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Nicotinic receptors: from protein allostery to computational neuropharmacology

by

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Abstract:

We propose an extension and further development of the Monod-Wyman-Changeux model for allosteric transitions of regulatory proteins to brain communications and specifically to neurotransmitters receptors, with the nicotinic acetylcholine receptor (nAChR) as a model of ligand-gated ion channels. The present development offers an expression of the change of the gating isomerization constant caused by pharmacological ligand binding in terms of its value in the absence of ligands and several “modulation factors”, which vary with orthosteric ligand binding (agonists/antagonists), allosteric ligand binding (positive allosteric modulators/negative allosteric modulators) and receptor desensitization. The new – explicit – formulation of such “modulation factors”, provides expressions for the pharmacological attributes of potency, efficacy, and selectivity for the modulatory ligands (including endogenous neurotransmitters) in terms of their binding affinity for the active, resting, and desensitized states of the receptor. The current formulation provides ways to design neuroactive compounds with a controlled pharmacological profile, opening the field of computational neuro-pharmacology.

Introduction: allostery from hemoglobin to brain receptors

The year 2021 is 50th Anniversary of the publication of the Book “Hemoglobin and Myoglobin in Their Reactions with Ligands”, by Eraldo Antonini and Maurizio Brunori, a milestone in the field of hemoproteins and Molecular Biology more generally. The authors offer an outstanding synthesis on the relationship between structure and function of the heme proteins and in particular on the cooperative binding of oxygen to hemoglobin. At the heart of the discussion, the views of Wyman-Antonini-Brunori are confronted with the more recent work of Max Perutz and John Kendrew on the crystallographic structure of hemoglobin and myoglobin. The final chapter gives a fair summary of the Monod-Wyman-Changeux two-state model of conformational selection and of the Koshland induced-fit model. Since then, further theoretical developments of allostery included, in particular, the issue of two vs

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multiple conformational states¹⁻⁴ (for review see ⁵). Also, an important additional aspect - which shall directly concern us in this paper - is the extension of the MWC model to brain communications⁶⁻⁷ and specifically to neurotransmitters receptors, with the nicotinic acetylcholine receptor (nAChR) as a model of ligand-gated ion channels (LGICs)⁸⁻¹² (for review see ¹³).

An original difficulty raised by this approach is that the physiological response of these receptors proteins has been, for years, mostly examined by electrophysiological methods in the microseconds to minutes time scale,¹⁴⁻²⁸ in addition of being investigated by the current biochemical and structural methods. Using strictly electrical measurements, the function is recorded as the transient gating of an ion channel, instead of -for instance- the activity of a catalytic site (in regulatory enzymes) or the direct binding of a ligand (as in hemoglobin). Moreover, in addition to the fast (millisecond timescale) all-or-none opening (activation) of the ion channel, the nAChR mediates an additional "high-order regulation" of the ionic response. In fact, prolonged exposure to exogenous acetylcholine causes, within seconds to minutes, a reversible decline of the conductance response to cholinergic agonists referred to as "desensitization"¹⁴ (for review see ¹³). Moreover, it was discovered with neurotransmitter receptors that in addition to the classical neurotransmitter - orthosteric – site, these proteins carry topographically distinct sites for a novel class of ligands called allosteric modulators. These modulatory ligands may stabilize the active conformation of the receptor and are referred to as positive allosteric modulators (PAMs) or if they stabilize the resting conformation, they are referred to as negative allosteric modulators (NAMs). Among them one may mention the benzodiazepines (PAMs of GABA_A receptors), the general anesthetics, or the anthelmintic ivermectin (PAM of nAChR)²⁹⁻³⁴ (for review see ³⁵). Last but not least, an important application of these modeling approaches is the development of new strategies for drug design.

1.Extension of the MWC model to pentameric receptors for neurotransmitters

In this first section, using nAChR as a model system we present an extension of MWC theoretical framework to the signal transduction mechanism mediated by synaptic receptors, which accounts for allosteric modulation and receptor desensitization in addition to ion-channel gating. Desensitization is modeled according to the initial triangular scheme by Changeux³⁶ without distinction between the fast and slow desensitized states as done in Ref¹⁶.

Therefore, the (molecular) mechanism of gating ions is limited to the reversible transitions between the resting, active and desensitized states of the receptor at thermodynamic equilibrium. Under these conditions, the change of the gating isomerization constant caused by ligand binding is expressed in terms of its value in the absence of ligands (L) and several “modulation factors”, which vary with orthosteric ligand binding (agonists/antagonists), allosteric ligand binding (PAMs/NAMs) or receptor desensitization. This new – explicit – formulation of such “modulation factors”, as we shall see, provides expressions for the potency, efficacy, and selectivity of the regulatory ligands, which opens to a theoretical understanding of the pharmacology of synaptic receptors including partial and silent agonism.

Definitions

Nicotinic acetylcholine receptors are pentameric ligand-gated ion channels (pLGICs) that convert a chemical signal, typically the local increase in the extracellular concentration of the neurotransmitter acetylcholine (ACh), into an ionic current through the post-synaptic membrane (reviewed in ¹³). At rest, the ion channel is closed. Binding of neurotransmitter elicits a rapid isomerization to the active state, here referred to as **gating**, which opens a transmembrane pore that allows the diffusion of ions at rates approaching tens of millions of ions per second. Prolonged exposure to high levels of agonist eventually results in a time-dependent decrease of the current termed **desensitization**, which involves the transition to a closed-channel form thought to be structurally different from the resting state.

Functional studies by electrophysiology have been highly instrumental to shed light onto the coupling between neurotransmitter or **agonist** binding and ion gating in nAChRs. By measuring the ionic current at different levels of agonist, whatever the mode of application, empirical **dose-response curves** can be derived that aim at establishing the correlation between the biological response of the receptor and the concentration of ligand in the perfusion. Typical dose-response curves show in cartesian coordinates a sigmoidal behavior that highlights the intrinsic or basal activity of the receptor at low agonist concentrations and the maximal response elicited at saturating conditions (Figure 2A) ~~together with the frequent occurrence of positive cooperativity between binding and gating~~. The concentration of ligand corresponding to half of the maximum response, which is indicated by the inflection point of the dose-response curve, is classically referred to as the ligand EC_{50} and is used as an indicator of **potency**; i.e. the lower the EC_{50} , the stronger the activity of the compound. Vice versa, if the response of the receptor in the presence of ligands is lower than the basal activity,

i.e. without ligands, ligand binding promotes receptor's inhibition and the ligand behaves as an **antagonist**. Some agonists, known as full agonists, are able to elicit the maximum response of the receptor. Others may elicit only a fraction of it and are referred to as **partial agonists**. Using single-channel electrophysiology, it has been shown that partial agonism is related to the probability of channel opening, which is lower than unity when partial agonists are applied at saturating conditions. In order to discriminate between full and partial agonists, a classification based on **agonist efficacy** has been used, which corresponds to the maximum open probability measured in single-channel experiments or the maximum gating current recorded in whole-cell experiments (see³⁷). Tetramethylammonium (TMA) is a typical example of partial agonist at the human muscle nAChR, which was shown to stabilize the open state of the receptor only for 78% of the time versus 94% by the endogenous neurotransmitter acetylcholine at same conditions.¹⁷ Most recently, a new pharmacological attribute termed **efficiency** has been introduced.³⁸ This attribute, which is distinct from potency and efficacy, was defined as the fraction of binding energy that is usefully converted into work (here conformational free energy) during receptor activation. The pharmacological relevance of this attribute remains to be clarified.

