# [ch:2-methods]Methods

## Bayesian data analysis

"Statistical evidence is part of the hot mess that is science, with all of its combat and egotism and mutual coercion." - McElreath (2020). Bayesian data analysis is the process of fitting a probability model to a set of data, and summarising the result with a probability distribution of the parameters of the model (Gelman 2014). At its most basic level, Bayesian data analysis is no more than counting the numbers of ways the data could happen, according to our assumptions. The more ways in which something can happen, the more plausible it is (McElreath 2020). Frequentist statistical approaches (such as t-tests and ANOVAs) can be thought of as special cases of Bayesian data analysis, which requires all probabilities to be defined by connection to the frequencies of events in very large samples.

We can use Bayes’ theorem to evaluate model given our observed data as follows:

where

* is the prior probability - how plausible is our model before we collect any data?
* is the posterior probability - in light of the data we have collected, how plausible is our model ?
* is the likelihood - the probability of observing the data if our model describes the true underlying data generating process.

Building and fitting a Bayesian model to data involves setting a prior probability for each parameter included in the model, and then updating the posterior probability based on the likelihood of the collected data. What does this look like in practise?

Imagine we have a coin, and we want to know if it is fair - that when we flip it, there is a 50 % chance that it will land heads up. Most coins are fair, which we can describe with a prior probability distribution which gives most weight to a 50 % chance of landing heads up (Figure [[ch2fig:coinflip]](#ch2fig:coinflip), black line). After flipping our coin ten times, we find that it landed heads up eight times, giving rise to the likelihood shown in red. When we calculate our posterior probability distribution, which is proportional to the product of the prior and the likelihood, we see it falls midway between the prior and the likelihood (purple line). Why? Ten coin flips is not particularly strong evidence, so even though our coin landed heads up eight times out of ten, there is still a good chance that the coin is fair - especially starting from the assumption that most coins are! However, if we flip the coin fourty more times for a total of fifty coin flips, the likelihood begins to dominate our posterior probability distribution, pulling it away from the prior. It is now clear that this is not a fair coin; the 95th percentiles of our posterior probability distribution fall between a 65 %–87 % chance of landing heads up. In actuality, the coin we have been flipping has an 80 % chance of landing heads up, well within our posterior probability distribution.

A lot of ink has been spilled on Bayesian methods and how they differ from frequentist approaches, but fundamentally they are more similar than they are different. In both approaches, the likelihood is the most important component of the analysis, and as samples sizes grow larger the likelihood becomes increasingly influential. However, they differ in two key ways. Firstly, instead of calculating a point estimate and a confidence interval, Bayesian analysis gives us the full probability distribution of our parameters of interest. Throughout this thesis, I have presented the full posterior probability distributions and expressed them in terms of their credible intervals (an example is given in Figure [[ch2fig:intervals]](#ch2fig:intervals). The credible interval is intuitive - it is simply the the range containing a particular percentage of probable values. For example, the 95 % credible interval is the central portion of the posterior distribution that contains 95 % of the values. We can say that given the observed data, the parameter we are estimating has a 95 % probability of falling within this range.

The second difference is the flexibility Bayesian analysis offers for incorporation of uncertainty from different sources, through a method known as multilevel or hierarchical modelling. Multilevel models seek to describe datasets which consist of clusters or groups of measurements that may differ from one another (**andrew\_gelman\_bayesian\_2014?**; McElreath 2020). As opposed to fitting each group individually, multilevel models allows for ’pooling’ of information between groups to give improved estimates and avoid averaging over experimental variability. Again, it is easiest to appreciate this with an example.

Imagine that instead of testing just one coin as in Figure [[ch2fig:coinflip]](#ch2fig:coinflip), we are interested in the differences between batches of coins. Instead of just modelling the variability inherent in the coin flip, we now need to capture any additional variability from batch to batch. We collect our data by flipping 100 different coins from 9 separate batches 50 times each, recording the number of heads in Figure [[ch2fig:mixed\_1]](#ch2fig:mixed_1).

