J Physiol 588.1 (2010) pp 45–58

SYMPOSIUM REVIEW

What single-channel analysis tells us of the activation mechanism of ligand-gated channels: the case of the glycine receptor

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Glycine receptors are, in several ways, the member of the nicotinic superfamily that is best-suited for single-channel recording. That means that they are ideal for testing ideas about how activation proceeds in a ligand-gated ion channel from the binding of the agonist to the opening of the channel. This review describes the quantitative characterization by single-channel analysis of a novel activation mechanism for the glycine receptor. The favourable properties of the glycine receptor allowed the first detection of a conformation change that follows the binding of the agonist but precedes the opening of the channel. We used the term 'flipping' to describe this pre-opening conformational change. The 'flipped' state has a binding affinity higher than the resting state, but lower than the open state. This increased affinity presumably reflects a structural change near the agonist binding site, possibly the 'capping' of the C-loop. The significance of the 'flip' activation mechanism goes beyond understanding the behaviour and the structure–function relation of glycine channels, as this mechanism can be applied also to other members of the superfamily, such as the muscle nicotinic receptor. The 'flip' mechanism has thrown light on the question of why partial agonists are not efficacious at keeping the channel open, a question that is fundamental to rational drug design. In both muscle nicotinic and glycine receptors, partial agonists are as good as full agonists at opening the channel once flipping has occurred, but are not as effective as full agonists in eliciting this early conformational change.

(Received 28 August 2009; accepted after revision 17 September 2009; first published online 21 September 2009)
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Introduction

It is tempting to consider glycine receptors as a 'minor' member of the nicotinic superfamily of ligand-gated ion channels. For instance, glycine channels do not have the profuse diversity of subunit genes that neuronal nicotinic receptors have, as only four subunit isoforms are known in man, α1–3 and β (mice and chickens have also α4). Any of the α subunits can form functional homomeric channels or $\alpha\beta$ heteromeric channels. Furthermore, the synaptic role of glycine is not as widespread as that of GABA, and glycinergic synapses are important only in

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This review was presented at a symposium on *Neurophysiology of inhibitory & excitatory amino acid receptors* which took place at the 11th International Congress on Amino Acids, Peptides and Proteins, Vienna, on 3 August 2009.

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DOI: 10.1113/jphysiol.2009.178525

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The Journal of Physiology

restricted areas of the adult nervous system, such as the spinal cord, brainstem and retina (for comprehensive surveys of the literature on the molecular properties, pharmacology and synaptic function of glycine receptors see Legendre, 2001 and Lynch, 2004). In addition, few of the agents that act on glycine receptors are specific and none is in therapeutic use. Glycine receptors can be activated by many common amino acids, such as β-alanine, taurine, sarcosine, β-aminobutyric acid, β-aminoisobutyric acid and GABA and these, except for β-alanine (which is somewhat intermediate, Lewis *et al.* 2003), have a very much lower efficacy than glycine, as they elicit a much smaller maximum current. The most potent antagonist is strychnine which is selective, has a competitive mechanism of action and a high affinity for both native and recombinant receptors (about 20 nM, Young & Snyder, 1973; Lewis *et al.* 1998). Picrotoxin, a mixture of picrotoxinin and picrotin which is a potent blocker of $GABA_A$ receptors, does block glycine receptors too and can help in identifying homomeric receptors, being at least 20-fold more potent on homomeric than on heteromeric channels (Pribilla *et al.* 1992; Burzomato *et al.* 2003). The $GABA_A$ competitive antagonist gabazine also blocks glycine receptors (Wang & Slaughter, 2005). Because of its low affinity (0.2 mM), and its fast dissociation from the glycine receptor (Beato *et al.* 2007), it has proved useful in probing the time course of glycine in the cleft of glycinergic synapses onto motoneurones (Beato, 2008). Relatively few compounds that act on glycine channels are sufficiently subunit-selective to be of any use as experimental tools, even though recent work has identified promising candidates such as 5,7-dichlorokynurenic acid (Han *et al.* 2004), ginkgolides and cannabinoids (Hawthorne *et al.* 2006; Yang *et al.* 2008). Glycine channel activity can be modulated by many of the agents that affect $GABA_A$ channels, such as zinc, steroids, alcohol and general anaesthetics (Lynch, 2004), but none of these is selective for glycine receptors. Finally, inherited defects of the glycine channel are one of the causes of human congenital startle disease or hyperekplexia (together with defects in other components of glycinergic synapses, such as the glycine transporter and receptor-clustering proteins such as gephyrin and collybistin; Harvey *et al.* 2008). Human hyperekplexia is a rare, mostly autosomal dominant disease, and it can be successfully treated with benzodiazepines, which presumably increase the signal at GABAergic synapses, compensating for the decrease in glycinergic transmission (Bakker *et al.* 2006).

Nevertheless, it happens that glycine receptors have properties that make them particularly useful for the investigation of channel-opening mechanisms. This sort of study ultimately relies on the analysis of single-channel recordings, because these give greater resolution and avoid some of the ambiguities of interpretation that whole-cell current measurements are subject to (Colquhoun, 1998). Although most investigations have been done with muscle-type nicotinic acetylcholine receptor channels, glycine receptors are better in two ways. Firstly, unlike nicotinic channels, they are not blocked by the agonist itself and that makes investigations with high agonist concentrations simpler, particularly for low-potency agonists (Lape *et al.* 2008). Secondly, valuable information on mechanism comes from the concentration dependence of single-channel activations: longer bursts made of several openings become increasingly common, replacing shorter ones, as agonist concentration is increased (and more binding sites on the channel become bound). At low concentrations, partially liganded shorter bursts are more common (and therefore easier to characterise) in glycine receptors than they are in muscle nicotinic recordings. In addition to that, glycine receptors express well in heterologous systems, where they produce channels that resemble native ones (Takahashi *et al.* 1992; Beato & Sivilotti, 2007), have a high conductance (86 pS for α1 homomers and 44 pS for $\alpha1\beta$ heteromers (Bormann *et al.* 1993) and, at least in cell-attached recordings, rarely open to subconductance levels (Beato *et al.* 2004; Burzomato *et al.* 2004). This relatively high conductance makes it easier to obtain single-channel records with good signal-to-noise ratio, in turn ensuring good time resolution in detecting and measuring channel openings and closings. These characteristics make glycine channels (at least in their α1 or $\alpha1\beta$ form) a useful system for testing our ideas about the molecular mechanisms of receptor activation in this superfamily, by fitting mechanisms to single channel records.

