It is more than 30 years since the advent of the gigaohm seal patch clamp technique, which enabled routine recordings of currents generated by the activity of a single ion channel molecule in its native environment. The muscle nicotinic acetylcholine (ACh) receptor (nAChR) is one of the best ligand-gated channels for single channel recording because of its sizable conductance, robust expression, and consistent subunit composition, both at the native neuromuscular junction and in heterologous expression systems. A specific application of single channel recording is kinetic analysis, the purpose of which is to understand how a channel activates in response to an appropriate stimulus. In a recent issue of The Journal of General Physiology, Mukhtasimova et al. report on their kinetic analysis of nAChR recordings at substantially improved temporal resolution. We shall discuss the implications of this achievement for our knowledge of how these ligand-gated channels work. In brief, the major findings confirm that there is a short-lived preopening conformation of the receptor, followed by the actual opening, and that partial agonists are partial because of a deficiency in the earlier preopening step.

A uniquely useful method

We shall discuss the achievements reported in Mukhtasimova et al. (2016) in detail below, but first we shall give an overview of the technique to demystify it for nonspecialist readers. In the case of the nAChR, the aim of kinetic analysis is to examine how the channel activates in response to the binding of the neurotransmitter ACh. A quantitative reaction mechanism is postulated, i.e., a scheme that shows the number of discrete states in which the system can exist, and the pathways that connect them. For each agonist binding and conformational change step, rate constants are estimated by analyzing sequences of openings and shuttling from recordings in different experimental conditions. Single channel recording is uniquely suitable for this: it may be the oldest of the single molecule techniques, but it remains unsurpassed both in the recording length and the time resolution that can be achieved and produces records containing tens of thousands of channel openings and shuttling at a temporal resolution of the order of tens of microseconds. It is the richness of information in these data that allows us to study the behavior of ion channels at a level of detail that is unique among proteins.

The information contained in the mechanism is useful and important for several reasons. For each step, rate constants define the frequencies of the forward and backward reactions, and their ratio gives the equilibrium constant. The values of the rate constants and the equilibrium constant reflect the height of the energy barriers for the reactions and the difference in free energy between the states they connect. For a ligand-gated channel, the mechanism specifies how many agonist molecules must bind to activate it maximally and the number of conformational changes that separate the binding events from channel opening. It also tells us how effective the bound agonist is at opening the channel and thus measures what pharmacologists call agonist efficacy.

Because kinetic analysis can dissect the binding from the gating steps, it can also estimate the channel’s affinity for its agonist and how it increases as the channel gates. Single channel kinetics is the only method that can answer this question and therefore solve the “binding-gating” problem (or the equivalent affinity/efficacy problem; Colquhoun, 1998). Last but not least, estimating rate constants allows us to predict channel behavior in nonequilibrium conditions, such as those at the synapse, for example. The channel’s trajectory can be mapped through the different states in response to different neurotransmitter concentration pulses (Colquhoun and Lape, 2012), and the time course of a synaptic current through the channel can be predicted.

This quality of information comes at a price, however. Kinetic analysis is slow and laborious, and its success cannot be guaranteed, even for channels with good signals (i.e., a high conductance). For instance, some channel–agonist combinations show pronounced modal behavior, which can make it impossible to assemble the set of data required, unless the modes can be clearly classified at all agonist concentrations needed. Of course, in whole-cell recording we would not even be aware that this heterogeneity is there. In other channels, the pattern of activity is such that the segments of data to be analyzed, typically clusters of many openings...
isolated by long desensitized shut times, do not contain enough binding and unbinding events to allow good estimates of the binding reaction. In the jargon of the method, clusters contain too few bursts/activations. This occurs in channels such as ELIC and α2 glycine receptors, where one is forced to supplement single channel data with macroscopic experiments such as agonist concentration jumps (Krashia et al., 2011; Marabelli et al., 2015).

