability of changing affinity even when agonists are present.

3) Calculate E_n (efficacy) by using Eq. 3. With knowledge of K_d, of an agonist and the constants M and E_o for the receptor, one can estimate efficacy without expressing the receptor in a cell or making any response measurements.

There may be another way to estimate M. In mouse endplate AChRs, the affinities of the active and desensitized states are similar. Hence, if K_d and the desensitized binding equilibrium dissociation constant can both be measured experimentally for a series of ligands, it might be possible to estimate M directly, by using Eq. 2.

Aside from the CRC, it is useful to know E_o for a receptor. To make a simple analogy, a ligand is like a deposit (of energy) into your bank account and the response is the account balance after the deposit. The allosteric constant is the starting balance. One needs to know all three energies, including the starting one, in order to understand quantitatively the energy of the ligand action. In AChRs some genetic diseases are caused by mutations of residues that are distant from the agonist binding sites (Engel and Sine, 2005), and many of these do nothing more than change the allosteric

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constant E_0. This may be true generally for other receptors. Such a change in the allosteric constant will influence efficacy, EC_{50} and the CRC without affecting affinity.

Eq. 3 can be rearranged to solve for the allosteric constant. For the case of ACh and WT AChRs we calculate $E_0=(20)(123\ \mu M)^{1.9}=6.2x10^{-7}$, which is similar to the E_0 value estimated by the arduous mutation-extrapolation method. All one needs to know is M, E_n and K_d for a given receptor to estimate the allosteric constant and the effect of mutations that change the level of constitutive activity.

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DISCUSSION

Scientists have been making CRCs for decades for many different receptors, including the muscle AChR. If the affinity-efficacy correlation is general, why hasn't it been observed previously? I think there are several reasons. The correlation is between E_n and K_d, whereas the corresponding CRC empirical parameters are efficacy and EC_{50}. Often efficacy is normalized to the response generated by a full agonist and not even measured. The confounding factors mentioned above can reduce the accuracy of the efficacy and EC_{50} estimates. Further, EC_{50} is a ratio of K_d and E_n and, hence, tends to hide a correlation between these equilibrium constants. Beyond these technical considerations, advances in our concept of receptor operation serve to rationalize the affinity-efficacy correlation. We are now certain that in AChRs agonists activate receptors by binding more tightly to the active conformation, and that the low vs. high affinities are correlated exponentially because the energy (conformation) changes that undergird each binding step are related linearly (Fig. 4).

The AChR is a typical receptor so it is possible that a correlation between affinity and efficacy is a more-general phenomenon. If so, the potential impact on CRC analyses is significant. Once the allosteric constant E_0 and the correlation constant M have been calibrated for a given receptor, then a complete dose-response profile can be calculated for an arbitrary partial agonist from an experimental estimate of either efficacy *or* affinity. A rapid screening system could be used, in which a cell is exposed to a series of different drugs in rapid sequence to estimate only efficacy, rather than to a series of concentrations of one drug. In cases where affinity is easier to measure, it may be possible to estimate the efficacy of a ligand without having to express the receptor in cells and measure a physiological response. The method also allows for estimates of the allosteric activation equilibrium constant in wild-type receptors and the effect of mutations on this constant.

M is the fundamental reason for the affinity-efficacy correlation. Although this correlation has been revealed in AChRs, it is important to establish whether or not it holds for more ligands and

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other receptors. Perhaps receptor modulators, too, will show a correlation. For a given receptor, E_o will definitely be the same for all ligands and evidence from AChRs suggests that M will be the same for many. It may turn out that all receptors in a given family share a common M (set by structural changes only at the agonist binding site) but perhaps not E_o (set by many amino acids throughout the protein, by natural selection). Once a receptor has been calibrated, high-throughput CRC analysis can commence. If affinity-efficacy correlations turn out to be common, widespread knowledge of E_o and M values may impact significantly the future of dose-response analysis.

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AUTHORSHIP CONTRIBUTION

Participated in research design: Auerbach, A.
Conducted experiments: Auerbach, A.
Contributed new reagents or analytic tools: Auerbach, A.
Performed data analysis: Auerbach, A.
Wrote or contributed to the writing of the manuscript: Auerbach, A.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Example CRC. Top, a CRC simulation. Peak macroscopic responses (left inset) were fitted by the Hill equation (solid circles and line) to estimate efficacy (0.34) and EC_{50} (173 μM). Single-receptor interval durations (right inset; R* is up) were fitted across concentrations to estimate rate constants (s^{-1}) for agonist dissociation/association (1735/[L]•88) and for the forward/backward activation conformational change (1003/2013). The K_d and E_2 estimates were the same with either method. Bottom, CRC equations. L is the ligand, R is the resting receptor and R* is the active receptor. K_d is the equilbrum dissociation constant for agonist binding to R, and E_n is the activation equilibrium constant with n bound ligands. The equations assume activation of receptors with <n bound ligands is negligible and that all sites are equivalent and independent; response is the probability of a single receptor being active, EC_{50} is the [L] that gives a half-maximal response, efficacy is the maximum response to the ligand.