Small-molecule compounds that potentiate or reduce the response of the receptor when co-applied with agonists (but that are sometimes able to elicit gating currents on their own) have been also reported (reviewed in ³⁵). They are referred to as **positive** or **negative allosteric modulators** (i.e. PAMs and NAMs). The term “allosteric” refers to the topographical location of their binding site on the receptor that is distinct from the neurotransmitter-binding or orthosteric site. From a pharmacological viewpoint, these compounds are particularly interesting because they act as non-competitive binders and can be used in co-application for the development of fine-tuned pharmacological strategies, possibly opening to personalized medicine. In addition, their binding sites have not been evolved for function and are potentially more druggable (reviewed in ³⁵). Recent structural analyses on the glycine receptor anionic channel (GlyR) have revealed the location of (at least) seven distinct allosteric sites, which span the entire receptor structure and are located mostly at the subunit-subunit interfaces including the ion transmembrane pore.³⁹ In the most recent cryo-EM structure of the cationic nAChR $\alpha 7$ solved in the presence of PNU-120596, this potent and selective PAM was found to bind to the transmembrane domain of the receptor at the interface of adjacent subunits⁴⁰. PAM binding to nAChRs was shown to increase peak currents with or without significant effect on the current decay rate, which is associated with receptor desensitization. The first drug shown to be a PAM in nAChR $\alpha 7$ was 5-

hydroxyindole (5HI). Its relatively modest potentiation, i.e. approximately a 2-fold increase in peak currents, required high doses of drug and left the current decays essentially unaltered. This compound became the prototype for a class of PAMs that is referred to as **type I**.⁴¹ Another class of more efficacious PAMs that increase peak currents but strongly decelerate the current decay rate is referred to as **type II**.⁴² Compounds like PNU-120596⁴³ or TQS⁴⁴ are potent type-II PAMs at nAChR $\alpha 7$. Remarkably, type-II PAMs were found to reactivate previously desensitized receptors.⁴⁵

One last class of unconventional modulatory ligands at nAChR is the one formed by **silent agonists**. Silent agonists are weak partial agonists that produce very stable and long-lived desensitized states, which make the receptor insensitive to subsequent agonist applications even after prolonged washout periods. A prototypical example of this class is NS67040, which is a silent agonist at $\alpha 7$ nAChR.⁴⁶ The ability of silent agonists to antagonize the effect of other drugs or even modulate the endogenous mechanisms of synaptic plasticity make these compounds particularly interesting as neuropharmacological agents.

General formulation

A theoretical interpretation of gating based on the MWC model provides interesting insights on the allosteric modulation of signal transduction mediated by nAChRs. Assuming pre-existing open-channel (O) and closed-channel (C) states in reversible equilibrium both in the presence and absence of neurotransmitter, receptor activation may be modeled by a simple thermodynamic cycle with four states; see Figure 1. As shown by Auerbach and coworkers,²² the great advantage of this scheme, which was proven valid by single-channel electrophysiology of endplate nAChRs,⁴⁷ is that it allows expressing the liganded isomerization constant (L') as a function of the unliganded isomerization constant (L) via the ligand-binding affinities for the open (O) and closed (C) states of the receptor as

$$L' = \left(\frac{K_{d,C}}{K_{d,O}} \right)^n L \quad (1)$$

with $K_{d,O}$ and $K_{d,C}$ being the equilibrium dissociation constants for the open and closed states of the receptor, and n the number of agonist molecules bound; see Appendix for the derivation.[†] The result of Eq.1 has a number of fundamental implications, which are presented below.

[†] Note that the notation in Eq.1 is different from the one in (Indurthi et al, 2021) and was changed here to make it consistent with the original formulation of the MWC model (Monod

Agonist vs antagonist

First, Eq.1 states that in the presence of modulatory ligands (i.e. agonists or antagonists) the gating constant depends (exponentially) on the differential binding affinity of the ligand for open versus closed. Therefore, if ligand binding to the open state is preferred ($K_{d,o} < K_{d,c}$), binding and gating are cooperative and the activity of the receptor is higher than basal. Vice versa, if ligand binding to the closed state is more favorable ($K_{d,c} < K_{d,o}$), the probability of gating is lower in the presence of ligands and receptor's function is inhibited. If so, the MWC model of gating predicts that any ligand that binds stronger to the open state is an agonist, whereas those that prefer binding to the closed-channel state including one of the many desensitized states behave as antagonists.

Efficacy

Second, since the maximum open probability depends entirely on the liganded gating constant, i.e. $p_o^{max} = L'/(L' + 1)$, the result of Eq.1 implies that agonist efficacy is determined by the differential binding affinity of the ligand for the open versus the closed states of the receptor as

$$p_o^{max} = \frac{L e^{-n\beta\Delta\Delta G_b}}{1 + L e^{-n\beta\Delta\Delta G_b}} \quad (2)$$

with $\Delta\Delta G_b$ being $\Delta G_b^O - \Delta G_b^C$, ΔG_b^O and ΔG_b^C the standard free energy of agonist binding to the open and closed states of the receptor, n the number of agonist molecules bound, and β the inverse temperature; see Appendix. Since the higher the binding affinity for open versus closed, the more negative the $\Delta\Delta G_b$, Eq.2 states that efficacy (p_o^{max}) correlates with the selectivity of agonist binding to the open state. As noted by Rubin and Changeux,⁴⁸ the latter opens to an understanding of full versus partial agonism. In fact, the result of Eq.2 predicts that partial agonism arises from a lack of selectivity for the open state, which results in non-exclusive ligand binding events; confront the predicted efficacy of ACh versus TMA for the muscle-type nAChRs in Figure 2B (compare the intersections of the dashed vertical lines for ACh and TMA with the black thick line). In addition, Eq.2 indicates that p_o^{max} depends on the value of the unliganded isomerization constant L , which quantifies the (intrinsic) equilibrium

et al, 1965). Here, the fully liganded and the unliganded gating constants are referred to as L' and L , respectively, instead of E_n and E_0 as customary done by electrophysiologists. The equivalent formulation in terms of E_n and E_0 is given in the Appendix.

of the receptor between its resting and active-open conformations, i.e. the gating probability. Therefore, Eq.2 predicts that gain-of-function mutations or modulations of the transmembrane potential,¹⁷ which leave agonist binding unaltered but affect the intrinsic gating probability, may turn a partial agonist into a full agonist; see in Figure 2B the predicted increase in efficacy for TMA in a gain-of-function mutant with a more favorable (intrinsic) gating free energy by 1 kcal/mol (compare the intersection of the dashed vertical line for TMA with the black thick line for the wild-type and the red dashed line for the mutant).

Potency

Third, in the limit of the thermodynamic cycle in Figure 1, an analytical expression of the agonist EC_{50} can be derived that links agonist potency with its binding affinity for the receptor; see Appendix for details. In the case of full agonists (i.e. $L' \gg 1$), this expression simplifies as

$$EC_{50} = \frac{K_{d,o}}{\sqrt{L}} \quad (3)$$

This insightful result indicates that potency is proportional to the agonist-binding affinity for the active state only and is modulated by the intrinsic gating propensity of the protein; the higher the unliganded gating constant L , the lower the EC_{50} . The result of Eq.3 has the following implications. First, potent agonism requires strong binding to the active state, i.e. the higher the affinity, the stronger the potency (Figure 2C). Second, potency arises from an interplay between agonist binding and the (intrinsic) ability of the protein to open the ion channel, which implies that gain-of-function or loss-of-function mutations will modulate agonist potency allosterically (Figure 2C), while preserving the structure and the chemical interactions at the agonist-binding site. Third, if the value of L is known e.g. via an efficiency analysis,³⁸ Eq.3 provides access to the agonist binding affinity for the active state via an experimental determination of its EC_{50} , i.e. from a dose-response curve. For illustration, using EC_{50} values from single-channel electrophysiology of the muscle-type nAChR for the full agonists acetylcholine (43 μM), nornicotine (72 μM), and dimethylphenylpiperazinium (246 μM) taken from Ref.²² and the unliganded gating constant measured by Nayak *et al* for the adult type ($6.6 \cdot 10^{-7}$),³⁸ Eq.3 yields binding affinities for the open state of 34 nM , 58 nM , and 200 nM , respectively. In addition, using the same value for the un-liganded gating constant, Eq.3 predicts that an agonist potency of 1 μM would require sub-nanomolar affinity (0.8 nM) for the open state. Last, the result of Eq.3 opens to *in-silico* predictions of the

agonist EC_{50} via ligand-binding affinity calculations in the active state, whose relevance is discussed below.

Selectivity

Fourth and last, using the result of Eq.3, an expression for the selectivity coefficient of agonist binding to one receptor (α) over another (β) is obtained by computing the ratio between the corresponding EC50 values as

$$K_{\alpha,\beta} = \frac{K_{d,o}(\alpha)}{K_{d,o}(\beta)} \times \sqrt{\frac{L(\beta)}{L(\alpha)}} \quad (4)$$

Interestingly, Eq.4 states that agonist selectivity depends not only on the (relative) binding affinity for the open state of the two receptors but also on the ratio between their unliganded isomerization constants. Considering that different synaptic receptor types or subtypes feature unliganded isomerization constants that differ by six orders of magnitude or more,³⁸ this result has fundamental biological and pharmacological implications on the selective activation by neurotransmitter-binding events under physiological conditions.

Altogether, the MWC interpretation of gating based on the thermodynamic cycle in Figure 1 predicts that the full pharmacological profile of a bioactive compound targeting neurotransmitter receptors (i.e. potency, efficacy, and selectivity) depends not only on its binding affinity for the protein, but also on the selectivity of binding the active versus the resting state of the receptor, as well as the intrinsic propensity of the ion-channel to gating. The latter opens to a quantitative understanding of the key pharmacological attributes of regulatory ligands by binding affinity determinations at the different physiological states of the receptor, as we shall see below.

