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COMMENTARY

The surprising difficulty of "simple" equilibrium binding measurements on ligand-gated ion channels

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Neurotransmitter-gated ion channels (NGICs) are a large group of integral membrane proteins that are opened by the binding of neurotransmitters to an extracellular domain. Like other classes of ligand-gated ion channels, NGICs harness the free energy of ligand binding to drive the conformational change that opens the channel pore. Models such as the Monod–Wyman–Changeux (MWC; Monod et al., 1965) network of coupled binding and conformational equilibria (Fig. 1 A, left) provide a framework for understanding the thermodynamic basis for this transduction process. Investigations of NGICs have skewed heavily toward electrophysiological measurements of the closed–open conformational exchange (gating). In such experiments, ligand binding is detected indirectly via its effect on channel gating. However, some experimental preparations are not amenable to measurement of channel current and some mutations lock channels into closed conformations. Nonetheless, channel behavior can still be studied by measurement of the ligand binding stimulus itself. Thus, methods for characterizing protein–ligand binding (Jarmoskaite et al., 2020; Wells, 1992) are an important experimental tool for studying NGICs. However, binding measurements on NGICs are not only relatively rare, but are, as the authors discovered, also deceptively difficult to perform correctly and suffer from a lack of uniformity in the experimental protocols used. As a result, there are significant differences in the inferred binding energies and/or mechanisms from different groups. To reconcile these apparent discrepancies, in an earlier issue of the *Journal of General Physiology*, Godellas and Grosman (2022) took a rigorous deep dive into the multiple facets of equilibrium binding experiments on NGICs. They show that careful consideration of the experimental design is critical to avoiding the numerous pitfalls that can easily ensnare the unwary investigator. After establishing appropriate methods for binding measures on acetylcholine receptors (AChRs), the authors address two important mechanistic questions for these channels: (1) are the binding sites identical and independent? and (2) are perturbations in the transmembrane domain (TMD) propagated to the distant binding sites in the extracellular domain (ECD)?

Experimental approach to quantifying equilibrium ligand binding to NGICs: Pitfalls, limitations, and solutions

Godellas and Grosman (2022) employ a classical technique that requires two separate experiments. Fig. 1 illustrates simulations for both experiments in the simplest possible non-trivial case of a receptor with two identical and independent binding sites, but the phenomenology is valid for any number of sites such as the five sites explored by the authors. In the first experiment, binding of a labeled ligand is directly monitored as a function of its applied concentration (Fig. 1 A). In the second experiment, binding of an unlabeled test ligand of interest (ligand A) is inferred from its displacement of the previously characterized labeled ligand (ligand B; Fig. 1 B). Analysis of this equilibrium competition-binding concentration–response relation depends on the properties of the receptor and both the labeled and unlabeled ligands.

The assays in Fig. 1 appear deceptively straightforward, but as pointed out by the authors, are in fact fraught with potential artifacts that can distort the data curves if not properly corrected for or avoided. First, it is noteworthy that the authors spent the effort to obtain well-defined equilibrium binding curves with clear saturation, as required for reliable analysis of these data. Next, we consider four additional types of errors which the authors went to great lengths to avoid through careful experimental design choices and rigorous verification of assumptions.

Potential pitfall #1: Failure to attain equilibrium

It seems unnecessary and perhaps trivial to note that binding reactions must reach equilibrium before measurements are recorded in an equilibrium binding assay. Nonetheless, validation that equilibrium has been reached is often not reported. Here, the authors carefully titrate two experimental parameters likely to affect the rate of equilibration. They find that for AChRs in their preparation ligands can require surprisingly long incubation times of 24–48 h and relatively high incubation temperatures of 37°C to reach equilibrium. Thus, experimental protocols that differ in these regards may contribute to apparent discrepancies in reported binding parameters. Importantly, they show that artifactual results due to a failure to reach equilibrium