Mechanisms: a user's guide

What is the purpose of fitting a mechanism to the data? A mechanism represents the process of channel activation as a chemical reaction that takes several reversible steps. First and foremost it is a way of writing down in a clear and compact form what you think physically happens when the channel is activated and as such it should help in formulating this question and thinking about it.

The modern age for modelling ligand-gated ion channels began in 1957 when del Castillo and Katz working at University College London on frog endplate nicotinic receptors proposed 'as a working hypothesis, that the receptor ... reacts by a ... two-step process, first forming an intermediate inactive compound which is then changed into an active, depolarizing form... whose nature and transformation are, at present, unknown' (del Castillo & Katz, 1957). At the time, the existence, let alone the nature, of ion channels as aqueous pores in the membrane was indeed controversial (reviewed in Hille, 2001). Starting from a simple, reasonable assumption of what the physical process must entail (in modern terms,

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binding of the agonist A to the receptor R, followed by agonist-bound channel AR opening to AR*), the del Castillo–Katz mechanism

$$A + R \underset{k_{off}}{\overset{k_{on}[A]}{\rightleftharpoons}} AR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR^* \qquad (1)$$

elegantly accounts for a range of observations that include the effects of partial agonists (agonists that are less effective at keeping the channel open once they are bound) and competitive antagonists (compounds that bind but cannot produce the subsequent conformational change that opens the channel).

A reaction mechanism, like that of del Castillo & Katz, postulates that the receptor–channel molecule can exist only in one or another of a relatively small number of discrete states. For example it may be open (AR*) or shut (R and AR), or it may have 0, 1, . . . *n* molecules of ligand A bound to it. Looking at the sharp transitions between shut and open states provides perhaps the most direct evidence ever for the assumption of all chemical kinetics that there are discrete states and that the time taken to move from one state to another can be very small. Of course, the postulated 'discrete states' are an approximation: they represent minima in the energy profile. Any sufficiently deep well represents a state that, for practical purposes, can be considered discrete. The rate for the transitions between the states (the number on the arrows in the reaction diagram) represents the *frequency* of the transitions and the next step in understanding the channel activation is to get values for these rates, and hence for the equilibrium constants for each reaction step. In the simple scheme in eqn (1), a binding step, described by an equilibrium dissociation constant K_d (the ratio between k_{off} and k_{on}) is followed by an opening or gating step, described by an equilibrium constant for the conformational change, often referred to as efficacy E (the ratio between the opening rate β and the closing rate α; note that in this review we use the term gating to refer only to the final shut–open transition, and not to the overall activation process).

Mechanisms like this have an immediate usefulness for the interpretation of single-channel records, as the average time that is spent in any of the states is given by the reciprocal of the sum of the rates of exit from the state. For instance, in the del Castillo–Katz mechanism, the mean lifetime of the open-state AR* is the reciprocal of the closing rate α, and is not affected by the binding rate constants (or by the concentration of A). As we will see, this is a first approximation, for two reasons. Firstly, our experimental observations do not measure the lifetimes of each individual state, because we can only know whether the channel is open or closed, but cannot tell which of the open or shut states the channel is visiting (typically there are several open and shut states in a mechanism). Secondly, the simple interpretation would work only if we could be sure that we can measure each single opening or closing in isolation, a condition that is hardly ever met in practice, where things are complicated by the finite bandwidth of our recordings, i.e. by the fact that we will miss the shortest events. If we do not detect a shutting between two openings, because it is too short, we will think that we have one long opening rather than two shorter ones (this is the missed event problem, see below).

The mechanism in eqn (1) also makes predictions that, with even greater caution (see Colquhoun, 1998), can be useful in the interpretation of macroscopic dose–response curves. The maximum open probability at equilibrium will be $E/(E+1)$, a function of efficacy alone, but the EC_{50} will depend on both the binding and the gating steps. If you want to know whether a mutation affects binding or gating, then EC_{50} measurements cannot tell you the answer. Single-channel analysis is the only way that is known to separate the reaction steps in the mechanism. That is why we need to estimate the rate constants (and hence the equilibrium constants) for each step in the mechanism and to find how each value is affected by mutations.

Over the years, the del Castillo–Katz mechanism provided a conceptual frame for interpreting new observations, such as the voltage dependence in both the decay of the endplate current and the apparent open time of the channel (Magleby & Stevens, 1972; Anderson & Stevens, 1973). It also gave a basis for clear thinking about what to expect experimentally, see for instance the prediction that channel openings occur in bursts if the binding step is not much faster than the opening step (Colquhoun & Hawkes, 1977). Mechanistic thinking about the receptor has allowed the application of linear-free energy relations to the investigation of how the perturbation introduced by agonist binding spreads to the channel gate (Grosman *et al.* 2000; Auerbach, 2007; Purohit *et al.* 2007). Over time, the simple 1957 mechanism evolved to incorporate the presence of two agonist binding sites for acetylcholine (Katz & Thesleff, 1957; Adams, 1975), the existence of monoliganded (Colquhoun & Sakmann, 1981) or unliganded openings (Jackson *et al.* 1990; Purohit & Auerbach, 2009) and the possibility that the two binding steps are different either because the binding sites are physically different to start with or because the affinity of the sites appears to change as binding progresses (Colquhoun & Sakmann, 1985; Sine *et al.* 1990). This review aims to examine how in our own work we have applied the mechanistic approach to glycine receptors.