Allosteric modulation

A straightforward extension of the thermodynamic cycle in Figure 1 opens to a theoretical interpretation of gating in the presence of allosteric modulators; see Figure 3A. In the limit of the MWC theory, this generalization provides an insightful expression of the liganded gating constant in the presence of orthosteric agonist (A) and allosteric modulators (M) as

$$L'_M = \left(\frac{K_{d,C}^M}{K_{d,O}^M} \right)^m \left(\frac{K_{d,C}^A}{K_{d,O}^A} \right)^n L \quad (5)$$

with m being the number of modulators bound and K_d^M the corresponding dissociation constants; see Appendix for the derivation. The result of Eq.5 indicates that in the presence of allosteric ligands an additional modulation factor appears, which depends on the number of modulators bound and their affinity for the receptor in its open and closed states. The latter has the following implications. First, since the allosteric modulation factor depends (exponentially) on the binding affinities for open versus closed, Eq.5 predicts that the modulator will be a PAM, if it binds preferentially to the open state, vice versa it will be a NAM. Second, if the allosteric modulation factor is as large as the one introduced by orthosteric agonist binding, the modulator will be able to elicit detectable gating currents on its own and might then be referred to as an allosteric agonist or “ago-PAM”.⁴⁶ Third, based on Eq.5 the co-application of agonist (A) with allosteric modulators (M) affects the value of the liganded gating constant. Therefore, pre-incubation with PAMs or NAMs is predicted to alter both the potency and the efficacy of a given orthosteric agonist, which is consistent with electrophysiology results of $\alpha 7$ nAChRs activated by acetylcholine in the presence of the positive allosteric modulator ivermectin.²⁹ Fourth, the result of Eq.5 indicates that the value of the liganded gating constant and therefore the pharmacological attributes can be accessed, in principle, from the binding affinity of all ligands (i.e. agonist and modulators) for the open and closed states of the receptor, which opens to rational allosteric drug design.

The present interpretation of gating based on the MWC theory highlights that receptor’s modulation by allosteric ligands (PAM and NAM) is mediated by a mechanism that is common to that by orthosteric agonists, antagonists, or partial agonists, i.e. the same two-state isomerization, such that the two are conceptually indistinguishable. As in the case of orthosteric ligands, PAM and NAMs may non-exclusively bind to any of the two states and in most cases exhibit no intrinsic activity in the absence of orthosteric ligands. Yet, the intrinsic affinity of some PAMs for the active-state conformation may be such that they cause by themselves the opening of the ion channel, as highlighted by the allosteric agonist 4BP-TQS at $\alpha 7$ nAChR⁴⁹ or ivermectin at the glutamate-gated chloride channel GluCl.³³ More generally, since PAMs and NAMs most often do not elicit gating current on their own but potentiate or inhibit the agonist-elicited response, their pharmacological action can be interpreted as that of weak-efficacy ion channel openers that one may qualify as “allosteric partial agonists”. In light of this, the joint application of multiple ligands binding to

topographically distinct orthosteric and allosteric sites offers a rich pharmacological strategy for an exquisite regulation of the overall receptor's response.

Desensitization

A second generalization of the MWC model of gating to account for receptor's desensitization was developed by introducing an additional isomerization of the protein to a distinct closed-channel state that preserves high affinity for the agonist; see Figure 3B. Following the MWC paradigm, the fast-desensitized state (D) was assumed to be in reversible equilibrium with the open-channel state (O) in the presence and the absence of ligands via the desensitization constants D' and D , respectively. In this framework, the following expression of the "effective" gating constant including desensitization is obtained

$$L'_D = \frac{L'}{1 + L'D'} \quad (6)$$

see Appendix for the derivation. Introducing the expressions of the liganded gating constant and the liganded desensitization constant in Eq.6, it yields

$$L'_D = \gamma_D \left(\frac{K_{d,C}}{K_{d,O}} \right)^n L \quad (7)$$

with

$$\gamma_D = \frac{1}{1 + \left(\frac{K_{d,C}}{K_{d,D}} \right)^n L D} \quad (8)$$

see Appendix. The results of Eqs.7-8 indicate that receptor's desensitization introduces an additional modulation factor (γ_D), whose value depends on: the binding affinity change from resting to desensitized; the number of agonists bound; and the gating (L) and desensitization (D) constants in the absence of ligands. Albeit there exists no mean to access the value of D experimentally, a closer look at Eq.8 reveals that the value of γ_D is bound between 1 and 0 (see Appendix), which implies that desensitization may at most reduce the "effective" liganded gating constant (L'_D). In addition, by developing the expression of γ_D in terms of binding, gating and desensitization free energies, one finds that when ligand binding is strongest to the resting state or it is strongest to the desensitized state but does not compensate for the (conformational) free energy cost of desensitization, the effect of desensitization on gating is negligible. By contrast, when ligand binding to the D state is strong enough, desensitization "antagonizes" gating to an extent that depends on the selectivity of binding to the D state; see Appendix for details. In this case, Eq.7 predicts that receptor's desensitization

has a direct effect on the pharmacological attributes of a modulatory ligand such as potency and efficacy, which both depend on the value of L' (see above). Interestingly, Eq.7 predicts that in case of particularly strong binding to the D state, receptor's desensitization may decrease agonist efficacy below the experimental detection limit without altering the binding affinity for the active state (Figure 4). This latter would result in a formal lack of gating currents upon agonist application, which would explain the modulation by non-conventional agonists that are known as silent agonists. Thus, the present MWC interpretation of gating predicts that silent agonism would be associated with strong selectivity of agonist binding to the desensitized state of the receptor.

2. Towards a computational neuropharmacology

A fundamental result of the extended MWC model of gating presented above is that this formulation provides a quantitative understanding of the modulation of neurotransmitter receptors by conventional and unconventional ligands. A second important result is that it opens to predictions of the pharmacological attributes of drug candidates via the determination of conformation-dependent binding affinities. Although these quantities are hardly accessible experimentally, the emerging framework for allosteric drug design is suited for modeling and simulations, with some caveats. First, high-resolution structures of the receptor-ligand complex should be known in all the physiological states, i.e. resting, active, and desensitized, with atomic resolution. Second, specialized free energy approaches for accurate binding affinity predictions in large and flexible multimeric proteins like nAChRs are needed. In this second section, the road to translate the emerging theoretical framework into actual “means” for the discovery of allosteric drugs acting on synaptic receptors is briefly presented.

Structural background

Until recently, high-resolution structures of intact nAChRs were absent, forcing the field to rely on low-resolution data⁵⁰ or high-resolution data of homologous receptors.⁵¹ Thanks to a number of improvements in cryo-EM and *ad hoc* measures to enhance the expression levels, the biochemical stability, and particle alignment, several high-resolution structures of nAChRs have been recently deposited.⁵² They include: structures of the cortical $\alpha 4\beta 2$ nAChR in complex with nicotine⁵³ in two different stoichiometries;⁵⁴ structures of the ganglionic $\alpha 3\beta 4$ nAChR in complex with nicotine or the antagonist AT-1001;⁵⁵ the structure of the

muscle-type nAChR in complex with α -bungarotoxin;⁵⁶ and most recently structures of the $\alpha 7$ nAChR in complex with α -bungarotoxin, epibatidine plus PNU-120596, or epibatidine alone.⁵⁷ The structures of $\alpha 4\beta 2$ and $\alpha 3\beta 4$ with nicotine bound are remarkably similar and feature a funnel-like transmembrane pore narrowing at the intracellular end consistent with the desensitized state. The structures bound to α -bungarotoxin show a closed-channel form with a constriction point in the mid of the transmembrane pore (at position 9'), which is analogous to that seen in GlyR with strychnine or GABAR with bicuculline and therefore consistent with the resting state. The structure of $\alpha 7$ nAChR with epibatidine plus PNU-120596 illuminates an open-channel conformation consistent with a physiologically active state, which interestingly could not be captured with epibatidine alone, i.e. in the absence of PNU-120596. Although this structure could not illuminate the binding site of PNU-120596, another more recent structure of $\alpha 7$ nAChR also solved in complex with it revealed that this type-II PAM binds to the inter-subunit allosteric site in the transmembrane domain similar to ivermectin in GlyR.⁴⁰ These structures altogether provide evidence for a gating mechanism in nAChRs where the extracellular domain becomes more compact during activation as a consequence of the global un-twisting and un-blooming of the quaternary receptor structure, and the TM domain expands via translation of the M2 helices away from the channel axis to open the pore. These observations are remarkably consistent with early predictions based on normal-mode analysis⁵⁸ and MD simulations of prokaryotic and lower eukaryotic homologues.⁵⁹⁻⁶⁰ Moreover, the availability of structural information for the different physiological states of nAChR in complex with orthosteric and allosteric ligands establishes an unprecedented structural framework for conformation-dependent binding affinity determinations by modeling and simulations.