How does kinetic analysis work?
The single channel currents that we see in a recording are approximately square pulses of random duration, as the channel molecules open and shut. If we record at steady-state (for a ligand-gated channel this means at a constant agonist concentration), the channel has a constant probability of exiting the state it is in. Consequently, its lifetime in each individual state is exponentially distributed, in a manner similar to that of the decay of a radioactive isotope, and the mean lifetime of that state is the reciprocal of the sum of the rate constants for exiting that state. In the simplest situation of one open and one shut state, we could measure the mean duration of the open events and in principle obtain from that the rate constant for shutting, and vice versa.

In the real world, the situation is more difficult. A simple two-state shut–open mechanism is not enough to describe any real ligand-gated channel, which can typically visit at least two open states and far more shut states. Its behavior therefore can be described as an aggregated Markov process, as all the shut (or open) states are indistinguishable from each other in the experimental record. The general theory for coping with this type of stochastic process has been developed since 1977, largely by Hawkes and Colquhoun (see Colquhoun and Sigworth [1995], Colquhoun and Hawkes [1995], and http://www.onemol.org.uk/?page_id=175).

The theory predicts that the distributions of observable properties, such as shut and open times, will be mixtures of many exponential components. Their time constants are not the reflection of a single rate constant, but are found from the eigenvalues of a matrix that contains a subset of rate constants. Furthermore, in real life, recordings have limited temporal resolution, and the openings and shuttings measured from the experimental record are extended by our failure to detect, respectively, brief shuttings and openings. This distortion is a real problem because brief events are common in ion channel records; Mukhtasimova et al. (2016) found that increasing resolution from 22 to ~8 µs doubled the number of shuttings shorter than 100 µs that were detected and measured.

The first estimate of ACh efficacy on nicotinic receptors (Colquhoun and Sakmann, 1985) was obtained by an approximate method for coping with missed short events. A systematic approach became possible after the exact solution to the missed event problem was found (Hawkes et al., 1990, 1992; Colquhoun et al., 1996), enabling the calculation of what is actually observed, rather than what would have been observed in the absence of bandwidth limitations. Thus, we can calculate the probability density of the observed record, given a mechanism and a set of rate constant values. This probability is known as the likelihood. Fitting data consists of finding a set of free parameters, the rate constants, which maximize this likelihood. In other words, we choose the rate constant values that make the observations most probable. This is the method of maximum likelihood.

Two programs are available for doing this: HJCFIT (developed by Colquhoun and co-workers at University College London [UCL]: http://www.onemol.org.uk/?page_id=331#hjcfit), which uses the exact solution to the missed event problem found by Colquhoun et al. (1996) and Hawkes et al. (1992); and QuB (developed by Qin, Auerbach, and Sachs at Buffalo), which uses a different, approximate method of missed event correction (Qin et al., 1996). QuB is used by Mukhtasimova et al. (2016) in the present paper. Nowadays, it is usual to fit simultaneously several experimental datasets that include recordings obtained at different agonist concentrations. This gives more information than fitting single concentration records (Colquhoun et al., 2003). The Sine laboratory was the first to use simultaneous fits, using QuB to pinpoint the kinetic steps affected by a congenital myasthenia mutation (Ohno et al., 1996).

Within each recording, segments of openings and shuttings are selected for analysis if they are likely to result from the activity of one individual channel molecule. This is done by choosing groups of openings delimited by long shuttings (e.g., desensitized periods at the higher agonist concentrations and shuttings likely to represent rebinding at the lower agonist concentrations). The selected segments (which no longer contain information on desensitization) are then “idealized,” i.e., each open and shut time is measured, either by a threshold crossing method followed by a correction for filtering distortion (as in the Mukhtasimova et al. [2016] paper) or by time-course fitting (Colquhoun and Sigworth, 1995). Models of increasing complexity are postulated and fitted to these data, and the resulting mechanism estimate is used to predict several aspects of the observations, e.g., the distributions of apparent open and shut times (which are extended by missed brief events) and (in HJCFIT only) conditional distributions and correlations between nearby apparent open and shut times. The process is repeated until the features of the data are well described and a minimal adequate model with its associated rate constants is identified.
The activation of nicotinic receptors