Back to glycine: the startle disease mutation K276E

In our lab, work on glycine receptors started in 1996, when our colleagues Michele Rees and Mark Gardiner from the UCL Paediatrics Department identified a new

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human startle disease mutation, in the M2–M3 loop of the α1 subunit. In recombinant receptors, this mutation, K276E, produced the typical 'startle phenotype', a large decrease in both the potency of glycine and its maximum effect (Lewis *et al.* 1998). These effects are very similar to those of other startle mutations, such as K271L/Q. This, the first startle disease mutation to be discovered (Shiang *et al.* 1993), is located nearby, at the extracellular end of M2, the pore lining domain, and not only produces the startle phenotype, but had been reported also to decrease the average channel conductance (Langosch *et al.* 1994). Our first exploratory single-channel recordings showed that the new K276E mutation did not affect channel conductance, but reduced the duration of apparent openings (Lewis *et al.* 1998). As we saw above, the simplest explanation for this is that the mutation must affect the channel gating step, rather than the agonist binding. This was not an obvious conclusion at the time. The structural information available was limited to a 9 Å structure of the *Torpedo* nicotinic receptor (Unwin, 1993), and the position of the M2–M3 loop with respect to the binding domain was unknown. Indeed, it was still thought possible that this domain was part of the agonist binding site, and any mutation here would have to impair receptor function by impairing glycine binding. Our data, however, showed that the binding site could not be grossly distorted in the mutant, as the mutation did not change the affinity of strychnine (whose binding site must overlap that of glycine, because strychnine is a competitive antagonist). Both the decrease in apparent open time and the macroscopic startle phenotype can be explained by a change in agonist efficacy E, the effectiveness with which the channel opens once it binds the agonist. This was in agreement with the observations of Lynch *et al.* (1997), that a startle phenotype could be produced by mutations at several positions, in either M1–M2 or M2–M3 and suggested that these loops could serve as a hinge in the channel opening. M1–M2 is unlikely to participate directly in agonist binding as it is on the cytoplasmic face of the membrane.

However plausible our conclusions, they had to be taken with caution, as they relied in part on interpreting the absolute magnitude of the maximum response observed in a macroscopic dose–response curve in an oocyte. This is a measurement that is affected not just by agonist efficacy, but also by other factors that can be changed by a mutation and are difficult to quantify in an oocyte, such as the rate and extent of desensitisation and the level of channel expression (Colquhoun, 1998). The other evidence for an effect on channel gating relied on interpreting apparent open times in the absence of an established mechanism. This is a potential problem and the reason is clear if we look at the del Castillo–Katz mechanism in eqn (1) again (even though, as we shall see, a realistic activation mechanism for glycine is much more complicated than that). Once the open channel AR* has closed, the channel – now in AR – can either open again, or lose the agonist molecule and go back to the resting unbound state, R. If the opening rate is fast enough, the channel will often return to AR* after pausing a very brief time in AR and will continue to oscillate between AR and AR*, opening and closing until the agonist dissociates (Colquhoun & Hawkes, 1977). This is known to happen in other ligand-gated channels, such as the muscle nicotinic receptor, where openings occur in groups or *bursts*. The duration of bursts is determined not only by the average length of an opening (i.e. by the channel gating) but also by the number of times that the channel reopens before the agonist dissociates and that depends on the *binding* step, i.e. on how tightly the agonist binds to the low-affinity, resting state of the channel. In our hyperekplexia recordings, if some or all of the apparent open times that we measured were really channel bursts, rather than single openings (because we missed the short shut times between the openings), they could be shorter either because the openings are shorter (i.e. the mutation reduces the stability of the open channel through an effect on gating) or because there are fewer openings per burst. The latter could occur if the mutation speeds up agonist dissociation and therefore affects binding rather than gating. At the time we concluded that it was likely that K276E affected mostly the transduction from agonist binding to gating. Obtaining stronger evidence for this conclusion would require a full correction for the limited recording bandwidth. The only way to solve this problem (and therefore to identify the role of this domain) is to establish a detailed mechanism for glycine activation, so that we could include in our analysis a full correction for missed events.

Fitting mechanisms to glycine channels

We therefore set out on a systematic investigation of the single-channel behaviour of the glycine receptor in its wild-type form, by obtaining recordings of channel activity at a range of glycine concentrations at equilibrium. There had already been a report (Twyman & Macdonald, 1991) that the behaviour of glycine channels is too complex to be explained by a simple del Castillo–Katz scheme with a single open state, even if the scheme is adjusted for two or three binding sites. In outside-out patches from mouse spinal cord neurones, the length of openings and bursts was seen to be concentration dependent, with longer events becoming more common as glycine concentration increases. A similar thing happens for muscle nicotinic receptors (Colquhoun & Sakmann, 1985), but for glycine the effect occurs over a wider concentration range. As Twyman & Macdonald (1991) insightfully point out, this must mean that the channel can open not only when saturated by the agonist, but also when only some of its

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binding sites are occupied, and that the resulting bursts look different. A simple sequential mechanism with one open state is therefore not sufficient.

Some practical problems were encountered early in our work. The native channels in this study are likely to be $\alpha 1\beta$ heteromers, so we started investigating in a systematic way how to express recombinant heteromeric channels that were as pure as possible, given that the α subunit can also form functional homomeric channels. Homomers can be identified by their higher conductance, but this is not always possible at low agonist concentrations, when the openings may not be long enough to reach full amplitude (Colquhoun & Sigworth, 1995). This means that if we want to use low concentration records in our analysis, we have to eliminate contamination by homomeric channels. Earlier work (including our own) had just assumed that a 1:4 or a 1:10 ratio of α to β subunits in the transfection mix was sufficient to do this, but we found that in HEK293 cells the ratio needed is more extreme, as high as 1:40 (Burzomato *et al.* 2003). In the same work we investigated the number of α subunits in the heteromeric pentamer, by inserting a reporter mutation (L9′T, Labarca *et al.* 1995) in the channel lining M2 domain of the α or the β subunit. This was found to have a greater effect on the agonist EC_{50} when inserted in the α subunit. This suggests that α is the more numerous subunit in the pentamer, if the effect of mutations in the different subunits is equivalent (as shown for the muscle nicotinic receptor and for some neuronal nicotinic receptors by Labarca *et al.* 1995 and Boorman *et al.* 2000; but see contrary results for $GABA_A$ receptors by Chang *et al.* 1996 and for some neuronal nicotinic receptors by Groot-Kormelink *et al.* 2001, 2006). If we assume that α is the principal binding subunit, this finding implies that there are three agonist binding sites in the heteromeric glycine receptor. Receptor stoichiometry is still controversial. Our finding of three α copies agrees with the results of Kuhse *et al.* (1993), but later data by the same group (Grudzinska *et al.* 2005) favour two α subunits in the receptor. Nevertheless, what matters is the number (and nature) of the agonist binding sites and Grudzinska *et al.* (2005) conclude that there is a minimum of three agonist binding sites. Some of the uncertainty may derive from differences between expression systems, as we find that in oocytes heavy homomeric contamination persists even with extreme (1:40) α:β transfection ratios (P. Krashia, unpublished observations). None of these problems would have been apparent in experiments with whole-cell currents.