Computational background

The calculation of the (absolute) free energy of binding by simulation remains a daunting task.⁶¹ Even more so, binding affinity calculations in flexible allosteric proteins that not only change their quaternary structure in response to ligand binding but also adapt their conformation locally, e.g. by modulating the degree of opening of the C-loop at the orthosteric site, are difficult to converge and must account for the conformational free energy of the protein. Despite the intimidation introduced by the size, flexibility and the transmembrane nature of nAChRs along with all challenges posed by absolute binding free energy simulations (including the lack of automated implementations), recent success stories

speak for an increasing level of maturity. First, alchemical free energy calculations by Aldenghi *et al* showed that binding affinity predictions with a mean absolute error of 0.6 kcal mol⁻¹ from experiments could be obtained for a set of 11 inhibitors of a bromodomain-containing protein.⁶² Second, the introduction of enhanced sampling in binding free energy calculations via funnel meta-dynamics was recently shown to yield binding affinity predictions with a RMSE <1 kcal mol⁻¹ from experiments for 12 diverse ligands on five GPCRs.⁶³ And, even more accurate predictions could be obtained on the muscarinic receptor M2 using a more realistic neuronal membrane model.⁶⁴ Third, analysis of the binding energetics of nine regulatory ligands of the ionotropic glutamate receptor revealed the existence of “hidden” contributions to the free energy of binding associated with closure of the ligand-binding domain, which need to be accounted for to obtain a significant correlation with experimental apparent affinities.⁶⁵ In light of this, the availability of automated implementations of rigorous binding free energy methods in combinations with enhanced sampling strategies and/or conformational free energy calculations in the apo state of the receptor offer an exciting prospective towards the establishment of a computational neuropharmacology of brain receptors. In addition, the increasing availability of specialized datasets of ligands with known modulatory activities such as GRALL,³⁹ which includes a large number of agonists, antagonists, positive and negative allosteric modulators at GlyR $\alpha 1$ with a structural annotation of their putative binding site, or ACRALL⁶⁶ with approximately 5000 modulatory compounds targeting two brain nAChRs ($\alpha 7$ and $\alpha 4\beta 2$) and the muscle-type nAChR ($2\alpha\beta\gamma\delta$) emerge as invaluable resources for calibration and validation.

Conclusion

Despite additional progress is needed, the synergistic combination of a new theoretical understanding of the pharmacological attributes of regulatory ligands as presented here, improved structural determinations of LGICs in different functional states and at physiologically relevant conditions, and accurate and efficient predictions of ligand binding affinities by Molecular Dynamics emerge as a promising avenue for the rational design of neuroactive compounds. This new field that is a direct result of a MWC interpretation of gating is referred to as computational neuropharmacology and is expected to lay the foundation of a novel paradigm for drug design in allosteric proteins, opening to the discovery of neuroactive compounds with controlled pharmacological profiles.

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Figures

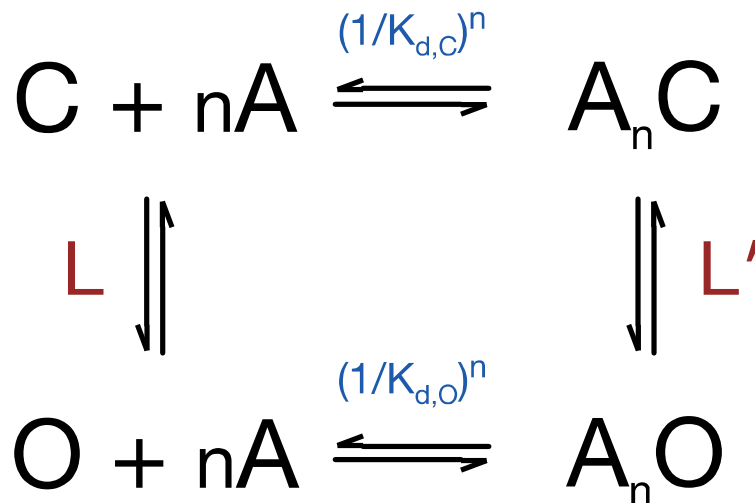


Figure 1. The Monod-Wyman-Changeux (MWC) model of gating by pentameric neurotransmitter receptors. Agonist molecules are indicated as A. The open and closed states of the receptor are named O and C. n is to the number of agonists bound in the fully liganded state, e.g. two in the muscle-type and five in the neuronal $\alpha 7$ nAChR. The fully liganded and unliganded isomerization constants for opening/closing the ion channel are named as L' and L , respectively. $K_{d,c}$ and $K_{d,o}$ correspond to the equilibrium dissociation constants for the agonist in the open and closed states. As shown by the arrows, the MWC model of gating assumes reversible equilibrium between the open and closed states of the receptor in the presence and the absence of ligands.

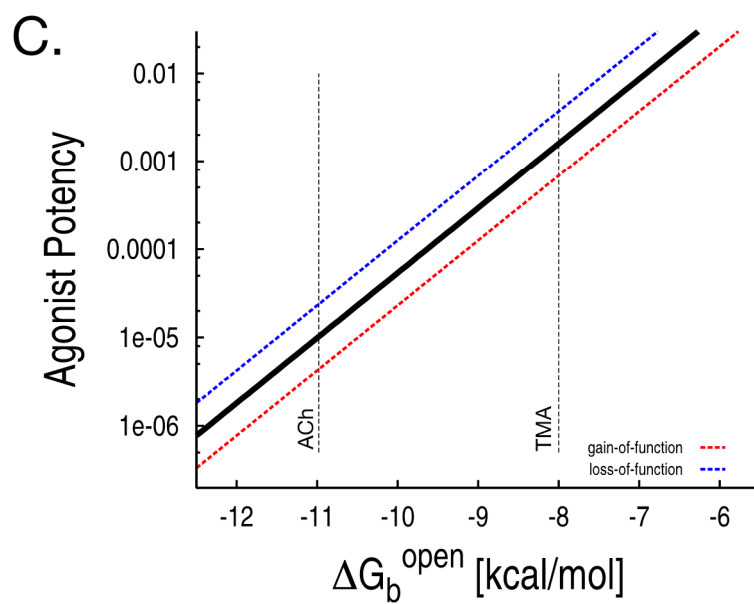
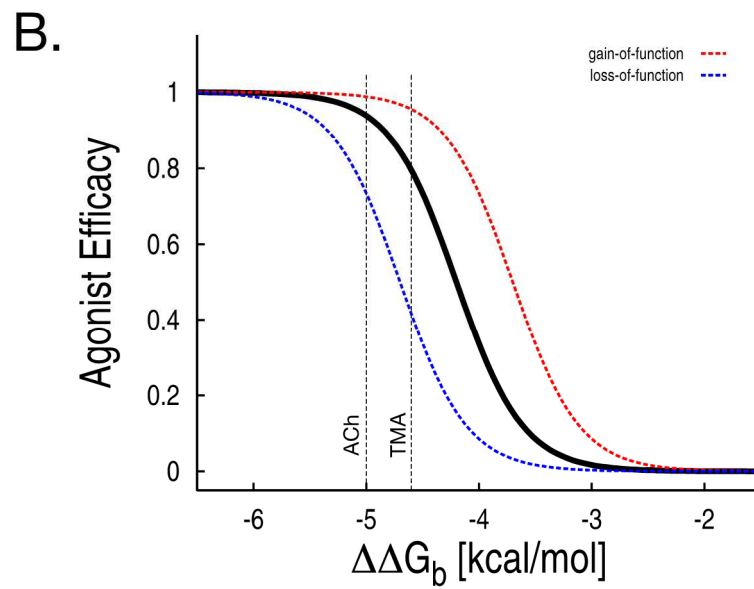
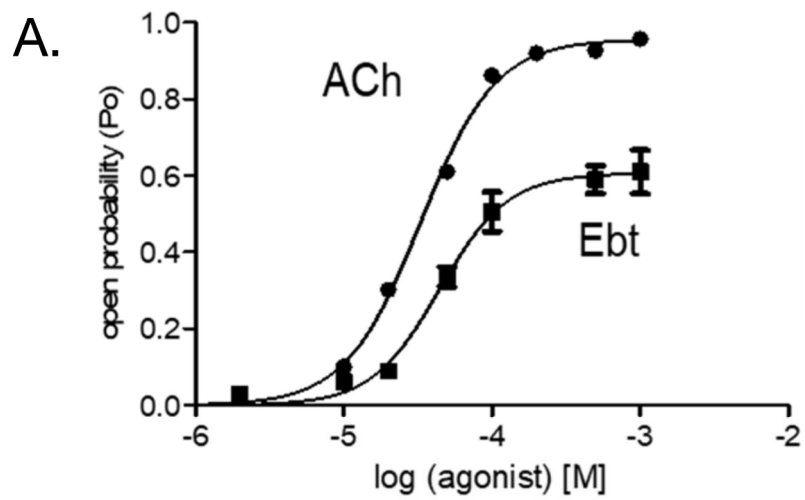