We could fit this dataset with a single or fixed-level model by estimating the probability of a coin landing heads up individually for each batch as below:

where is the number of times coin lands heads up, is the unique log-odds for landing heads up for each batch of coins , and is our prior. This would give us a separate posterior probability distribution for each batch, but would not be able to tell us anything about the global population of all nine batches. The logit transformation here converts the prior from real values to probabilities between zero and one.

Instead, in addition to estimating the probability separately for each batch, we can estimate a population parameter (across batches) and a parameter which corresponds to the variability between batches. We can do this with the multilevel model below:

where is again our prior, but this time it has two separate parameters: one for the population average probability of landing heads up , and one for the standard deviation of that probability between batches .

Fitting this model to the data in Figure [[ch2fig:mixed\_1]](#ch2fig:mixed_1), we can see that while the overall population average probability of a coin landing heads up when we flip it is very close to 50 %, there is between group variability which we are able to capture using a multilevel model. For a more detailed - and eloquent - overview of Bayesian data analysis and multilevel modelling, Chapter 1 of McElreath (2020) is an excellent introduction.

## Concentration response processing and presentation

Raw spectrographic images and current traces were pre-processed in Python and Clampfit (Axon) before analysis with R. Where applicable, all experimental data points are displayed in each figure. To help visualise uncertainty and prevent some data points being hidden, they are arranged with a small amount of horizontal jitter; vertical position remains unaffected.

We fit our fluorescence quenching data with the following equation:

where represents corrected fluorescence intensity, and are values, and is the remaining variance. For the fluorescence quenching data, was fixed to the value obtained from W311\*-GFP+SUR1 unroofed experiments (0.1) as explained in more detail in chapter [[ch:4]](#ch:4). Current inhibition data were fit to the same equation but with representing normalised current magnitude, instead of , and instead of .

We used the brms package in R to perform a non-linear fit to equation [[eq:hill]](#eq:hill) reformulated as a multilevel model. The parameters in the equation were supplied as:

In this case, each group of measurements is the set of current inhibition or fluorescence quenching values obtained from a single excised patch or unroofed membrane. Essentially, the (or ) parameter for each concentration-response experiment can be described as the combination of a population parameter that is an estimate of the construct-specific value (b), and an additional ’random’ component that varies between experiments on the same construct (dexperiment).

## MWC model equations and fitting

The concerted MWC-type model fitted to the patch-clamp fluorometry data was formulated as follows:

When no ligand is present (i.e. when ), equation [[eq:mwc\_gating]](#eq:mwc_gating) becomes:

We can use this to normalise the predicted changes in the open fraction to an observed change in current:

We used the brms package in R to fit a multilevel model to equations [[eq:mwc\_binding]](#eq:mwc_binding) and [[eq:normalised\_po]](#eq:normalised_po). First, we normalised the fluorescence quenching data by the determined from W311\*-GFP+SUR1 unroofed experiments (0.1). We then corrected it by transforming each data point as as described in more detail in Chapter [[ch:3]](#ch:3).

The parameters in the equation were supplied as:

Each of the three parameters was modelled as a combination of a population parameter and an additional random component . Each combined set of current inhibition and nucleotide binding measurements from one excised patch was grouped as one experiment. The remaining variance was allowed to vary between fluorescence and current data.

The alternate single-binding model was formulated as follows:

The extra length of these formulas when compared to equations [[eq:mwc\_binding]](#eq:mwc_binding) and [[eq:normalised\_po]](#eq:normalised_po) do not represent any additional complexity; just an unfortunate consequence of the lack of exponents of which make it impossible to simplify further. Parameters were supplied and fitted as in equation [[eq:mwc\_priors]](#eq:mwc_priors).