Since the invention of patch clamping, steady-state single-channel records have provided the richest source of functional information, as the number of components that can be distinguished in the open and closed time distributions is much greater than the number of components that can be resolved in macroscopic responses and it directly provides a minimum value for the number of open and closed states. Indications can also be gleaned on which of the steps in the mechanism are concentration dependent and, by looking at open–shut correlations, on how open states and closed states are connected. Once you have a mechanism, you try to estimate the values of the rate constants for each of the steps from the time constants of the distributions, including approximate corrections for missed events.

This approach gave us much of our understanding of the activation of muscle nicotinic receptors (Colquhoun & Sakmann, 1985), but suffers from several limitations. Analysing distributions of dwell times means that the information contained in the *order* of events in the original records is not used. Furthermore, it is often impossible to be sure about how many components a distribution contains and an exact correction for the missed event problem is only possible *after* a mechanism is postulated (Hawkes *et al.* 1990, 1992). Finally, the relation between the time constants and areas of the experimental dwell-time distributions and the values of the mechanism rate constants (that we wish to estimate) is not straightforward. Even in the absence of missed events, each time constant is not the expression of a single rate constant but the reciprocal of the minus eigenvalues of the appropriate submatrix of the transition matrix $\boldsymbol{Q}$ (areas are considerably more complicated, see Colquhoun & Hawkes, 1995). That makes it difficult to estimate rate constant values one by one, in a piecemeal approach.

The modern approach is to fit mechanisms to the whole sequence of openings and closings recorded for a set of experimental conditions: the procedure is shown diagrammatically in Fig. 1. Analysis programs that do that have been developed by David Colquhoun and co-workers in London http://www.ucl.ac.uk/Pharmacology/dc.html (the software we used in our work) and by Tony Auerbach, Fred Sachs and colleagues at Buffalo http://www.qub.buffalo.edu. In the case of ligand-gated channels, we need to fit stretches of data at several different agonist concentrations (after idealisation, in our case by time course fitting with the SCAN program). Idealised data are analysed initially to choose portions of the recording that are likely to reflect the activity of a single ion channel molecule. In practice this means dividing the records at low agonist concentrations into bursts (groups of openings separated by shuttings during which the channel has lost its bound agonist) and those at high concentrations into clusters (groups of bursts delimited by desensitised intervals). These data, together with an estimate of the time resolution (i.e. the shortest event that can be unambiguously resolved), is input into HJCFIT, the fitting program ('HJC' stands for Hawkes, Jalali and Colquhoun, from the 1990 and 1992 papers which showed how to calculate the distributions of *apparent* open and shut times, which are what we actually observe). In order to calculate the likelihood of the data (Colquhoun &

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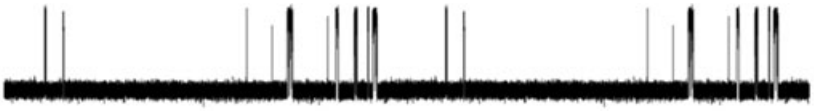


Idealised channel records

10 μM Gly

#	ms	pA
1	3.047	0
2	0.064	2.43
3	0.035	...

30 μM Gly

#	ms	pA
1	5.120	0
2	1.312	2.48
3	0.044	...

100 μM Gly

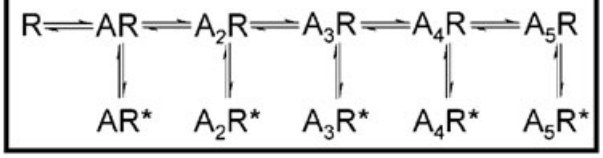
#	ms	pA
1	9.323	0
2	2.562	2.51
3	0.050	...

1000 μM Gly

#	ms	pA
1	2.211	0
2	5.322	2.47
3	0.539	...

Resolution = 30 μs

Mechanism and starting guesses for rate constants



Input into HJCFIT program:
Chooses rate constant values to maximise likelihood of observed sequence of events (exact missed event correction)

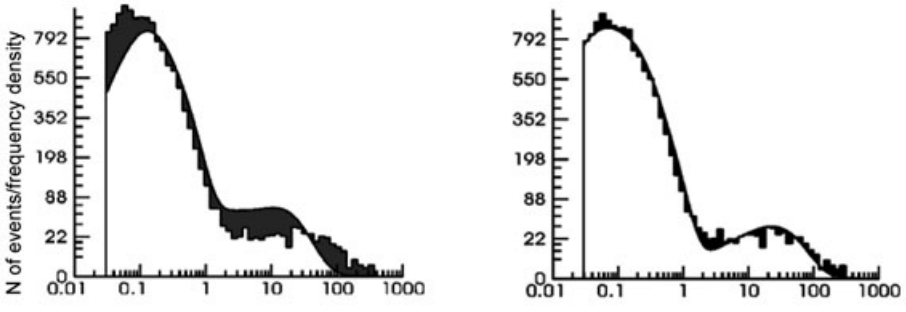
Output at convergence: rate constant estimates and maximum likelihood value

$\alpha_3 = 7000 \pm 1260$

$\beta_3 = 129000 \pm 5120$

$k_{+2} =$ . . . . .