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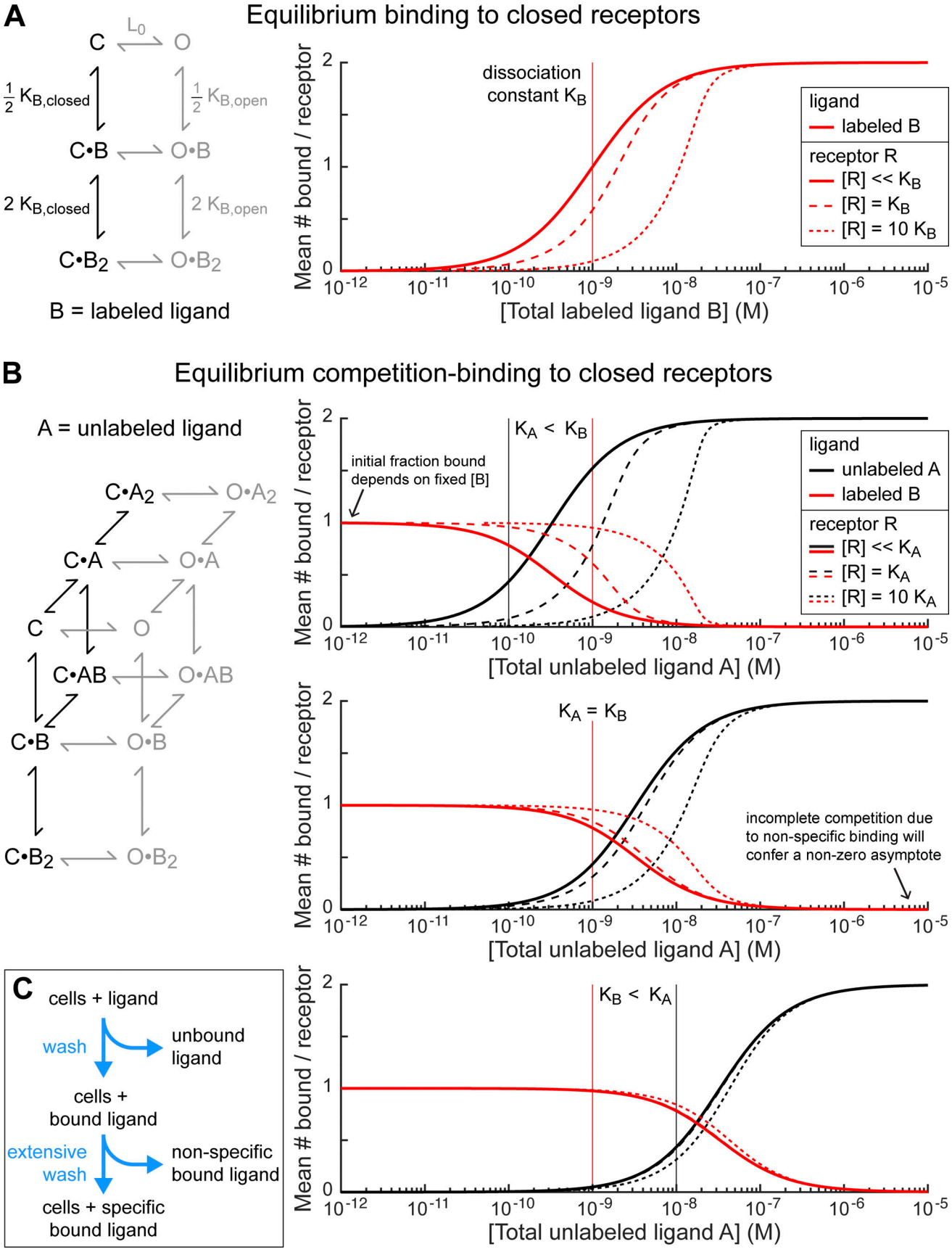

Figure 1. **Effects of ligand depletion in equilibrium binding and binding–competition experiments. (A)** Left: MWC model for binding of labeled ligand B to a receptor containing two identical and independent sites. $K_{B,closed}$ and $K_{B,open}$ are dissociation constants for closed (C) and open (O) states, respectively, and

L_0 is the equilibrium constant for the closed–open conformational change. Gray shading of open states indicates that they are not appreciably populated for NGICs (1) in the absence of ligand, and (2) in complex with antagonists or inverse agonists. Right: Simulations showing number of bound B ligands per receptor at equilibrium as a function of the total applied labeled ligand concentration for closed channels ($K_B = K_{B,closed}$). Dashed curves indicate distortions due to ligand depletion when the receptor concentration approaches or exceeds K_B. Data for this type of experiment is shown in Fig. 6 of Godellas and Grosman (2022) (note linear versus logarithmic y-axis scale). **(B)** Left: MWC model for competitive binding between labeled ligand B and unlabeled ligand A to a receptor containing two identical and independent sites. Gray shading as in A. This is a simplified version of the five-site model explored by the authors (Fig. 1 in Godellas and Grosman [2022]). Right: Simulations as in A showing displacement of labeled ligand B (red) at fixed concentration by increasing concentrations of unlabeled ligand A (black) for closed channels ($K_A = K_{A,closed}$). Note that binding of unlabeled ligand A is not directly measured in experiments but inferred from displacement of labeled ligand B. Dashed curves indicate distortions due to ligand depletion when the receptor concentration approaches or exceeds K_A at three different K_A:K_B ratios. Data for this type of experiment is shown in Figs. 7, 8, 10, 12, and 14 of Godellas and Grosman [2022]). **(C)** Protocol used by the authors for physical separation of unbound, non-specifically bound, and specifically bound labeled ligand.

may be misinterpreted as evidence for non-identical or non-independent sites. This is potentially a very serious artifact that may lead to an incorrect conclusion regarding the binding mechanism. Thus, it is essential that the incubation time and temperature necessary to reach equilibrium is verified for each unique combination of labeled and unlabeled ligand and receptor.