The nAChR is one of the best understood ion channel in terms of molecular function, and it is impossible to do justice here to the contributions of so many biophysicists (for reviews see Sine [2012] and Auerbach [2013]). We will therefore sketch out only the most relevant features. The channel opens with high open probability when it is bound to two molecules of the neurotransmitter ACh, which is a highly efficacious agonist (Colquhoun and Sakmann, 1985; Sine et al., 1990). ACh and other nAChR agonists tend to be small cationic molecules, which bind also to the open pore of the channel and block it at high concentration (Sine and Steinbach, 1984; Colquhoun and Ogden, 1988). Even if a blocked state is added to the reaction scheme, this is a complication for kinetic analysis, because the agonists have low affinity for their site of action in the pore, and bind and unbind very quickly. High concentration work becomes difficult because block introduces additional brief shuttings, and the unresolved shuttings reduce the apparent amplitude of openings.

Binding of ACh to the two binding sites, at the extracellular interface of the αε and αε subunits, stabilizes the open state by a total of ~10 kcal/mol because agonist affinity is 6,600-fold higher in the open channel (Auerbach, 2013). Unliganded, spontaneous openings of the nAChR do occur (Jackson, 1984), but in wild-type receptors they are both very short and very infrequent, with an equilibrium constant <10⁻⁶, and gain of function mutations have to be introduced to measure them efficiently (Purohit and Auerbach, 2009). Openings of singly liganded nAChRs can also be detected at sufficiently low agonist concentration (Colquhoun and Sakmann, 1985; Sine et al., 1990). In retrospect, these might be openings of singly primed receptors (see the end of this section).

These findings can be summarized by the simple scheme in Fig. 1 A (Colquhoun and Sakmann, 1985), which extends that first proposed by Del Castillo and Katz (1957), by allowing two agonist molecules to bind and monoligated openings to occur. The gray sections, which cannot be estimated directly from data from wild-type channels, emphasize the overall similarity of this scheme with a classical Monod-Wyman-Chance mechanism. In the scheme in Fig. 1 A, the channel goes from resting to open (R → O) in one step, and differences in agonist efficacy result from differences in the rate constants for this step. The mechanism allows only one type of opening to occur from each of the three levels of agonist binding (e.g., unliganded, monoligated, and diliganded). Because of that, this scheme cannot explain the finding that the spontaneous unliganded activity of gain-of-function mutant nAChRs is complex, with several open and shut states (Grosman and Auerbach, 2000; Mukhtasimova et al., 2009).

To cope with this problem, Mukhtasimova et al. (2009) proposed a different scheme (“Primed,” Fig. 1 B), in which the channel is allowed to occupy a new set of shut intermediate states, which connect resting and open states and provide a gateway to opening. Irrespective of whether any agonist has bound, the shut nAChR channel can be resting, singly primed, or doubly primed (R, P’ or P”). The simplest physical interpretation of the scheme is that each of the two binding sites can change its conformation (e.g., prime) independently. The black section of the scheme shows the actual pathway fitted (binding-priming-binding-priming), which was chosen in the 2009 analysis on the basis that it was well defined with wild-type nAChR data. Choosing a plausible subset of the scheme is a necessary approximation because the number of rate constants in the general scheme is too large for them all to be well-defined by the data.

The need to explicitly add intermediate states in the activation mechanism of a ligand-gated ion channel arose first from the kinetic analysis of another channel of the nicotinic superfamily, the α1β glycine receptor. In some ways, the glycine channel is a better subject for this technique because its activity is much more concentration dependent than that of nAChRs and is not complicated by block. We proposed the “flip” mechanism to account for the complexity of the shut time distribution of this channel (Burzomato et al., 2004) and subsequently adapted this scheme to fit nAChRs (Fig. 1 C;
Increasing resolution

As we discussed above, single channel data are distorted by the finite temporal resolution of the technique. Although analysis takes that into account, there is no doubt that increasing temporal resolution will increase the information we can extract from the data. Sine and co-workers have increased resolution by almost threefold, pushing kinetic analysis of nAChRs to its technical limits.