The predictions from fits of two different mechanisms (continuous lines) are compared with the observed shut time distribution (histogram). The area of residuals is shown in black.



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Hawkes, 1995), and in order to correct for missed short events, it is necessary to postulate a mechanism at the outset (the adequacy of this mechanism will be judged later from the quality of the fit). The program calculates the likelihood of the set of data, given the chosen mechanism and the estimated resolution, and optimises the values for the rate constants (from the starting guesses) until it converges to a maximum value for the likelihood and outputs a set of rate constant values (Colquhoun *et al.* 2003; Hatton *et al.* 2003).

The next step is to assess whether the fit to the data is good. The actual data in different displays (dwell-time distributions, P_{open} curves, open–shut time correlations) are examined, together with the predictions calculated from the mechanism and the best-fit rates for the same distributions or plots (see Colquhoun *et al.* 2009). Figure 1 (bottom) shows one such display for a shut time distribution and the fit of two different models to the data. The difference between the observations (histogram) and the predictions of the model (continuous line), highlighted by the dark fill, is much greater for the plot on the left and suggests that the model fitted on the left is worse at describing the data. The continuous line superimposed on the histogram was not fitted to the histogram, but is the predicted HJC distribution that is calculated from the output of HJCFIT. The good match of the HJC distribution with the observed histogram shows that the model fitted on the right is capable of describing the data.

The first glycine receptor we investigated with this approach was the $\alpha 1$ homomeric receptor (from rat subunits expressed in HEK293 cells and recorded in the cell-attached configuration; Beato *et al.* 2004). Homomers contain five possible agonist binding sites, but how many of these participate in activation? We found that the binding of three glycine molecules is sufficient for the channel to reach its very high maximum open probability (98%), but could not determine whether a fourth and a fifth glycine molecule *can* bind to the other sites. If they do bind, they cannot further increase the channel open probability. Maximum activation at incomplete binding saturation may be a property common to other homomeric receptors in this superfamily, given the similar conclusions reached for the homomeric α7-5HT3 chimeric receptor (Rayes *et al.* 2009). This elegant work expressed mixtures of control and binding-defective subunits and identified the number of mutant subunits in each opening in the single-channel record by adding a 'reporter' conductance mutation.

We also applied direct mechanism fitting to the heteromeric $\alpha 1\beta$ glycine receptor, and systematically attempted to fit a number of physically plausible reaction schemes to sets of records. Each set contained four records obtained at four different concentrations from the foot of the P_{open} concentration curve to its top. Most of the 30 schemes that we tested failed to provide a good description of the data and it became clear that, in order to obtain a reasonable fit, the mechanisms had to incorporate several specific features (Burzomato *et al.* 2004).

First of all, the receptor can bind several agonist molecules and our data, in particular the value of the slope of the P_{open}–concentration curve (3.4), suggests at least three. Openings can occur with measurable frequency when one, two or three glycine molecules are bound and there are three open states. Similar results were obtained for homomeric channels (Beato *et al.* 2004), whose burst distributions have several components. As the agonist concentration is increased, the longer burst components (which reflect higher levels of ligation) become more prevalent, but their time constant does not change.

So far, our work confirmed the insights of Twyman & Macdonald (1991). However, we next found that the mechanism they proposed, which is the simplest mechanism that can be constructed with three binding steps and three open states, is not sufficient to describe the data. The three bound shut states in this scheme are not enough to account for the observed shut time distributions of $\alpha 1\beta$ channels. In order to achieve good fits, we had to add more shut states, and this posed the question of how to connect these states to the mechanism. Shut states in addition to the ones required by simple sequential schemes had already been postulated in order to explain the time course of recovery of $GABA_A$ currents from paired-pulse desensitisation and the concentration dependence of the biphasic decay of currents elicited by fast agonist applications (Jones & Westbrook, 1995). $GABA_A$ receptors have two agonist binding sites and the scheme proposed by Jones & Westbrook incorporates two binding steps, two open states and two additional shut states (which they term desensitised) connected to the resting shut states.

A similar scheme with three binding steps (Fig. 2) described our data from heteromeric receptors very well, but the similarity with $GABA_A$ receptors is limited to the shape of the mechanism. For the $GABA_A$ receptor, the additional shut states are relatively long (tens of milliseconds) and it is the rapid entrance into these states that gives the burst (and therefore the current in response to a short, synaptic-like transmitter pulse, see Wyllie *et al.* 1998) its long duration and relatively low open probability. This does not occur for glycine receptors, as first pointed out by Legendre (1998), who modelled the macroscopic kinetics of zebrafish glycine channels,

Figure 1
A diagrammatic example of how an activation mechanism is fitted to sets of single-channel data with the HJCFIT program.

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testing various schemes with two binding sites and two open states on the time course of currents evoked by fast applications of glycine. The 'reluctant gating' scheme that Legendre proposed connects both open states to the fully saturated resting state, one directly and one via an additional (reluctant) shut state, which is relatively short lived (sub-millisecond) and explains well both the sigmoidal rise of current onset after a step rise in agonist concentration and the biphasic decay of the current after the glycine pulse ends.

The results of our fits of the Jones and Westbrook-type model confirmed Legendre's finding that the maximum P_{open} of glycine channels is high. Consequently, the additional shut states needed to explain the equilibrium behaviour of heteromeric channels have to be short (in fact we found them to be short enough to be often missed in recordings). This also means that the open probability during a burst is high and this is relevant for the response to a short, synaptic-like application of transmitter. Note that in our channel analysis, long-lasting desensitised states are used to excise individual clusters of openings, but are not characterised or incorporated in the mechanism. This procedure can be justified by testing it with simulations (Colquhoun *et al.* 2003). Long desensitised states occur for glycine receptors exposed to long agonist pulses and must be separately characterised by appropriate concentration jump protocols with long applications (Legendre *et al.* 2002; Beato *et al.* 2007; Beato, 2008; Pitt *et al.* 2008).