Potential pitfall #2: Failure to distinguish between unbound, specifically bound, and non-specifically bound ligand

For measurement of the labeled ligand B, it is imperative to experimentally distinguish between unbound and bound ligand. Here, Godellas and Grosman (2022) wisely selected [^{125}I] radioactively labeled α-bungarotoxin (α-BgTx) as their labeled ligand. This choice allowed them to take advantage of the very slow dissociation and low non-specific binding of α-BgTx (Lee, 1970) to physically separate unbound and bound ligand by simply rinsing the cells with buffer to remove unbound ligand (Fig. 1 C). Signals proportional to the unbound and bound ligand were then obtained from the radioactivity present in the supernatant and pellet, respectively. Note, for ligands that dissociate more quickly, an alternative approach, such as a scintillation-proximity assay (Udenfriend et al., 1985), may be necessary. It is also necessary to distinguish between specific binding to the receptor binding sites and non-specific binding to the cell membrane, other proteins, or other places on the receptor. The use of α-BgTx again allowed the authors to physically remove non-specifically bound ligand via extensive cycles of resuspending, washing, and pelleting the cells (Fig. 1 C). In addition to minimizing non-specific binding by choosing to monitor [^{125}I]-α-BgTx, they further corrected for any residual non-specific binding by estimating its contribution in cells lacking AChRs. As noted by the authors, in the competition assays non-specific binding is readily apparent as a non-zero asymptote of the binding signal at the highest concentrations of the unlabeled ligand (Fig. 1 B). The take-home message is that appropriate choice of labeled ligand can be essential to reliably separate bound versus unbound ligand in equilibrium binding experiments.

Potential pitfall #3: Ligand depletion

Consider a generic dissociation reaction of a ligand B from a receptor R (Eq. 1):

$$R \cdot B \overset{K_B}{\leftrightarrow} R + B. \quad (1)$$

The equilibrium constant for this reaction, which is the dissociation constant K_B of the ligand, depends on the concentrations of unbound receptor $[R]$, unbound free ligand $[B]_{free}$, and receptor–ligand complex $[R{\cdot}B]$ (Eq. 2):

$$K_B = \frac{[R][B]_{free}}{[R \cdot B]}. \quad (2)$$

While the total applied concentration of B ($[B]_{total}$) is generally under experimental control, the free concentration of B is the thermodynamically relevant quantity. However, binding to the receptor depletes the amount of available free ligand such that $[B]_{free} < [B]_{total}$. As a result, binding curves in which the bound fraction is plotted against $[B]_{total}$ are distorted, shifting progressively to the right, and becoming steeper as the receptor concentration $[R]$ increases (dashed curves in Fig. 1, A and B). These effects can produce significant errors in estimates for the binding parameters when the receptor concentration is on the order of or greater than the ligand dissociation constant. Godellas and Grosman (2022) carefully considered this issue and adjusted their receptor expression to render this effect negligible. Nonetheless, we feel that the graphical depiction of the potential severity of the effects of ligand depletion under several different conditions, as illustrated in Fig. 1, complement the authors' textual description.

Ideally, the receptor concentration would be controlled (e.g., purified receptor protein) so that the free and bound concentrations of ligand can be computed for a given binding dissociation constant. However, for typical cell-based expression systems the receptor concentration is usually not known exactly. In such cases, the difference between the applied and free ligand concentrations is often assumed to be negligible, which greatly simplifies analysis of the binding data. This approximation is valid when the receptor concentration is much less than the ligand's dissociation constant, which is often the case for studies involving heterologous expression of NGICs in cells. Nonetheless, this assumption should not be taken for granted, and the receptor concentration should at least be estimated to assess potential issues due to ligand depletion.

