Decades ago as a beginning graduate student, one of us (K.L.M.) was studying short-term synaptic plasticity at the neuromuscular synapse. The responses were generally consistent from day to day, but there was some variability. At that time, K.L.M. naively thought that if it were possible to directly study the macromolecular building blocks underlying neuromuscular transmission, the variability would disappear. The advent of recording from single ion channels (Neher and Sakmann, 1976; Hamill et al., 1981; Magleby and Pallotta, 1983; Colquhoun and Sakmann, 1985) showed just how naive this wishful thinking was. Single-channel current records, which indicate the opening and closing transitions of a channel by step changes in the current, revealed that successive open- and closed-interval durations were stochastic in nature, with the interval durations ranging many orders of magnitude, and predictable only in terms of probabilities. In retrospect, such stochastic variation of single ion channel gating was predictable from the observed exponential decays of macroscopic ionic currents recorded from cells when large numbers of open channels were closing (Hodgkin and Huxley, 1952; Magleby and Stevens, 1972); the exponential decays arose from exponentially distributed effective open durations (burst durations).

With further analysis, the stochastic variation in ion channel gating was found to be far more complex. Channels can gate in multiple open and closed kinetic states, with the mean lifetimes of the various kinetic states ranging over many orders of magnitude (Magleby and Pallotta, 1983; Colquhoun and Sakmann, 1985; McManus and Magleby, 1988). A further addition to variability was the observation at the single-channel level that channels can switch between different modes of gating for constant experimental conditions, with the duration of the gating in the various modes ranging from milliseconds to minutes (glutamate-activated channel, Patlak et al., 1979; AChR channel, Auerbach and Lingle, 1986; fast chloride channel, Blatz and Magleby, 1986; BK channel, McManus and Magleby, 1988; embryonic AChR channels, Naranjo and Brehm, 1993; BK channels in drosophila, Silberberg et al., 1996; GABA\textsubscript{A} channels, Lema and Auerbach, 2006; NMDA receptors, Zhang et al., 2008; AMPA receptors, Prieto and Mollmuth, 2010; and GluA3 receptor channels, Poon et al., 2011). Modal shifts in gating are typically identified by abrupt changes in mean open probability (Po), mean open-interval duration, and/or mean closed-interval duration. Whereas modal gating can be described by discrete-state Markov models (Blatz and Magleby, 1986; Silberberg et al., 1996; Popescu and Auerbach, 2004) with shifts among different collections of kinetic states, the mechanism underlying modal gating has remained an enigma.

This commentary now addresses a recent paper by Vij, Purohit, and Auerbach (Vij et al., 2015) that provides novel insight into the mechanism underlying modal gating for adult mouse endplate acetylcholine receptor (AChR) channels. Their study was possible in large part because they engineered a form of stable modal gating that could be readily studied. This is in contrast to the more typical transient modal gating identified by mode shifting, which can be difficult to study because of its stochastic variability of occurrence and duration. We start with examples of transient modal gating from the study of McManus and Magleby (1988) and then present an example of stable modal gating from the paper of Vij et al. (2015).

Fig. 1 shows two examples of transient modal gating (McManus and Magleby, 1988). Fig. 1 A presents a short excerpt of a continuous recording of current through a single BK channel, divided into three consecutive traces. Channel opening and closing is indicated by upwards and downward steps in the current, respectively. The down arrow indicates a shift from normal mode gating to brief open mode gating. The upward arrow 590 ms later signals a return to normal mode gating. The modal shifts were readily identified by the stability plot in Fig. 1 B, which presents a moving average of the mean duration of open intervals taken 50 at a time. The abrupt decrease in the mean open-interval duration from 2.8 to 0.5 ms indicates a shift from normal mode gating to brief open mode gating, and the abrupt return indicates a shift back to normal mode gating. Another example in Fig. 1 of mode shifting from the same
BK channel presented in A and B is depicted in C and D. The BK channel shifts from normal mode gating to (flicker) buzz mode gating with a mean open-interval duration of 0.05 ms, and then back to normal mode gating. The stability plot for this channel also indicated mode shifts to and from an intermediate mode with a 2.0-ms mean open duration (not depicted). 96% of the time was spent in normal mode gating, with infrequent entries into the other three modes.