The Mukhtasimova et al. (2016) paper is first of all a tutorial in good experimental working practice in single channel recording. Single channel conductance was increased by selecting potassium as the permeant ion and by recording in the absence of extracellular calcium. The size of the currents was increased also by using a higher holding potential (−120 rather than −70 mV). Noise reduction was addressed by increasing seal stability using a fluoride-based extracellular solution, by pulling electrodes from nonfilamented glass (it is left to the reader to guess how many pipettes had to be discarded because they did not fill properly), and by keeping only the very best patches (below 160 fA root-mean-square noise at 5 kHz; again, we can only guess how many patches had to be discarded).

This increased the effective cut-off frequency from 10 to 23 kHz, a level previously matched only by using thick-walled quartz pipettes (which have to be fabricated with a specially designed puller; see Parzefall et al. [1998]). The best way to grasp the impact of this is to measure its effect on the threshold for detecting a channel event: the resolution. The authors found that at 10 kHz (the bandwidth with their usual recording conditions), the shortest opening or shutting that could be identified by their threshold-crossing idealization software was 22 µs. But at a cut-off frequency of 25 kHz (close to the new bandwidth of 23 kHz), the detection limit was much lower, e.g., 8 µs. This is remarkable. Bringing the detection threshold down to these levels does matter; the lifetime of the fully bound primed intermediate for nAChRs is estimated by Mukhtasimova et al. (2016) to be 4.4 µs, compared with previous estimates of 8 µs and 10 µs for the flip intermediate in glycine and muscle nicotinic receptors, respectively (Lape et al., 2008).

A second good aspect of this paper is that it questions and verifies the quality of parameter estimates, checking whether the rate constants were well-defined, by measuring the effects on the fits of systematically changing and fixing the value of each rate constant. An alternative approach (Colquhoun et al., 2003; Burzomato et al., 2004) is to repeat the simulations many times to produce the distributions of the parameter estimates. In addition to that, data were simulated from the mechanism in the Mukhtasimova et al. (2016) paper, using the set of values obtained and experimental-like noise, and these simulated data were refitted. This allowed the authors to detect a bias in the direct fit, which overestimated the fully liganded opening and shutting rate constants, and these estimates were adjusted by cycles of simulation and refitting until the values obtained in the fit matched those originating the data. This overestimation is attributed by the authors to problems introduced by noise for very brief events idealized with threshold crossing. Finally, the values obtained were verified again by checking that they predict accurately the mean burst duration (this is calculated by a method that assumes no missed events, but this measurement is relatively robust to recording bandwidth).

Agonist efficacy: The role of intermediates is confirmed

The high-resolution data thus obtained were used by Mukhtasimova et al. (2016) to analyze the effects of the full agonist ACh and the partial agonist carbachol (CCh). The scheme fitted (Fig. 2 A) is different from the primed scheme in Fig. 1 B (Mukhtasimova et al., 2009), as it contains only one level of priming and is therefore not a subset of the original full primed model. It differs from the flip model (Fig. 2 B) only in that the two binding steps to the resting conformation are allowed to differ.

ACh and CCh have different efficacy, and if we dissect out block, the fits predict maximum open probabilities of 91% and 50%, respectively, as calculated by the expression

$$\frac{\theta_2 P_2}{\theta_2 P_2 + P_2 + 1}$$

where $\theta_2$ and $P_2$ are the equilibrium constants for fully liganded opening and priming, respectively (in our notation these are called $E$ and $F$, respectively; Fig. 2, compare A and B).