Figure 2. Pharmacological attributes of modulatory ligands in muscle-type nAChRs. (A) Dose-response curves of nAChRs from the motor end-plate exposed to increasing concentrations of the full agonist acetylcholine (ACh) and the partial agonist epibatidine (Ebt) by single-channel electrophysiology (taken from Ref.²²). (B) Agonist efficacy versus the differential binding affinity for active versus resting ($\Delta\Delta G_b$) as predicted by Eq.2 with two ligands bound. Numerical results were obtained assuming an intrinsic gating free energy of **8.4** kcal/mol as measured by Nayak et al.³⁸ This analysis predicts that a change in the selectivity of binding to the active state as small as 0.4 kcal/mol turns a full agonist like ACh (p_o^{max} of 0.94) into a partial agonist like tetramethylammonium or TMA (p_o^{max} of 0.78)[‡]. (C) Potency versus the binding affinity for the active state (ΔG_b^o) as predicted by Eq.3. This analysis predicts that gain-of-function (red) or loss-of-function mutations (blue) producing a change in the intrinsic gating free energy of ± 1 kcal/mol, increase/decrease the EC50 of ACh by a factor of two.

[‡] Differential binding affinities, i.e. $\Delta\Delta G_b = \Delta G_b^o - \Delta G_b^c = RT \ln(K_{d,o}/K_{d,c})$, for ACh and TMA were evaluated using Eq.1, which yields $K_{d,o} = K_{d,c} \sqrt{L/L'}$, along with dissociation equilibrium constants for the resting conformation (i.e. 40.1 μM for ACh and 3000 μM for TMA) and maximum open-channel probabilities (i.e. 0.94 for ACh and 0.78 for TMA) obtained from single-channel electrophysiology of the muscle-type nAChR (Lape 2008). Maximum open-channel probabilities were used to access the liganded gating constant as $L' = p_o^{max}/(1 - p_o^{max})$. The un-liganded gating constant, $L = \exp(-\Delta G_0/RT)$, was accessed using an intrinsic gating free energy of 8.4 kcal/mol as measured by Nayak et al. for the same receptor (Nayak 2019).

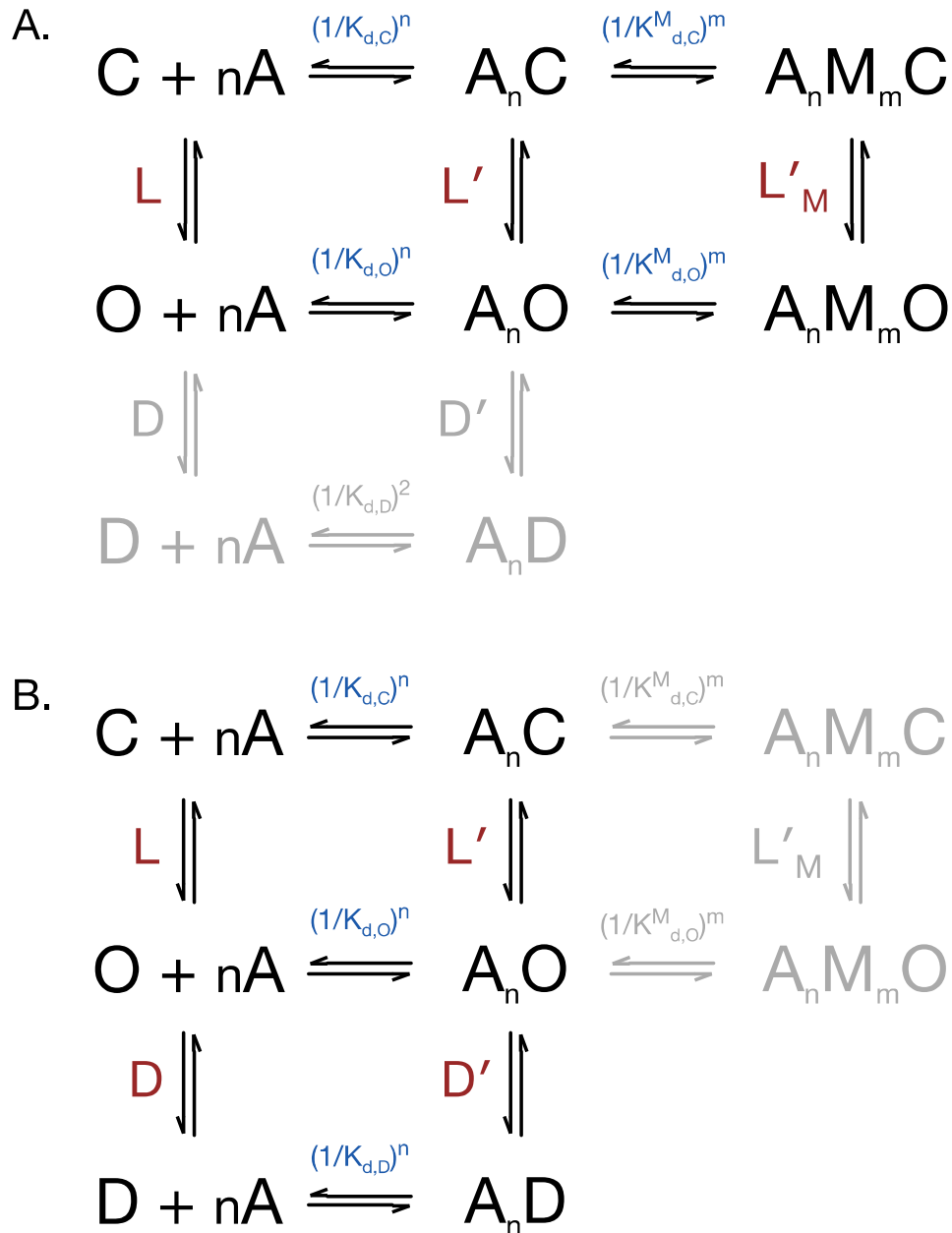


Figure 3. Extensions of the MWC model of gating to include allosteric modulation (top) and receptor desensitization (bottom). The terminology is the same as in Figure 1. In addition, allosteric modulators are indicated as M with m being the number of bound modulators in the (fully) liganded state. The desensitized state of the receptor is indicated as D, with D and D' being the liganded and unliganded desensitization constants. Consistent with Figure 1, $(1/K_{d,D})$ corresponds to the binding affinity of the agonist for the desensitized state, while $(1/K_{d,i}^M)$ is used to express the binding affinity of the modulator for the i -th physiological state of the receptor. The effective isomerization constant in the presence of allosteric modulators is referred to as L'_M . As shown in the Appendix, these straightforward extensions of the MWC model of gating are useful to express the fully liganded isomerization constant in the presence of allosteric effectors (L'_M) or including desensitization (L'_D), which opens to more accurate predictions of the pharmacological attributes of the regulatory ligands.