# [ch:4]MWC modelling

## Modelling nucleotide regulation of the KATP channel

The complex regulation of KATP channel activity by nucleotides and phosphoinositides has led to a wide range of scientists seeking to unify the constellation of structural and functional studies into one mechanistic framework, which is capable of explaining each aspect of channel regulation. The importance of KATP channels in regulating insulin secretion, responding to cardiac stress, and protecting against seizures is one driving force behind the search for a functional model (Peter Proks and Ashcroft 2009). Another aim is more holistic; hoping that increasing our understanding of how the KATP channel is regulated by the interplay of its ligands may shed light on other ion channels or proteins functions (**garfinkel\_modeling\_2017?**). In any case, the primary goal of constructing a mathematical model of the KATP channel is to explain as much of the diversity of channel function as possible, while keeping the model as simple and biologically relevant as possible; a balancing act between completeness and complexity.

Previous attempts at modelling the function of KATP channel regulation have primarily focused on nucleotide inhibition (S. Trapp et al. 1998; D. Enkvetchakul et al. 2000a, 2001a; Markworth, Schwanstecher, and Schwanstecher 2000; Bernard Ribalet, John, and Weiss 2000; P. Drain, Geng, and Li 2004; Peter Proks et al. 2005; Lehong Li et al. 2005; Bernard Ribalet et al. 2006; Craig, Ashcroft, and Proks 2008), due to the relative ease of isolating the effects of nucleotide inhibition. There have been fewer attempts at incorporating activation by Mg-nucleotides (Bernard Ribalet, John, and Weiss 2000; Peter Proks, Wet, and Ashcroft 2010; Vedovato, Ashcroft, and Puljung 2015). The difficulty in quantifying phosphinositide regulation of the KATP channel means that in most cases where it is considered, it is implicitly included as a component of the intrinisc gating of the channel, rather than explicitly described (Baukrowitz et al. 1998; Fan and Makielski 1999; D. Enkvetchakul et al. 2000a), although there are some exceptions (Bernard Ribalet, John, and Weiss 2000; D. Enkvetchakul et al. 2001a; D. Enkvetchakul and Nichols 2003a).

What does a functional model of ion channel function look like? Broadly, a model attempts to categorise discrete conformational states of the channel, and describe the transitions between those states. In the simplest case, an ion channel can be described as fluctuating between an open state and a closed state (Figure [[ch4fig:simple\_model\_diagram]](#ch4fig:simple_model_diagram)). As these states exist in equilibrium, they can be described by an equilbrium constant () which is composed of the rate constant for the opening transition () divided by the rate constant for the closing transition ().

To relate this to empirical measurements of ion channel function, is equivalent to the of this two-state channel. Alternatively, in this simple two-state channel, and can be calculated directly by measuring the lifetimes of the closed and open states respectively from single-channel recordings (Reinhold Penner (auth.) 1995; Sivilotti and Colquhoun 2016). Of course, real ion channels are more complicated and two states are not sufficient to describe the complexity of the ligand regulation of KATP channels, which visit a multitude of conformational states. As our understanding of the channel grows, the more complex a model needs to be to fully account for all observed aspects of function.

One shortcoming of KATP channel functional models to date is that there are limited data directly measuring binding of nucleotides to the channel, and as such the nucleotide-bound conformational states and transitions of the channel have had to be inferred from electrophysiologcal measurements. Here, we hope to apply our correlated measurements of nucleotide binding and channel inhibition to reconcile the predictions of existing models of KATP channel inhibition by nucleotides.

### Restricting the subset of possible models

Functional models which have been proposed to describe KATP channel inhibition can be categorised into two groups; models in which each Kir6.2 subunit is able to change between open and closed conformations independently, and models in which opening and closing take place via a concerted mechanism of all four subunits (D. Enkvetchakul et al. 2000b, 2001b; P. Drain, Geng, and Li 2004; K. Fang, Csanady, and Chan 2006; R. Wang et al. 2007; Craig, Ashcroft, and Proks 2008; Peter Proks and Ashcroft 2009; Vedovato, Ashcroft, and Puljung 2015). The independent class of models are often referred to as Hodgkin and Huxley (HH)-like models, after the original model proposed to describe voltage-gated ion channels (Hodgkin and Huxley 1952). The concerted class of models are often referred to as Monod-Wyman-Changeaux (MWC)-like models, after the allosteric model formulated by Monod, Wyman and Changeaux to describe hemoglobin (Monod, Wyman, and Changeux 1965b).