We also tested another way of adding the shut states to the activation scheme, i.e. between the resting closed states and the open states (the example here has these additional, intermediate shut states also connected by binding steps).

This mechanism, which we termed 'flip' (Fig. 3), describes very well the single-channel data, despite a lower number of free parameters than the Jones & Westbrook-type mechanism.

If the Jones & Westbrook type model were a correct representation, then fitting it to data suggests that the affinity for binding to the resting conformation increases progressively by 65-fold from the first to the third glycine molecule that binds. This implies that a vacant binding site can sense whether the other ones are occupied or not, and change its affinity accordingly. That seems improbable because the binding sites are quite a long way apart (at least 40 Å apart if contiguous). However, the appearance of increasing affinity for the shut conformation can be produced, just as in oxygen binding to haemoglobin, if there exist two different shut conformations, one with a higher affinity for glycine that the other. In the presence of glycine, a shift of the equilibrium to the higher affinity conformation is expected. This additional, higher affinity shut state is explicitly incorporated in the flip model (Fig. 3), where the channel can exist in three different conformations, resting (R), flipped (F) and open (F*) with increasing affinity for the agonist. Note that the binding sites in this mechanism are assumed to be independent of each other and the binding affinity is the same for the first, second and third binding to the resting state (R). Likewise the affinity is the same for the first, second and third binding to the flipped conformation. The first two rows of the flip mechanism have thus exactly the same form as the Monod–Wyman–Changeux mechanism for the binding of oxygen to haemoglobin, where oxygen, just like the agonist, selects and stabilises the higher affinity

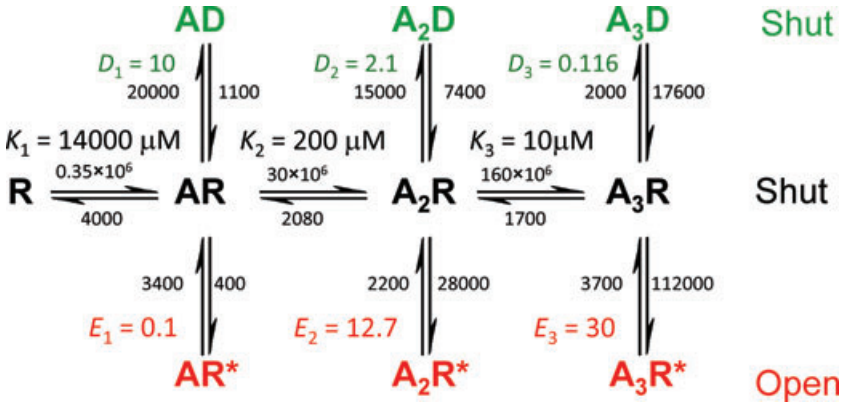

Figure 2
The extended version of a Jones & Westbrook-type mechanism (Jones & Westbrook, 1995) used to fit the single-channel activity of glycine heteromeric receptors. Up to three molecules of agonist A can bind to the resting receptor R (middle row, black). After binding glycine, the receptor can open (AR* states, bottom row, red) or desensitise (AD states, top row, green). The equilibrium constants are *K* (equilibrium dissociation constant), *E* (the equilibrium constants for the conformational change from resting to open, ratio between the opening and the closing rate) and *D* (the equilibrium constants for the conformational change from resting to desensitised, ratio between the rate of entry and that of exit from the desensitised states); numbers in small print shown by the arrows of the scheme are the values of the rates (or rate constants; units are s^{-1} or $s^{-1}\ M^{-1}$, as appropriate) obtained by Burzomato *et al.* (2004).

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form of the protein (Monod *et al.* 1965). All that has been added here is the opening reactions.

We could not get good fits with any mechanism if we allowed binding or unbinding from the open states, but, given how tightly the agonist binds to the open channel (for examples from muscle nicotinic receptors see Jackson, 1989; Grosman & Auerbach, 2001) estimating binding and unbinding rates for the open states is hard. The reason for the poorer fit when open states are connected directly is that the ratio of E_3/E_2 is constrained by microscopic reversibility to be the same as the ratio of E_2/E_1. Another possibility to explain the poorer fit when open states are joined is that microscopic reversibility is breached for the open–shut reaction, as might be the case if the presence of ions in the pore influenced opening and shutting rates (Läuger, 1995), a possibility suggested by our finding that the intracellular concentration of chloride strongly affects the time course of glycine channel deactivation (Pitt *et al.* 2008).

Given that the flipped conformations are not distal, unconnected shut states, but are on the path between resting and open states, it is tempting to attribute a physical meaning to these states, viewing them as an intermediate point in the process of activation. The channel has changed conformation in response to the agonist and is no longer resting but is not open yet. It is plausible that one (or more) such intermediate states should be visited in the channel trajectory from resting to open (Auerbach, 2005), as the wave of conformational change initiated in the extracellular domain by the binding of the agonist spreads to several groups of residues in succession until it reaches the channel and causes it to open (Grosman *et al.* 2000; Chakrapani *et al.* 2004; Purohit *et al.* 2007). In the nicotinic superfamily, the details of what happens near the binding site when an agonist binds are still uncertain, but it is well established that, in the acetylcholine binding protein, at least one of the loops that surround the site, loop C, moves to a 'capped' conformation when the site binds an agonist (usually a small molecule) but not when an antagonist is bound (Hansen *et al.* 2005). This domain closure occurs at the interface between subunits and is a smaller motion than that of the extracellular domain of AMPA receptor subunits (reviewed in Mayer, 2006). Our observations give no direct information about the structure of the 'flipped' conformation, but the idea that binding affinity is higher in the flipped conformation because the C-loop caps the binding site is very attractive.