The first notable result in the Mukhtasimova et al. (2016) paper is that this difference in efficacy between ACh and CCh is exclusively caused by a difference in
the priming step. The priming equilibrium is 10-fold more favorable for ACh, with equilibrium constants $P_2$ of 0.2 and 0.02 for ACh and CCh, respectively. Strikingly, the next step, the primed-open equilibrium, is almost identical for the two agonists, with $\theta_2$ values of 60 and 50, respectively. This is a strong confirmation of our hypothesis that the efficacy of agonists in this channel superfamily is determined exclusively by the initial step from the resting to the transient intermediate shut state (Lape et al., 2008). Our proposal came from the analysis of glycine and nAChR recordings with lower resolution (20–40 µs), and it is remarkable that the 8-µs high resolution data in the Mukhtasimova et al. (2016) paper confirm this pattern (see also Corradi and Bouzat [2014] for an extension to 5HT3 receptors).

Ever since Del Castillo and Katz (1957), it had been assumed that partial agonism resulted from a limitation of the shut to open conformation change. When this transition is dissected into two steps, shut-primed and primed-open, efficacy appears to be determined only by the initial step. This change in our view of agonist efficacy is probably the most concrete contribution brought about by the application of schemes with intermediates because it pinpoints the intermediate as the key state to be targeted in designing efficacious agonists. This view of efficacy is intuitively appealing because the early conformation change probably occurs close to the binding site, which is where differences in agonist structure can be detected. Satisfyingly, mechanisms with intermediates also account for the observation (Sine and Steinbach, 1986) that the shortest shut times measured with nAChR agonists of different efficacy are similar. As pointed out by Mukhtasimova et al. (2009), if these shuttings were sojourns in the resting state $A_2R$ of the scheme in Fig. 1A (Colquhoun and Sakmann, 1985), they would be terminated mostly by the channel opening, with rate constant $\beta_2$, which in this scheme would be efficacy dependent, so this class of shuttings should be rare (and longer) for partial agonists, contrary to observation. However, similar short shuttings are precisely what we expect to find if the short shuttings are sojourns in a primed/flipped intermediate state and their mean duration is determined by the opening transition, the rate of which is essentially agonist independent.

The present results differ from earlier ones in that they estimate a somewhat lower equilibrium constant for ACh-induced priming/flipping of saturated receptors (0.2 for ACh [Fig. 2] compared with 3.8 in Lape et al. [2008]), predicting that the fully liganded receptor will spend 18% of its time in the intermediate state and that the maximum probability of being open will be 91% (somewhat lower than the previous estimate of 96% in Lape et al. [2008]). Because of open channel block, we cannot measure maximum open probability.
directly from the data. The relatively low $P_2$ value of 0.2 is a result of the very fast unpriming found by Mukhtasimova et al. (2016), about 10 times faster than that estimated from lower resolution data (Lape et al., 2008).

The 20–80% rise time of a macroscopic nAChR current is predicted to be 110 µs, somewhat slower than that predicted by Lape et al. (2008), viz 55–97 µs. For these comparisons with earlier results, as Mukhtasimova et al. (2016) remark, we should consider that the experimental changes that have brought about the improved resolution are likely to change the channel kinetics somewhat. For instance, choosing potassium as permeant cation and a very negative holding potential changes deactivation (in opposite directions), and the absence of calcium may slow the effective opening rate constant (Magleby and Stevens, 1972; Gage and Van Helden, 1979; Sine et al., 1990).

Estimating rate constants with increased resolution
Mukhtasimova et al. (2016) find a somewhat faster doubly primed-opening rate, 125,000 s$^{-1}$ (compared with the value of 87,700 s$^{-1}$, found by Lape et al. [2008] with a 20-µs resolution). Both values are substantially higher than the earliest estimates of 31,000 s$^{-1}$ (Colquhoun and Sakmann, 1985) and 16,000 s$^{-1}$ (Sine et al., 1990); note that these are rates for the full resting-open transition and are therefore expected to be slower than the rate for the primed-open transition by a factor of $P_2/(P_2 + 1)$.