Ligand depletion also distorts the binding–competition curves, but in this case the effects depend on the relative values of the receptor concentration and the dissociation constants for both unlabeled and labeled ligands (Fig. 1 B). The authors do not have a method for directly measuring the free unlabeled ligand concentration, but they were careful to iteratively tune their

cell-based expression to achieve a receptor concentration that is less than the dissociation constant for α-BgTx to validate their assumption that the applied and free ligand concentrations are nearly equal. They also explored binding-competition exclusively with unlabeled ligands that have a lower affinity than the labeled ligand ($K_B < K_A$), in which case the distortion is mitigated (Fig. 1 B). In general, it is imperative to consider the ligand affinities and receptor concentration in each experiment to avoid artifactual distortion of the binding curves due to ligand depletion.

Potential pitfall #4: Non-identifiable binding parameters due to conformational change and site–site cooperative interactions

For NGICs, ligands that open the channel (agonists) bind more tightly to open (conducting) versus closed (non-conducting) channel conformations. This state-dependent binding severely challenges interpretation of equilibrium binding concentration–response relations because the additional parameters needed to describe the binding mechanism often cannot be constrained by the data (i.e., non-identifiable parameters; Middendorf and Aldrich, 2017; Bellman and Åström, 1970). In contrast, for NGICs that are predominantly closed in the absence of ligand, binding of ligands that either have no effect on the closed–open equilibrium (antagonists) or close the channel (inverse agonists) essentially lock the channel in a closed conformation for the duration of the experiment (Fig. 1, A and B). In such cases, the binding mechanism is greatly simplified, often allowing unambiguous estimation of the fewer required binding parameters. Thus, the authors evaluated binding-competition between labeled and unlabeled inverse agonists, which eliminates complications from conformational exchange between closed and open states. Their finding that the Hill coefficient for binding of inverse agonists is approximately equal to one is consistent with identical and independent sites in the closed conformation of the receptor. This observation addresses an important question in the field of NGICs, namely whether the binding sites are functionally identical and whether they act independently or cooperatively. However, in general one should consider whether multiple sites on a receptor are either non-identical or interact cooperatively (i.e., are non-independent). Such cases will require additional parameters to explain the binding mechanism, exacerbating the problem of parameter non-identifiability. The authors also investigated several unlabeled ligands that act as agonists, for which interpretation of the equilibrium competition–binding relations is less straightforward.

The critical importance of choosing an ideal labeled ligand

The above considerations make it evident that a key aspect of the experimental design is the choice of labeled ligand. The authors' judicious selection of [^{125}I]-α-BgTx has several advantages: (1) it is a weak inverse agonist which eliminates complication from closed–open gating conformational changes, (2) its slow dissociation allows simple physical separation of unbound, nonspecifically bound, and specifically bound ligand, (3) it has low non-specific binding in the cell preparation, and (4) its relatively high affinity with respect to the tested unlabeled ligands mitigate effects due to ligand depletion.

Comparison of binding curves for different mutants

Every perturbation (e.g., mutation) may differentially affect unlabeled and labeled ligand affinities and receptor expression. Thus, as the authors advise, it is imperative that after each perturbation, all the above assays and controls are performed including (1) reverification that the reaction has equilibrated, (2) redetermination of the labeled ligand binding curve, (3) re-estimation of the receptor concentration to avoid ligand depletion artifacts, and (4) redetermination of the unlabeled ligand competition–binding curve. The authors furthermore chose a fixed labeled ligand concentration that consistently achieved the same fraction of occupied receptors in the absence of unlabeled ligand. This choice simplifies direct comparison of binding–competition curves between mutants by avoiding complication from multiple effects on both labeled and unlabeled ligands.

Mechanistic insight into ligand binding to ACh receptors

With the methodology in hand to properly measure equilibrium binding curves for NGICs, Godellas and Grosman (2022) explored the effect of mutations in either the ECD or TMD on binding of the inverse agonist methyllycaconitine (MLA). Their results are consistent with MLA binding to independent and identical sites on each receptor, adding evidence for non-cooperative and functionally identical sites in closed homomeric α7-AChRs. They further show that extensive mutations in the TMD (differences between human α7-AChR and *Caenorhabditis elegans* β-GluCl) have little effect on MLA binding, whereas 13 mutations in the ECD (differences between human and chicken α7-AChR) alter MLA affinity. Their observations are consistent with the idea that the effects of mutations in the TMD do not propagate to the distant binding sites in the ECD, whereas mutations outside the binding site but within the ECD can alter binding. These data suggest that the effects of mutations are largely localized to either the ECD or TMD domains, an idea for which disparate conclusions have been reached based on measures of downstream channel current (e.g., Hatton et al., 2003; Purohit and Auerbach, 2009; Wang et al., 1997). Here, Godellas and Grosman highlight how careful direct examination of ligand binding can shed new light on NGIC mechanisms.

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