A crucial feature of our mechanism is that it has no need to postulate interaction between the agonist binding sites. A good fit is obtained when we assume that binding affinity depends only on the receptor conformation (i.e. resting, flipped or open), but is not influenced by how many ligands are already bound. A somewhat similar mechanism has been postulated recently for the muscle nicotinic receptor by Mukhtasimova *et al.* (2009) who also include pre-open shut states which they refer to as 'primed' rather than 'flipped'. They suggest that, before opening, one or both of the C-loops of the principal binding subunit α must move to cap the binding site and 'prime' the channel for gating. If only one site is primed, the open channel is relatively unstable and openings are brief, if both sites are primed, the classical long high P_{open} bursts appear. The structural interpretation is elegantly substantiated by showing that cross-linking the tip of

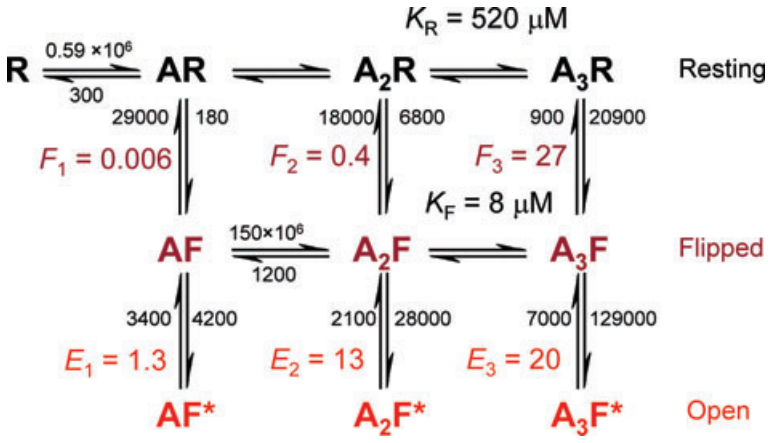

Figure 3
The 'flip' mechanism (Burzomato *et al.* 2004) used to fit the single-channel activity of glycine heteromeric receptors. R denotes the resting conformation (black, top row), F flipped (dark red, middle row, see text) and F* open (red, bottom row). The equilibrium constants are K_R and K_F (equilibrium dissociation constant for the resting states or the flipped states, respectively), F (the equilibrium constants for the conformational change from resting to flipped, ratio between the rate of entry and that of exit to the flipped states) and E (the equilibrium constants for the conformational change from flipped to open, ratio between the opening and the closing rates); the numbers in small print shown by the arrows of the scheme are the values of the rates (or rate constants; units are s^{-1} or $s^{-1}\ M^{-1}$, as appropriate) obtained by Burzomato *et al.* 2004).

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the C-loop to the other side of the binding site has an effect similar to that of adding agonist. Agonist binding facilitates priming, but is not necessary for it to occur, especially if the channel bears a gain-of-function mutation in the pore. In these mutant channels, both brief and long spontaneous openings are seen in the absence of agonist (see also Grosman & Auerbach, 2000) and addition of agonist produces a progressively larger proportion of long openings. The flip model can describe spontaneous openings by adding unliganded flipped and open states but would predict only one sort of opening (because, in its extended form, it only has one unliganded open state). On the other hand, the 'primed' mechanism can account for the existence of more than one sort of opening in spontaneously active mutants because the priming transition occurs independently in each binding site. But direct testing of the concerted *versus* independent flipping postulates is not possible with the resolution that is achievable now, because it is not possible to estimate all the rate constants in the primed mechanism. The flip mechanism as applied to the wild-type receptor (where spontaneous openings are too rare to be characterised) has 10 states and all of its 14 free rate constants could be estimated from single-channel data (Burzomato *et al.* 2004). The primed mechanism for the glycine receptor would have 28 states and more than twice as many free rate constants as the flip mechanism. Even when applied to the muscle nicotinic receptor (where there are two binding sites), the primed model cannot be fitted in its entirety because of the number of free parameters, and that forced Mukhtasimova *et al.* (2009) to fit only a somewhat arbitrary subset of the full primed model.

A more subtle question arises if we ask whether the flipping reaction is concerted (i.e. whether it occurs simultaneously for all binding sites). All we can say about that is that our mechanism, which postulates a converted flipping reaction, describes the data quite well. This question highlights the fact that – no matter how detailed – the mechanisms that we fit to our data are bound to be simplified approximations of the real activation process (which is likely to include several steps from agonist binding to channel opening, Purohit *et al.* 2007). However, we do not have structural 'snapshots' of the channel frozen at different points in its activation trajectory. Until then, analysing single-channel data by using mechanisms as detailed as can be robustly fitted and characterised is the best tool we have to identify the essential features of the physical behaviour of the channel molecule in time.

The mechanism of partial agonism

Fitting the detailed flip model to our glycine data has also allowed us to revisit one of the questions that gave rise to the del Castillo–Katz scheme, namely what makes an agonist partial or full. Until recently it has always been supposed that a partial agonist is partial because it is poor at opening the channel, i.e. that the open–shut equilibrium constant, E, is small, as proposed by del Castillo & Katz. In the flip model, overall agonist efficacy is split into two steps, the initial conformational change (flipping) and the actual opening of the channel. Thus a partial agonist could be poor either at opening the flipped channel (low E) or at causing the channel to flip (low F), or both. *A priori*, we did not know what to expect. It may be useful to look at another receptor superfamily, that of glutamate channels, where a compelling model for agonist efficacy has been proposed for AMPA channels, and see what this model would predict if it applied to our channel. For glutamate receptors, closure of a 'clamshell' ligand-binding core around the agonist is well documented structurally in constructs of the extracellular domains that do not include the channel. Functional data with macroscopic currents suggests that AMPA receptors go through a pre-opening conformational change (Zhang *et al.* 2008), but these channels have a relatively small conductance and verifying this conclusion by our sort of single-channel modelling is impracticable. For AMPA channels, the model for agonist efficacy comes from elegant work with a chemically related series of agonists and links efficacy to the degree of closure of the ligand-binding domain in the agonist-bound structures (Jin *et al.* 2003; Mayer, 2006; but see Zhang *et al.* 2008). This relation does not apply to the NR1 subunit of NMDA receptors (Inanobe *et al.* 2005) and may not hold for kainate receptors (see Mayer, 2005; Hald *et al.* 2007; Frydenvang *et al.* 2009; Fay *et al.* 2009). If we assume for the sake of argument that nicotinic superfamily channels behave like AMPA receptors, and that each agonist moves the extracellular domain to a different extent, depending on its overall efficacy, each agonist would be expected to give rise to a physically different flipped conformation in the bound channel. It would be reasonable to expect these different intermediates to connect to the open state with different opening rates. As a consequence, you might see that agonists differ both in the average durations of the sojourns in the flipped state and in the opening and closing rates they produce. This is not what we found (Lape *et al.* 2008).