The most intriguing observation that stems from the improved estimation of rate constants in the Mukhtasimova et al. (2016) paper is the emergence of a pattern in the priming rate constants. Primed states are favored, as the channel becomes fully bound, purely because priming becomes easier. The forward priming constant increases by ∼20-fold for both ACh and CCh, whereas unpriming is unchanged for CCh and, if anything, somewhat faster for ACh. In other words, agonist binding favors entry into the intermediate state but does not slow unpriming. This is new territory—our attempts to probe nAChR intermediates by fitting the flip mechanism gave estimates of monoliganded priming rate constants that were poorly reproducible across our three independent datasets (see Fig. 2 B), although we found a pattern similar to that seen by Mukhtasimova et al. (2016) for the better-resolved glycine receptor (Burzomato et al., 2004).

In Mukhtasimova et al. (2016), the primed conformation has a 23-fold greater affinity for ACh than the resting conformation (8 vs. 187 µM), entirely because of the association rate constant is faster, close to the maximum compatible with diffusion. This is comparable with the 65-fold increase in affinity for the flipped state found at UCL for the glycine receptor, which also resulted from an increased association rate constant (Burzomato et al., 2004). In the nicotinic receptor, we detected a smaller increase in affinity (about twofold), but our estimate of the association rate constant had a high coefficient of variation across experimental sets.

The physical basis for the observation that priming increases the association rate constant is obscure. It is well established that the binding pocket closes on the agonist as the channel activates, and one might have expected the higher affinity in the intermediate to result from a slower unbinding, rather than the faster binding inferred from fits. It may be that the schemes that we can robustly fit to the functional data are still insufficiently detailed to allow us to map completely its results to the physical reality of the receptor.

It would be surprising if a paper like Mukhtasimova et al. (2016), which ventures in new experimental territory, did not raise new questions. Perhaps the most puzzling finding is the 19-fold decrease in affinity estimated for the binding of the second ACh molecule relative to the first. Binding affinity to the resting conformation is high for the first molecule (10 µM) but decreases for the second ACh molecule (187 µM). Once the receptor primes, affinity returns to 8 µM. This result implies strong negative cooperativity, e.g., that the two agonist sites interact and that binding to one site depends on whether or not the other site is occupied. This change in apparent affinity (and hence the interaction) occurs in the resting receptor, in the absence of priming. It is hard to imagine how such a strong interaction between two binding sites that are 60 Å apart could occur in the absence of a conformational change. Note that if the sites are independent, i.e., do not interact, then $K_1$ and $K_2$ would appear to be the same (whether or not the sites were initially identical; see Appendix).

A general scheme for two binding steps would have to incorporate explicitly two different monoliganded states and allow the binding sites to be different before the agonist binds (Appendix, Scheme A2). In the flip model, the two monoliganded states can be collapsed into one because of the assumption that the two sites are identical and independent. A similar assumption was made by Mukhtasimova et al. (2009), but in Mukhtasimova et al. (2016) interaction between sites is allowed, so there should be two different monoliganded states (Appendix, Scheme A2). All the fitted schemes reduce the number of free parameters that they attempt to fit to the number that can be estimated from the data.

Choice of model in kinetic analysis
These considerations highlight the problem of model choice in kinetic analysis. As we have seen, the depth of information in single channel data can give numerical estimates for remarkably detailed schemes, with up to 14 or so free parameters, whereas even the best macroscopic methods can rarely give more than 3 or 4. But even that is not always enough.