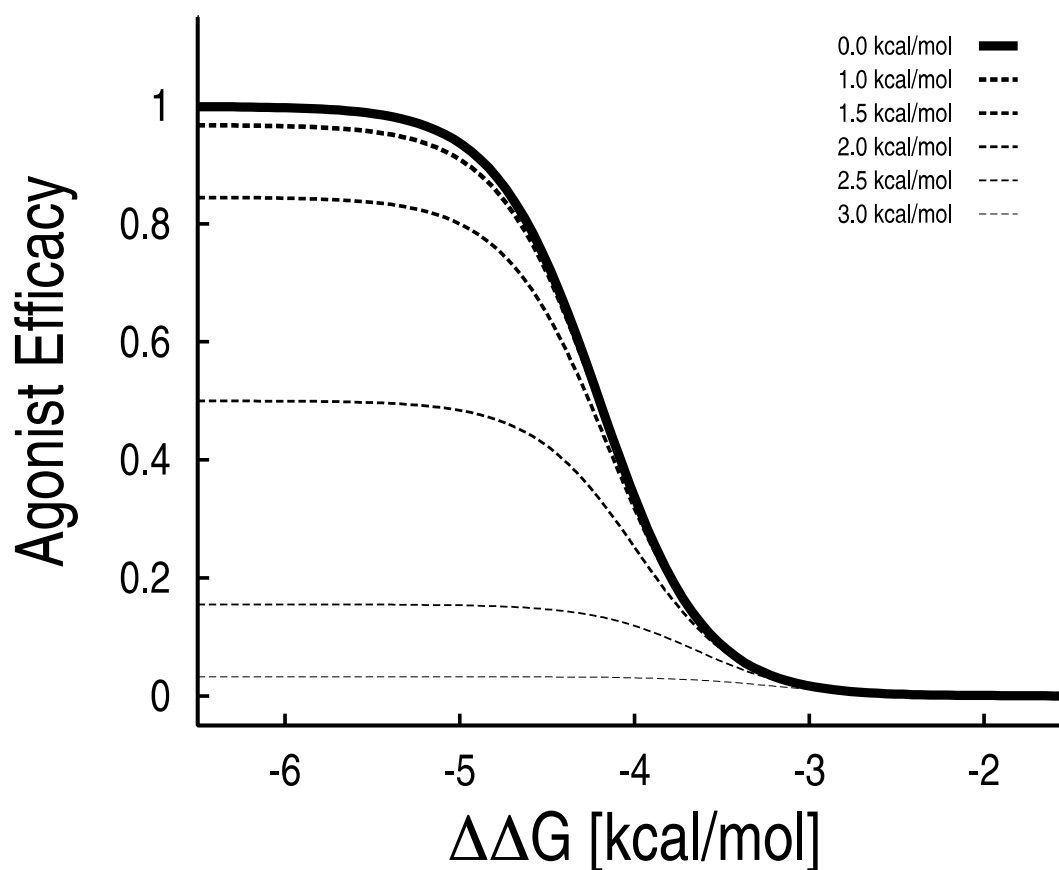
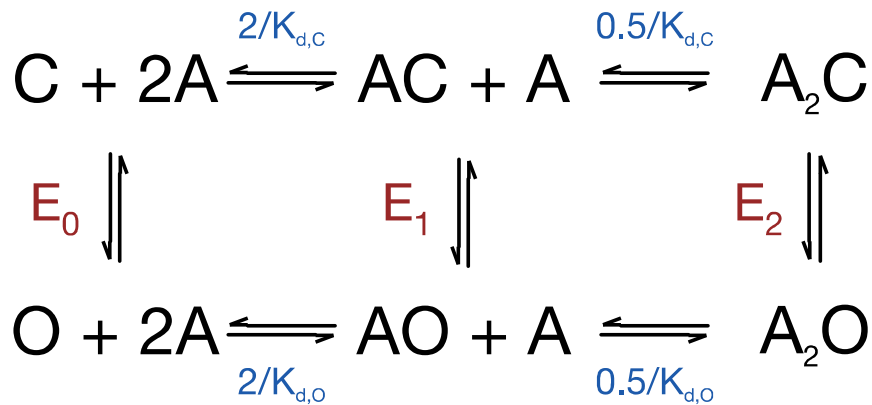


Figure 4. Effect of desensitization on agonist efficacy. The modulation of agonist efficacy by receptor desensitization was explored introducing the result of Eq.6 into Eq.2. For this purpose, the value of the unliganded gating constant (L) was taken from Nayak et al³⁸ (i.e. assuming $\Delta G_0 = 8.4$ kcal/mol) and that of the unliganded desensitization constant (D) assuming an arbitrary desensitization free energy of **12.4** kcal/mol, i.e. 4 kcal/mol above the active state. Numerical results obtained with increasing the binding selectivity for desensitized over resting from 0.0 kcal/mol (unselective) to 3.0 kcal/mol (selective) are shown as dashed lines. The data show that agonist efficacy decreases with increasing the selectivity for desensitized until it vanishes. The latter explains the phenomenon known as silent agonism.

Appendix

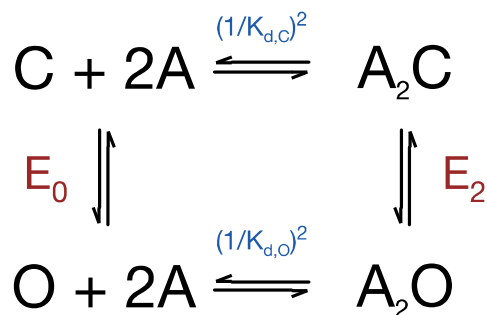
Allosteric modulation of pLGICs

One central tenet of the MWC theory of protein allostery (1965) is that the signaling molecule, here a neurotransmitter receptor, undergoes reversible transitions between discrete conformational states that are accessible even in the absence of ligands.⁶⁷ Assuming that the receptor may switch spontaneously between a lower energy closed-channel conformation (C) and a higher energy open-channel conformation (O) in the absence of ligands, and that two agonist molecules (A) are sufficient to shift the equilibrium towards the open state as in the muscle-type $\alpha 1\beta\gamma\delta$, the cortical $\alpha 4\beta 2$, or the ganglionic $\alpha 3\beta 4$ nAChRs, a MWC interpretation of gating is consistent with the thermodynamic cycle in Scheme 1.



Scheme 1. MWC model of gating by nAChRs. Based on the MWC theory, the closed-channel state (C) and the open-channel (O) states of the receptor are assumed to be in reversible equilibrium both in the presence and the absence of agonist (A). The equilibrium constants for gating (vertical legs) are indicated as E_n with n being the number of number of agonist molecules bound to the receptor. The equilibrium constants for binding (horizontal legs) are indicated as $1/K_{d,c}$ and $1/K_{d,o}$, and provide a measure of the agonist binding affinity for closed versus open.

Assuming that the two agonist-binding sites are equivalent and independent, i.e. they have approximately the same affinity for the agonist and that the strength of binding does not depend on the ligation state at the other site, Scheme 1 can be simplified as



Scheme 2. A simplified MWC model of gating by nAChR. Assuming that the agonist-binding sites are equivalent and independent, the horizontal legs in Scheme 1 can be fused into a single step.

without loss of generality. The advantage of Scheme 2 is that it provides an expression of the diliganded gating constant (E_2) as a function of the unliganded gating constant (E_0). In fact, by naming ΔG_0 and ΔG_2 the free energy of gating in the absence and the presence of bound agonists, and ΔG_b^O and ΔG_b^C the standard molar free energy of agonist binding to the open and closed states of the receptor, respectively, the thermodynamic cycle in Scheme 2 grants

$$2\Delta G_b^C + \Delta G_2 = \Delta G_0 + 2\Delta G_b^O$$

By separating out ΔG_2 and using the relation $\Delta G^\circ = -RT \ln K_{eq}$, one obtains

$$E_2 = \left(\frac{K_{d,C}}{K_{d,O}} \right)^2 E_0 \quad (\text{A.1})$$

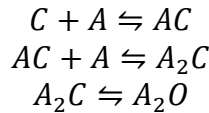
This fundamental result shows that there is cooperativity between agonist binding and gating and that the cooperativity factor depends on the agonist binding affinities for the open and closed states, and the number of agonists bound, i.e. in this case two. By expressing the cooperativity factor in terms of binding free energies, it yields

$$\frac{K_{d,C}}{K_{d,O}} = e^{-\beta(\Delta G_b^O - \Delta G_b^C)} = e^{-\beta\Delta\Delta G_b} \quad (\text{A.2})$$

with $\Delta\Delta G_b$ corresponding to the (binding) selectivity of the agonist for the open state. It follows that if agonist binding is selective for the open state, i.e. $\Delta G_b^O < \Delta G_b^C$, the cooperativity factor $K_{d,C}/K_{d,O} > 1$ and ion gating is favored in the presence of ligands. Vice versa, if agonist binding is selective for the closed state, $K_{d,C}/K_{d,O} < 1$ and the ligands behave as inverse agonists. Hence, the results of Eq.A.1 and Eq.A.2 indicate that the modulation of gating by ligand binding events depends on the sign and the size of the differential binding affinity for open versus closed, i.e. $\Delta\Delta G_b = \Delta G_b^O - \Delta G_b^C$. We note that if the number of agonist molecules that stabilize the active state is larger than two, e.g. as in homomeric $\alpha 7$ nAChRs, Eq.A.1 still holds but the exponent of the cooperativity factor is also larger than two. Thus, introducing n in Eq.A1, with n being the number of agonists bound, the result is generalized to an arbitrary number of ligands; see Eq.1 of the *Main Text*.