An example of stable modal gating from the paper by Vij et al. (2015) for adult mouse endplate AChRs expressed in HEK cells is shown in Fig. 2, which presents excerpts from their Fig. 2. The horizontal panel in Fig. 2 A characterizes normal mode gating. On the left is a continuous single-channel record displayed as seven traces (opening in this instance is downward) showing clusters of activity (C-O) separated by longer closed intervals indicating desensitized channels (D) resulting from the continuous presence of 30 µM ACh. A histogram of the number of clusters versus their Po shows a single, normal mode of activity with a mean Po of 0.7 (middle). The open and closed dwell-time distributions of the entire record are each described by a single-exponential component (right). With a single WT channel, only normal mode activity (plus a few isolated openings) was observed in the clusters for up to 30 min of recorded activity, indicating stability of the normal mode in the absence of mutations. In a previous study of AChRs expressed naturally in *Xenopus laevis* myocytes, a single putative shift to a higher Po mode was observed (Auerbach and Lingle, 1986), indicating that mode shifts in WT endplate AChRs are possible but rare.

Fig. 2 B shows examples of stable modal gating in an engineered mouse endplate AChR. Incredibly, the single-point mutation αP197A in the C loops that cap the ACh-binding pockets was found to induce four different stable modes of gating, with mean Po values of 0.1, 0.2, 0.67, and 0.96 (middle). The Po = 0.67 (±0.06) mode was similar to the WT mode (0.70 ± 0.06). The dwell-time distributions obtained from the entire single-channel record, which would include data from all four modes, were described by four closed- and three open-exponential components, suggesting that most of the kinetic properties underlying the different modes were clearly distinct. As was the case for normal mode gating (Fig. 2 A), Vij et al. (2015) did not observe mode switching within any of the clusters of gating for the mutation-induced modes in Fig. 2 B or in other patches, suggesting that the various mutation-induced modes in their experiments were stable. On this basis, their mutation-induced modal gating arises from different AChR channels gating in different stable modes within the same patch. A cluster of activity in a single stable mode would occur when one of the desensitized channels in the membrane patch recovered from desensitization for a brief period of time (mean of ~400 ms) before becoming...
desensitized once again. In this case, clusters of activity with the same or different modal behavior should occasionally overlap when two or more channels recovered from desensitization at overlapping times. The authors (Auerbach, A., personal communication) indicated that clusters of activity did occasionally overlap.

Before summarizing the experimental findings of Vij et al. (2015), it would be useful to briefly summarize some of the properties of adult endplate AChRs for those who do not follow this essential synapse. In mammals, neuromuscular transmission requires the presynaptic release of acetylcholine from the nerve terminals of motor neurons whose cell bodies are located in the spinal cord and brainstem onto AChRs located in the postsynaptic muscle membrane. Without such transmission, skeletal muscles become paralyzed, breathing stops, and death soon follows. The adult endplate AChRs studied by Vij et al. (2015) and references therein are each comprised of five homologous subunits: αβδε. Each AChR has two ACh-binding sites in the extracellular domain, one at the α-δ interface and one at the α-ε interface. At each interface, three aromatic groups (αY190 and αY198 in the C loop and αW149) play a major role in determining the ~175-µM ACh affinity ($K_d$) of the adult endplate AChR. In the absence of agonist, the gating equilibrium constant for the closed–open transitions is $7 \times 10^{-7}$, indicating that unliganded channels do open, but only with a very low probability (Jackson, 1984; Nayak et al., 2012; Purohit and Auerbach, 2013). Gating in the absence of agonist is consistent with the Monod–Wyman–Changeux allosteric gating mechanism. The addition of high concentrations of ACh activates AChRs to open with a Po of 0.96 (in the absence of desensitization).

In an extensive series of experiments like those in Fig. 2, Vij et al. (2015) found that: (a) WT channels gated in a single mode. (b) Point mutations at 10 of the 12 residues comprising loop C at the α-δ binding pocket produced stable modal gating. The number of stable modes ranged from two to four, depending on the site and identity of the substituted residue. (c) Point deletions in the C loop at the α-δ binding pocket could also produce modal gating. (d) C loop mutations at the α-ε binding pocket did not produce modal gating. (e) Partial agonists generated fewer modes than ACh. (f) Modal gating was not observed in the absence of agonist. (g) Removing a tryptophan sidechain in the δ subunit (δW57A) prevented modal gating. (h) ACh affinity at the α-δ binding pocket was increased in modes with increased Po and decreased in modes with decreased Po.