Figure 2. Affinity-efficacy correlation. K_d and E_2 were estimated from single-channel currents for different agonists of adult, mouse AChRs expressed in HEK cells. (Jadey and Auerbach, 2012; Jadey et al., 2011). On a log-log scale, K_d and E_2 are correlated linearly. ACh, acetylcholine; Ana, anabaseine; CCh, carbamylcholine; TMA, tetramethylammonium; Nic, nicotine; DMT, dimethylthiopyrrolidine; DMP, dimethylpyrrolidine; Cho, choline. The x-intercept is -5.64 and the slope is =1.95.

Figure 3. Cyclic model for binding and activation. Receptors can activate in the absence of ligands ($R \rightleftharpoons R^*$) and agonists can dissociate from active receptors ($L_nR^* \rightleftharpoons R^*+nL$). Linear activation schemes (Fig. 1) are good approximations when these two steps are uncommon. Dotted lines indicate that the binding of multiple agonists is sequential and not simultaneous. n is the number of

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(equivalent) agonist binding sites. E_o is the 'allosteric' constant and is agonist-independent. E_n is the activation equilibrium constant of a fully-occupied receptor. The low/high affinity ratio K_d/J_d is the 'coupling' constant and is agonist-specific. Ligands activate receptors when the coupling constant is >1 (Eq. 1).

Figure 4. The M energy landscape. The black and gray lines pertain to a partial and a full agonist. For both, the energy change in high-affinity binding (proportional to log J_d) is M-times greater than that in low-affinity binding (proportional to log K_d) (Eq. 2). M is the ratio of the lengths of the vertical arrows, and is the same for both agonists. In AChRs $M=\log(J_d)/\log(K_d)=1.92$, and this constant is the basis for the affinity-efficacy correlation shown in Fig. 2. The dashed lines indicate that the full agonist 'tilts' the unified energy landscape moreso than the partial one.

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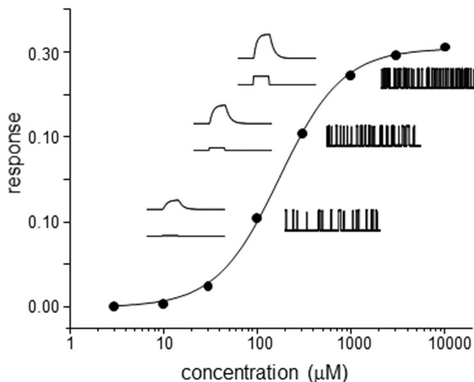
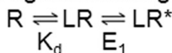
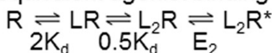
Figure 1



one agonist binding site



two equivalent agonist binding sites



$x = [L]/K_d$

$$response = \frac{xE_1}{1 + x + xE_1} \qquad \frac{x^2E_2}{1 + 2x + x^2 + x^2E_2}$$

$$EC_{50} = \frac{K_d}{E_1 + 1} \qquad \frac{K_d(1 + \sqrt{E_2 + 2})}{E_2 + 1}$$

$$efficacy = \frac{1}{1 + \frac{1}{E_1}} \qquad \frac{1}{1 + \frac{1}{E_2}}$$

Figure 2

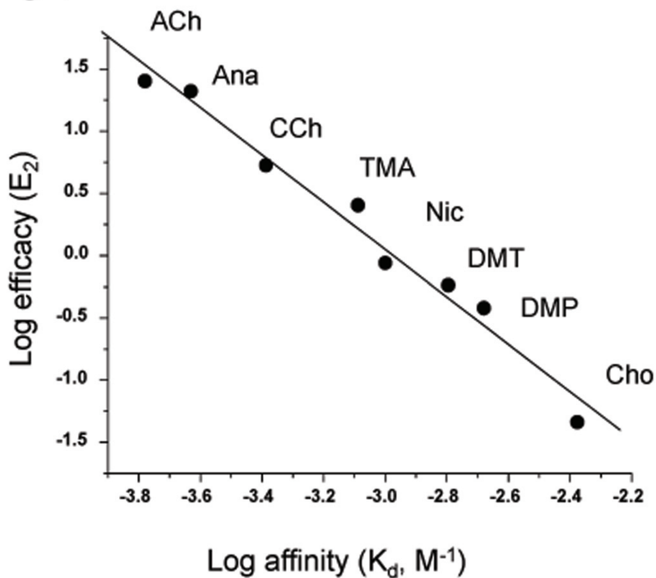


Figure 3

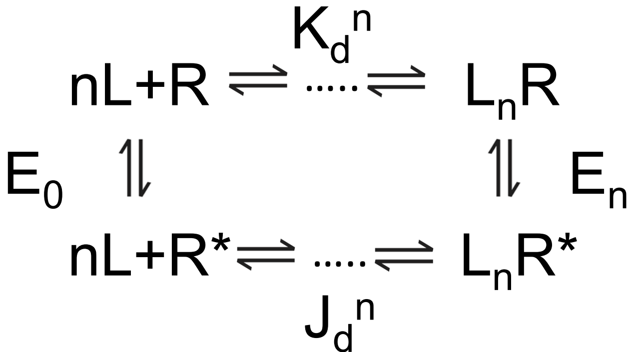


Figure 4