Agonist efficacy

Highly efficacious agonists promote a nearly maximum biological response at saturating concentrations of agonist. In order to quantify agonist efficacy at thermodynamic equilibrium, an expression of the open-channel probability must be derived. For this purpose, let us consider a pentameric receptor that is activated by the binding of two agonist molecules, e.g. the neuronal nAChR $\alpha 4\beta 2$ or the muscle-type nAChR. Assuming that the predominant pathway for ion gating involves the following chemical equilibria



with corresponding equilibrium constants

$$K_1 = \frac{[AC]}{[C][A]}$$

$$K_2 = \frac{[A_2C]}{[AC][A]}$$

$$K_3 = \frac{[A_2O]}{[A_2C]}$$

Naming the probability of the various chemical states as

$$p(C) = p_A; p(AC) = p_B; p(A_2C) = p_C; p(A_2O) = p_D$$

which obey the following constraints

$$\begin{aligned}
p_B &= K_1[A] p_A \\
p_C &= K_2[A] p_B \\
p_D &= K_3 p_C \\
p_A + p_B + p_C + p_D &= 1
\end{aligned}$$

a system of four linear equations with four unknown is obtained. Introducing the following expressions

$$\begin{aligned}
p_B &= K_1[A] p_A \\
p_C &= K_2[A] p_B = K_2 K_1 [A]^2 p_A \\
p_D &= K_3 p_C = K_3 K_2 K_1 [A]^2 p_A
\end{aligned}$$

into the last equation above, it yields

$$p_A = \frac{1}{1 + K_1[A] + K_2 K_1 [A]^2 (K_3 + 1)}$$

and

$$p_D = \frac{K_3 K_2 K_1 [A]^2}{1 + K_1[A] + K_2 K_1 [A]^2 (K_3 + 1)}$$

Based on Scheme 1, $K_1 = 2/K_{d,c}$, $K_2 = 0.5/K_{d,c}$ and $K_3 = E_2$. By factorizing $[A]^2$ from both numerator and denominator, it yields

$$p_D = \frac{E_2}{\frac{K_{d,c}^2}{[A]^2} + \frac{2K_{d,c}}{[A]} + (E_2 + 1)} \quad (\text{A.3})$$

which provides an expression for the open-channel probability as a function of the agonist concentration, i.e. a typical dose-response curve. In the limit of $[A] \gg K_{d,c}$, the result of Eq.A3 yields an expression for the open-channel probability at saturating conditions as

$$p_O^{max} = \frac{E_2}{E_2 + 1} \quad (\text{A.4})$$

The result of Eq.A4 indicates that the maximum response that can be elicited by agonist binding, as know as agonist efficacy, is solely dictated by the value of the liganded gating constant E_2 , as previously established. Introducing the results of Eq.A1 and Eq.A2, which derive from the MWC model, into Eq.A4 and expressing the dissociation constants in terms of the corresponding binding free energies, it yields

$$p_O^{max} = \frac{E_0 e^{-2\beta\Delta\Delta G_b}}{1 + E_0 e^{-2\beta\Delta\Delta G_b}} \quad (\text{A.5})$$

This fundamental result indicates that agonist efficacy depends on both the differential agonist-binding affinity for the open versus closed states of the receptor, i.e. $\Delta\Delta G_b$, and the unliganded isomerization constant, E_0 . Since the latter can be in principle accessed by an efficiency analysis,³⁸ Eq.A5 opens to an interpretation of the agonist efficacy and therefore of full versus partial agonism in terms of $\Delta\Delta G_b$; see *Main Text*. We note that the validity of Eq.A.4 and Eq.A.5 goes beyond the case where the receptor is activated by two agonist molecules. In this case, the fully liganded isomerization constant in Eq.A4 may be usefully replaced by E_n or simply L' as in the original formulation of the MWC theory, and the factor

2 in the argument of the exponential in Eq.A.5 with n , with n being the number of agonists bound; see Eq.2 in the *Main Text*.

Agonist potency

This interpretation of gating based on the MWC theory (Scheme 2) opens to a chemical understanding of agonist potency, which is understood as an apparent affinity of the ligand for the receptor and is typically expressed using $EC50$ values. Since the $EC50$ corresponds to the concentration of agonist required to elicit 50% of the maximum response, a formal expression of agonist potency is obtained using the results of Eq.A3 and Eq.A4 and imposing $p_o = \frac{1}{2} p_o^{max}$. Thus,

$$\frac{E_2}{2(E_2 + 1)} = \frac{E_2[A]^2}{K_{d,c}^2 + 2K_{d,c}[A] + [A]^2(E_2 + 1)}$$

$$K_{d,c}^2 + 2K_{d,c}[A] + (E_2 + 1)[A]^2 = 2(E_2 + 1)[A]^2$$

$$(E_2 + 1)[A]^2 - 2K_{d,c}[A] - K_{d,c}^2 = 0$$

and solving for $[A]$, it yields

$$EC50 = \frac{K_{d,c} (1 + \sqrt{E_2 + 2})}{(E_2 + 1)} \quad (A.6)$$

The result of Eq.A6 shows that agonist potency depends on the binding affinity for the resting state and the liganded gating constant. If $E_2 \ll 1$, i.e. there is little or no cooperativity between binding and gating, $EC50 \approx K_{d,c}$ so that agonist potency depends solely on the ligand-binding affinity for the resting state of the receptor as for a non-allosteric protein. In sharp contrast, if $E_2 \gg 2$, which holds for a typical full agonist of the glycine receptor or the muscle-type nAChR,¹⁷ Eq.A6 yields

$$EC50 = \frac{K_{d,c}}{\sqrt{E_2}} \quad (A.7)$$

which indicates that agonist potency also depends on the isomerization constant with bound ligands. Introducing the result of Eq.A1 into Eq.A7 and rearranging, it yields

$$EC50 = \frac{K_{d,o}}{\sqrt{E_0}} \quad (A.8)$$

which indicates that in allosteric proteins like the pentameric receptors investigated here, agonist potency depends on the ligand-binding affinity for the active state, $K_{d,o}$, and the unliganded isomerization constant, E_0 .

Agonist selectivity

Last, a MWC interpretation of gating opens to a quantitative understanding of the selectivity of agonist binding, which is critical for the selective activation of one receptor type or subtype over the others at physiological conditions. Using the result of Eq.A8, agonist selectivity may be expressed as a ratio between two apparent binding constants as

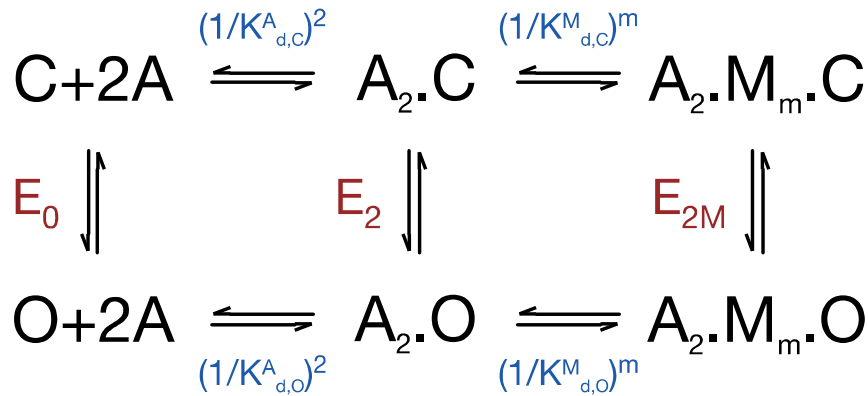
$$K_{\alpha,\beta} = \frac{EC50(\alpha)}{EC50(\beta)} = \frac{K_{d,o}(\alpha)}{K_{d,o}(\beta)} \times \sqrt{\frac{E_0(\beta)}{E_0(\alpha)}} \quad (\text{A.9})$$

where the indexes α and β indicate two receptor types (e.g. nAChR vs 5-HT₃R) or subtypes (e.g. nAChR $\alpha 4\beta 2$ or muscle type).

In the limit of validity of the MWC model, the results of Eq.A5, Eq.A8, and Eq.A9 open to a quantitative understanding of key pharmacological attributes such as efficacy, potency and selectivity of the regulatory ligands of brain receptors; see *Main Text*.

Modulation by PAMs and NAMs

A straightforward generalization of the thermodynamic cycle in Scheme 2, opens to an interpretation of gating in the presence of positive and negative allosteric modulators. Assuming that these modulators bind to a site that is topographically distinct from the orthosteric site and that their binding affinity depends on the state of the receptor, i.e. there is no cross talking with agonist binding, allosteric modulation can be consistently incorporated in the MWC model of gating by introducing an additional horizontal step as shown in Scheme 3.



Scheme 3. Generalization of the MWC model of gating to include allosteric modulation. Therein, m is the number of bound modulators, E_{2M} is the gating equilibrium constant with two agonists and m modulators, and $K_{d,o}^M$ and $K_{d,c}^M$ are the equilibrium dissociation constants in the open and closed states, respectively, which quantify the state-dependent binding affinity of the modulator (M).