**Figure 2.** Stable modal gating in adult endplate AChRs. (A) Stable normal-mode gating. (Left) Continuous single-channel recording displayed as seven traces obtained with on-cell patch-clamp recording from HEK cells with expressed WT mouse AChRs. Opening is downward. The clusters of activity arise from AChRs coming out of desensitization (D) to gate (C-O), and then returning to desensitization. (Middle) Histogram of the number of clusters versus their mean Po. Only a single normal mode of activity is seen with a Po of 0.7. (Right) Dwell-time distributions of shut- and open-interval durations are each described by a single exponential, indicating gating in two states (C-O). (B) Four stable modes of gating after the mutation P197A in the α subunits. (Left) Single-channel current record during continuous recording displayed as seven traces. (Middle) Gating in four different stable modes with mean durations of 0.01, 0.20, 0.67, and 0.96. (Right) The dwell-time distributions of the entire current record are described by multiple exponentials, indicating different kinetics for the different modes. See Fig. 2 of Vij et al. (2015) for further details.
These observations clearly focus the culprit for stable modal gating of AChRs on changes in the structure of the binding pocket for ACh at the α–δ interface. These findings of Vij et al. (2015) thus rule out many nebulous mechanisms for stable modal gating. For example, modal gating is not induced by: (a) cytosolic components that come into variable contact with the channel; (b) intramembrane proteins that come into contact with the channel; (c) fluctuating changes in the lipid composition in contact with the channel; (d) changes in internal tension/pressure in the lipid membrane holding the channel; (e) junk and/or impurities in the solution bathing the extracellular part of the channel that could get stuck in channel machinery; (f) the phase of the moon; and (g) cosmic rays. Yet another nebulous mechan-ism is that different mutations might produce different modal behavior arising from mutation-induced differences in translational or posttranslational modifications of the channel. For all of these nebulous mechanisms, it seems highly unlikely that only mutations at the α–δ binding pocket, but not the α–e binding pocket, would produce such modal gating.

Given the conclusion that modal gating in AChRs arises from mutation-induced structural changes in the α–δ binding pocket, how is it possible that a single-point mutation or deletion can produce from two to four different stable modes? In a tour de force discussion, Vij et al. (2015) systematically account (with suitable precautions) for their observations using a novel 2 × 2 model. A highly simplified version of their discussion follows. For WT channels, the α–δ binding pocket has two conformations, free and bound. The properties of these two conformations remain stable over time, producing a stable affinity for ACh, giving a stable Po, and consequently, a single stable gating mode. A mutation of the C loop of the α–δ binding pocket then produces from two to four different long-lasting conformations of the bound binding pocket, giving a corresponding number of long-lasting affinities and long-lasting modes. Why up to four modes in stable modal gating? Vij et al. (2015) propose that the α-subunit C loop and the δ-subunit β sheet can each assume two different conformations, giving rise to a 2 × 2 model, with up to four different stable conformational subtypes (α₁δ₁, α₁δ₂, α₂δ₁, and α₂δ₂), each with a different stable affinity that produces a different stable mode. The proposed stable conformations are not known, but perhaps molecular dynamics simulations could reveal such alternative stable conformations if they are present. If such alternative stable conformations of the binding pocket cannot be found, then it is possible that the different conformational subtypes might arise indirectly from different multiple stable conformations in other parts of the channel feeding back to regulate the conformations and affinities of the binding pocket in a mutation-specific manner. The authors conclude that the different stable modes arise from different stable affinities at the α–δ binding pocket, thus providing a mechanism for modal gating in AChRs 30 years after the initial observation.

**Limitations**

The modal gating studied by Vij et al. (2015) was not naturally occurring, but induced by mutations in the α–δ AChR binding pocket. Furthermore, the multiple different modes produced by single-point mutations were stable, so mode switching, a hallmark of modal gating, was not observed. The different modes in their study were defined by different Po’s, which, as expected, arose from different affinities for agonist at the mutated binding pocket, which would produce different Po’s and consequently, different modes of gating. This may sound like circular reasoning, so why is their paper such a profound step forward toward understanding modal gating? It is because single-point mutations or deletions in the binding pocket could produce stable gating in two to four different modes. Different modes of gating from the same channel, however stable, are a form of modal gating. Vij et al. (2015) then present a novel model consistent with their considerable data to advance a mechanism for modal gating. Hence, they provide a proposed mechanism to explain a previously mysterious phenomena. Additionally, they have developed a system that routinely produces different stable modes, which should allow further systematic study of the relationships between the proposed alternative conformations, affinity, Po, and gating kinetics. Finally, their system could perhaps be tweaked by additional mutations to decrease the stability of the modes so that the process of mode switching could itself be routinely observed and studied. In future studies, it will be interesting to examine to what extent their conclusions apply to transient modal gating in WT adult endplate AChRs and in other ion channels where modal gating has been reported.

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