Both the partial agonist taurine (which keeps the fully bound channel open approximately 50% of the time) and the full agonist glycine spend a very short time in the flipped state (about 8 μs). This happens largely because the mean lifetime of the flipped state is dominated by the opening rate and this is very fast for both agonists (Fig. 4*B*). The rate of leaving the flipped state for the resting state is about 6-fold faster for taurine than for glycine, so the flipped state structures must be somewhat different. However, most of the difference in the flipping equilibrium constant F stems from the rate of leaving

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the resting state for the flipped state, which reflects the structure of the resting state. Once the flipped state is reached, both glycine-bound and taurine-bound channels open at almost the same, high rate. The channels also stay open for much the same time, on average, for both agonists, so the gating equilibrium constant is much the same for the full and partial agonist, contrary to what has always been supposed until now. The deficiency in the partial agonist lay almost entirely in its ability to make the channel flip, and hardly at all in the ability of the channel to open once flipped. This is very clear if we express our findings as an energy diagram for the two agonists (Fig. 4*A*): once the intermediate (flipped) conformation is reached, the energy barrier and energy change to go to the open states are similar for both agonists. It is the first step, the flipping, that accounts for almost all the difference between full and partial agonists.

Taurine and glycine differ in the time they spend in the resting bound state (because the resting to flipped rate constant is different). In the saturated receptor the partial agonist takes a long time to produce a flip (almost 1.5 ms on average), whereas the glycine-bound channel only takes an average of about 50 μs to flip. Similar results were obtained for the effects of acetylcholine and tetramethylammonium on muscle nicotinic receptors. Work on this channel at negative potentials was technically much more difficult because of channel block by the agonist, but our finding that efficacy is mostly due to differences in flipping was confirmed when we analysed the effect of tetramethylammonium and acetylcholine at positive holding potentials (where block is effectively absent).

We have begun to apply the flip mechanism to study the effect of mutations, starting with the *spasmodic* αA52S mutation, known to produce startle disease in mice. The αA52 residue is in loop 2, near the interface between the extracellular part of the channel and its transmembrane domains, an area that may mediate the transduction of the binding perturbation in channel opening (Lee & Sine, 2005; Dougherty, 2008). In heteromeric channels, we found that A52S also impairs flipping (Plested *et al.* 2007). This effect is too small to result in a noticeable decrease in the maximum P_{open} (which is given by $F_3E_3/(1 + F_3 + F_3E_3)$, but is enough to increase the glycine EC_{50} by about 5-fold.

The examples discussed in our case history for the glycine receptor show that single-channel analysis can throw light on a variety of questions on the behaviour of the channel as a molecular nanoswitch activated by a neurotransmitter. These questions range from the precise role of particular amino acid residues in the protein, to the nature and time course of the conformational wave that couples binding to channel opening, to the number of agonist molecules that are necessary for full activation. As the mechanisms that we fit get closer to physical reality we can expect progress to be made on relating structure to function, a problem that has proved to be remarkably difficult.

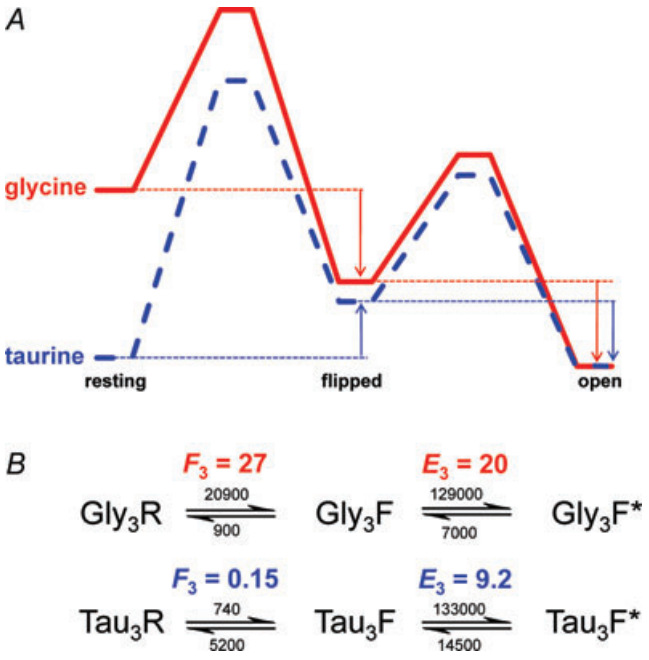

Figure 4
Energy diagram and activation mechanism for glycine heteromeric receptors fully bound to the full agonist glycine (red) or to the partial agonist taurine (blue). In the energy diagram (*A*), the main difference between the two agonists is in the first step, the transition from resting to flipped. This is downhill for glycine, but uphill for taurine. The calculations used a frequency factor of 10^{-7} s^{-1} (Andersen, 1999) and the lines are shifted vertically so they meet at the open state. *B*, the activation mechanism shows the values for the rates (numbers near the arrows, expressed in s^{-1}) and the equilibrium constants F_3 and E_3 for flipping and gating in the receptor saturated by glycine or taurine (abbreviated as Gly and Tau; values from Burzomato *et al.* 2004 and Lape *et al.* 2008).

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Acknowledgements

The work on glycine receptors was supported by the Medical Research Council (Project grants G9629178, G9819400 and Programme Grant G0400869). I am grateful to David Colquhoun for commenting on a draft of the manuscript.

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