Since agonist binding and allosteric modulator(s) binding are independent events, the order of the corresponding steps in Scheme 3 is arbitrary. Assuming microscopic reversibility at each step of Scheme 3, the gating constant in the presence of agonist and modulators is

$$E_{2M} = \left(\frac{K_{d,c}^M}{K_{d,o}^M} \right)^m E_2$$

Introducing the result of Eq. A1 in the equation above, it yields

$$E_{2M} = \left(\frac{K_{d,c}^M}{K_{d,o}^M} \right)^m \left(\frac{K_{d,c}^A}{K_{d,o}^A} \right)^2 E_0 \quad (\text{A.9})$$

Interestingly, the result of Eq.A9 indicates that allosteric modulators binding introduces an additional cooperativity factor that depends on the number of modulators bound (m) and the ratio between their binding selectivity for the active state; confront the result of Eq.A9 with that of Eq.A1. Thus, when the modulator binding affinity for the open state is higher, ligand

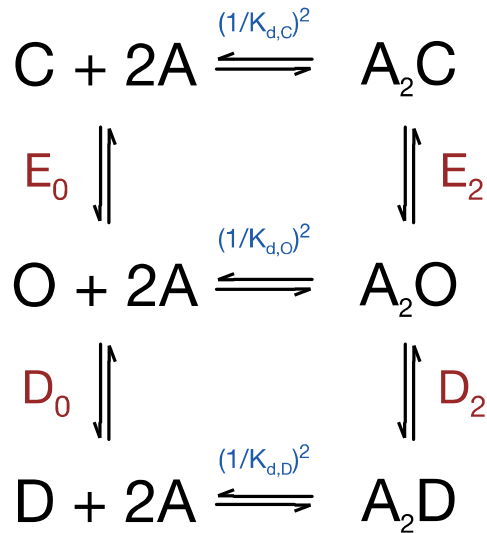
binding favors gating, i.e. $E_{2M} > E_2$, and the ligand behaves as a positive allosteric modulator (PAM). By contrast, when the binding affinity for the closed state is higher, ligand binding disfavors gating i.e. $E_{2M} < E_2$, and the ligand behaves as a negative allosteric modulator (NAM). Expressing Eq. A.9 in terms of binding free energies, it yields

$$E_{2M} = e^{-\beta[2\Delta\Delta G_b^A + m\Delta\Delta G_b^M]} E_0 \quad (\text{A.10})$$

with $\Delta\Delta G_b^A$ and $\Delta\Delta G_b^M$ being the binding selectivity of the agonist and the modulator for the active state, e.g. $\Delta\Delta G_b^M = \Delta G_{b,O}^M - \Delta G_{b,C}^M$. The result of Eq.A10 shows that the fully liganded gating constant and therefore both agonist efficacy (Eq. A4) and potency (Eq. A7) in the presence of modulators can be evaluated from the knowledge of the binding free energy of all ligands in the resting and active states, and the unliganded gating constant that is accessed from an efficiency analysis.³⁸ As before, to account for the specificity of the different receptor subtypes, the number of agonist molecules bound can be usefully generalized to n in Eq.A.9 and Eq.A10; see Eq.5 in the *Main Text*.

Desensitization

A second generalization of the MWC model of gating allows introducing the effect of receptor's desensitization, possibly to an early or fast-desensitized state that is thought to be in equilibrium with the resting and active forms.¹² For this purpose, an extra step must be introduced in the vertical direction of Scheme 2, which corresponds to an additional isomerization of the protein to a distinct closed-channel state that preserves high affinity for the agonist. Following the MWC paradigm, the fast-desensitized state, here termed D, is assumed to be in equilibrium with the open-channel state (O) both in presence and absence of ligands via the desensitization constants D_2 and D_0 , respectively; see Scheme 4.

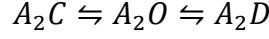


Scheme 4. Generalization of the MWC model of gating to include receptor's desensitization.

Based on Scheme 4 and in analogy with previous developments (see above) the diliganded desensitization constant can be expressed in terms of the unliganded desensitization constant via the ligand binding affinities for open versus desensitized as

$$D_2 = \left(\frac{K_{d,O}}{K_{d,D}} \right)^2 D_0 \quad (\text{A.11})$$

which indicates that the stronger the binding selectivity for the D state, the higher the probability of desensitization. To include the effect of desensitization on the modulation of gating by ligand binding events, we aim for an expression of the “effective” gating constant that accounts for this additional isomerization to a distinct closed-channel state. For this purpose, we first express the probability of the open state at saturating agonist concentrations, i.e. the right hand side of Scheme 4, and then use this result to obtain an expression of the “effective” gating constant including desensitization. Thus, considering the following chemical equilibria



and the corresponding equilibrium constants

$$E_2 = \frac{[A_2O]}{[A_2C]}$$

$$D_2 = \frac{[A_2D]}{[A_2O]}$$

the probability of resting (p_C), active (p_O), and desensitized (p_D) obey the following constraints

$$\begin{aligned} p_O &= E_2 p_C \\ p_D &= D_2 p_O \\ p_C + p_O + p_C + p_D &= 1 \end{aligned}$$

Thus, solving for the open-channel probability, it yields

$$p_O = \frac{1}{1 + \frac{1}{E_2} + D_2} \quad (\text{A.12})$$

which corresponds to the maximum open-channel probability (p_O^{max}) with desensitization. If receptor’s desensitization is marginal (i.e. $D_2 \rightarrow 0$), Eq. A12 states that p_O^{max} solely depends on the liganded gating constant consistent with Eq.A4 and desensitization can be safely neglected. By contrast, if desensitization is important, D_2 is large and the open-channel probability vanishes. By equalizing the result of Eq. A12 with that of Eq.A4 as

$$\frac{1}{1 + \frac{1}{E_2} + D_2} = \frac{E_2^{eff}}{1 + E_2^{eff}}$$

an expression for the diliganded “effective” gating constant including desensitization is obtained

$$E_2^{eff} = \frac{E_2}{1 + E_2 D_2} \quad (\text{A.13})$$

Reassuring enough, this result shows that when receptor’s desensitization is marginal (i.e. $D_2 \rightarrow 0$), $E_2^{eff} = E_2$ and desensitization can be neglected. Vice versa, when the desensitized state is thermodynamically more stable than the active state (i.e. $D_2 \rightarrow \infty$), $E_2^{eff} = 1/D_2$ and the probability of gating is controlled by the desensitization reaction. Since agonist potency and efficacy both depends on the value of the liganded gating constant, the result of Eq. A13 opens to an interpretation of the pharmacological attributes including desensitization. Introducing the results of Eqs. A1 and A11 into Eq. A13, it yields

$$E_2^{\text{eff}} = \gamma_D \left(\frac{K_{d,c}}{K_{d,o}} \right)^2 E_0 \quad (\text{A.14})$$

with

$$\gamma_D = \frac{1}{1 + \left(\frac{K_{d,c}}{K_{d,D}} \right)^2 E_0 D_0} \quad (\text{A.15})$$

This fundamental result shows that receptor's desensitization introduces an additional cooperativity factor, γ_D , which depends on the binding affinity change from resting to desensitized, the number of agonists bound (i.e. here two), and the unliganded gating and dissociation constants. Albeit there exists no mean (yet) to access the value of D_0 experimentally, a closer look at Eq. A15 reveals that the value of γ_D is bound between 1 (i.e. no cooperativity,) and 0 (i.e. anticooperativity), so that desensitization may solely reduce the value of “effective” diliganded gating constant. By further developing the expression of γ_D in terms of binding, gating and desensitization free energy changes, it yields

$$\left(\frac{K_{d,c}}{K_{d,D}} \right)^2 E_0 D_0 = e^{-\beta[2\Delta\Delta G_b^{\circ}(R \rightarrow D) + \Delta G^{\circ}(R \rightarrow D)]} \quad (\text{A.16})$$

which shows that the value of γ_D is dictated by the interplay between the binding selectivity for the D state and the desensitization (free) energy. Interestingly, this last result implies that: (i.) if ligand binding is strongest in the resting state (i.e. $\Delta\Delta G_b^{\circ}(R \rightarrow D) > 0$), $\gamma_D \sim 1$ and desensitization can be neglected; (ii.) if ligand binding is strongest in D but does not fully compensate the cost of the isomerization reaction to reach the D state (i.e. $|\Delta G^{\circ}(R \rightarrow D)| > |\Delta\Delta G_b^{\circ}(R \rightarrow D)|$), $\gamma_D \sim 1$ and desensitization can be neglected; (iii.) if ligand binding is strongest in D and the binding energy compensates the cost of desensitization, $\gamma_D < 1$ and desensitization and gating are anticooperative. As noted above, a generalization of the results of Eq.A.11, Eq.A.14, Eq.A.15 and Eq.A.16 to go beyond the case with two bound agonists is obtained by replacing the factor 2 with n , with n being the number of agonists bound in the fully liganded active state; see Eq.7 or Eq.8 in the *Main Text*.

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