Models are useful only in so far they are a sufficiently good approximation of the actual physical re-
ality of the receptor’s conformations. It is sobering to pause and consider what a truly realistic mechanism in principle should include. It’s self-evident that states with different numbers of ligand molecules bound must have physical existence and that open and resting states should exist for all of them. We should also add at least one level of (shut) intermediates. The resulting model is too complex to be fitted as such. The very general full primed mechanism (Fig. 1 B) has 9 shut and 9 open states, with 35 connections and 66 rate constants, of which 16 can be set by microscopic reversibility, leaving 50 free parameters to be estimated (Colquhoun et al., 2004). This is far more than can be done with existing methods. This means that some restricted subset of the full primed model always has to be chosen to get a well-defined fit (Mukhtasimova et al., 2009; Colquhoun and Lape, 2012). Attempts to choose the best subset are discussed by Colquhoun and Lape (2012). It makes sense to eliminate states that are rarely visited because the experimental record will contain little information about the rates of entry to, and exit from, such states. The problem is that such states have to be identified from indeterminate fits. The question of how to choose the best subset of an over-parameterized model is still not completely solved.

Clearly there is a conflict between ensuring that the mechanism used is simple enough to be fitted robustly and including sufficient detail to try to relate the kinetic steps to the physical reality of channel activation. These problems only stress the importance of increasing the information we can extract from data, by improving temporal resolution, as Mukhtasimova et al. (2016) have done, or improving our analysis techniques.

Although we must remain aware of these limitations, it is important to stress that the broad picture that is emerging is consistent and compelling. The channel activates by passing through (at least) one set of intermediate states, which differ from the resting states because of their increased agonist affinity. Mechanisms that incorporate intermediates describe well the activation of many channels in the nicotinic superfamily, including isoforms and mutants of the glycine receptor, the prokaryotic model channel ELIC, and the GABA and 5HT₃ receptors.

Evidence from other lines of work also supports the existence of such intermediates. Over many years, the Auerbach laboratory has systematically mutated a vast proportion of nAChR residues to carry out linear free energy analysis. The ϕ values thus obtained are clustered in a few, maybe four, groups, and their values decrease in a gradient from the binding site to the channel gate. The simplest interpretation of these findings is that the clusters move sequentially during activation (reviewed in Auerbach [2013]). The same group proposed that the increase in agonist binding affinity that occurs with activation requires two correlated, but distinct, conformational changes in the binding site (“catch” and “hold”; Jadey and Auerbach, 2012).

It is impressive and encouraging that there is this degree of convergence, given that the results outlined above stem from work performed with three different idealization programs and two different global fitting maximum likelihood methods with different missed event corrections.

The future
As we have seen, the Mukhtasimova et al. (2016) paper sets new standards for kinetic analysis in nAChRs and their relatives. New tantalizing glimpses of the details of the activation process as the channel travels through the intermediate states between binding and opening have emerged from the improved resolution, giving us new questions to address.

How can we progress further? The first possibility is obviously to attempt to further improve temporal resolution, but there are few weapons that remain to be deployed, and it is hard to know how much of an improvement can still be achieved with present technology. We can work on the experimental conditions, for instance mutating the channel to achieve a higher conductance or using thick-walled quartz glass pipettes. Recording with these has achieved 6-µs resolution (Parzefall et al., 1998), even without some of the other refinements used by Mukhtasimova et al. (2016). We will then probably be close to the limit of the response of existing amplifiers, whose rise time is estimated to be ~5 µs with the internal 100-kHz filter. Other areas of possible improvement lie in the analysis. For idealization, it has been suggested that time course fitting can gain some further resolution compared with threshold crossing (Colquhoun and Sigworth, 1995), but this advantage has never been quantified. For the mechanism fitting itself, we have seen that the schemes that we are fitting are at the edge of what is possible to estimate in terms of the number of free parameters, so improving our fitting methods could also help. Possibilities here include the use of a likelihood that can deal with open–shut correlations in the mechanism, such as the one used in HJCFIT. As Bayesian Markov Chain Monte Carlo approaches become more computationally feasible (even with missed event correction), they are also likely to become more useful, as they systematically give us the distributions of the estimators and flag parameters that are poorly identifiable or whose point estimate is biased less laboriously than simulations (Epstein et al., 2016). These may also help us by putting on a more rational basis the choice of the model to be fitted, which, as we have seen, is critical.

Only time and effort will tell how much these measures can help. However, as we have seen, much of
the progress of the last decade has come simply by extending our work to more channels and tracing the common features of activation across the super-family. Kinetic analysis of single channel recording has given us more insight than any other functional method into the activation of ligand-gated channels. More is to come.

**APPENDIX**

**Binding to the resting state**

In the scheme fitted in the Mukhtasimova et al. (2016) paper (Fig. 2 A), the two binding steps to the resting conformation are written as shown in Scheme A1. The rest of the states are omitted here, to aid clarity.

![Scheme A1](image)

Here we shall explore the consequences of writing the binding steps as in Scheme A1.

Scheme A2 shows the general model that describes two binding sites. The two sites are denoted as a and b and they may (or may not) be different initially and they may (or may not) be independent. Independence implies that binding to the a site is the same, whether or not the b site is occupied and vice versa. Although the two models differ kinetically, Scheme A2 can be condensed to Scheme A1 at equilibrium:

![Scheme A2](image)

If the sites are independent, i.e., binding to site a is the same whether or not site b is occupied, Scheme A2 reduces to Scheme A3. Good fits to nAChR data have been obtained with the assumption of independence.

To reduce the most general Scheme A2 to Scheme A1 (fitted in Mukhtasimova et al. [2016]), we take the total rate of leaving each state, so, for example, \( k_{+1a} \) in Scheme A1 is actually \( k_{+1a} + k_{+1b} \) in Scheme A2, and similarly for other rate constants. This means that Scheme A1 \( K_1 \) is, in terms of Scheme A2,

\[
\frac{k_{+1a} + k_{+1b}}{k_{-1a} + k_{-1b}}.
\]

And Scheme A1 \( K_2 \) is actually

\[
\frac{k_{+2a} + k_{+2b}}{k_{-2a} + k_{-2b}}.
\]

If the two sites are independent, the order of binding is irrelevant and the model reduces to Scheme A3. In this case, the \( K_1 \) and \( K_2 \) estimated with Scheme A1 both become

\[
\frac{k_{+a} + k_{+b}}{k_{-a} + k_{-b}}.
\]

In words, independence of the binding sites implies \( K_1 = K_2 \), regardless of whether the a and b sites are initially identical or not.

When fitting Scheme 1, Mukhtasimova et al. (2016) found \( K_1 = 10.4 \) µM and \( K_2 = 187 \) µM. The two equilibrium constants differ by a factor of 18. The foregoing argument shows that this implies that the two sites are not independent. It says nothing about whether or not the a and b sites are identical.

In contrast with Mukhtasimova et al. (2016), we have found good fits to both nicotinic and glycine data without the need to postulate interaction between the binding sites, in either the resting conformation or in the flipped conformation. Because the binding sites are quite a long way apart and no global conformation change has occurred, this was what would be expected on physical grounds, and it was one of our reasons for preferring the flip model.

However, the flip model also makes a physically implausible assumption of a different kind. It assumes that the change from testing to flipped conformation was concerted—the whole molecule flips in a single reaction step. Because the change from resting to flipped is envisaged as a change in shape that is localized to a
region quite close to the binding site, it’s hard to imagine one site interacting sufficiently strongly with the other to make the conformation change concerted. In the original primed model (Mukhtasimova et al., 2009), this objection is removed by postulating that the initial preopening conformation change occurs independently at each binding site. In the Mukhtasimova et al. (2016) fitting, the reduced primed model works at the price of postulating a physically implausible interaction between the binding sites in the resting conformation.

This conflict is unlikely to be resolved until such time as it becomes possible to estimate all the rate constants in the full primed model, with binding as in Scheme A2. That is not yet possible. The full model has too many free parameters for even single channel recording to identify (see Choice of model in kinetic analysis).

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