Conceptually, an MWC-like model is easier to reconcile with the structure of KATP given that each inhibitory nucleotide binding site is composed of domains from two adjacent subunits; it is hard to imagine how nucleotide binding could lead to an indepedent conformational change in one subunit alone (Craig, Ashcroft, and Proks 2008). Empirically, the two types of model make testable predictions about channel behaviour and nucleotide binding. In a concerted model, each nucleotide binding event contributes the same amount of energy towards closure of the pore, such that each subunit binding a nucleotide will have an additive effect on the probability of the channel closing. In an independent model, as each subunit is free to change its conformation independently, the stochiometry of nucleotide binding is less clear. Most formulations of an independent model have suggested that KATP channel behaviour is most consistent with a single nucleotide binding event being sufficient to drive closure of the channel (S. Trapp et al. 1998; Markworth, Schwanstecher, and Schwanstecher 2000; Lehong Li, Geng, and Drain 2002; Kun Fang, Csanády, and Chan 2006).

A number of studies have examined the kinetics of single KATP channels to determine which model best describes nucleotide inhibition (P. Drain, Geng, and Li 2004; Kun Fang, Csanády, and Chan 2006; R. Wang et al. 2007; Craig, Ashcroft, and Proks 2008). (P. Drain, Geng, and Li 2004) examined single channel currents in patches excised from *Xenopus* oocytes injected with a mixture of Kir6.2DC and Kir6.2DC-N160D,T171A subunits. The T171A mutation appears to eliminate the interburst closures of Kir6.2DC by dramatically slowing the rate at which the ATP-sensitive inhibitory gate closes. The authors classified the single channel stoichiometry by assessing the sensitivity of currents to inhibition by spermine, which is provided to a subunit by the N160D mutation. An exponential relationship between the mean burst time of the channel and the number of mutant subunits incorporated into it fit the predictions made by a concerted model of inhibition.

R. Wang et al. (2007) and Craig, Ashcroft, and Proks (2008) constructed tetrameric concatemers of Kir6.2 subunits to precisely control the stochiometry of the resulting channels. The authors introduced mutations which affected either nucleotide binding (K185E (R. Wang et al. 2007; Craig, Ashcroft, and Proks 2008)) or mutations which altered intrinsic gating (C166S, T171Y (R. Wang et al. 2007)) into a fixed proportion of Kir6.2 subunits in the concatemerised channels. This selective disruption of individual subunits resulted in changes in ATP-dependent inhibition which could only be explained by a concerted model of KATP channel inhibition. However, as these experiments relied on introducing an additional physical linker between Kir6.2 subunits, the observed concerted gating behaviour may in part be due to the concatemerisation.

## Implementing an MWC model

### A simple case

The simplest case of an allosteric MWC model for an ion channel is shown as Scheme I in Figure [4.3](#ch4fig:mwc_model_diagrams). This simple case assumes a channel composed of a single monomer with a single binding site for ligand . The channel is restricted to two functional states, open and closed. These two states exist in an equilibrium described by L, which is equivalent to . Ligand binds to the protein with a microscopic affinity constant . The ligand differentially stabilises the open and closed states by a constant . When is unity, the ligand binds equally to both states and so does not influence the conformational changes of the channel. When , the ligand preferentially stabilises the open state, whereas when the ligand instead preferentially stabilises the closed state. therefore represents *transduction* of nucleotide binding to channel gating, and vice versa.

For KATP inhibition, each monomer in Scheme I represents a subunit of Kir6.2, and in our case the ligand is TNP-ATP. The equation describing the expansion of Scheme I to account for four identical subunits is shown in Chapter [[ch:2-methods]](#ch:2-methods). Importantly, in an MWC model, cooperativity between subunits is not due to the incorporation of an additional parameter, but a phenomenon which arises naturally due to the energetic coupling between ligand binding and channel gating described by the transduction parameter .

### The role of PIP2

Of course, nucleotide inhibition is not the only ligand regulation of KATP channels. If we assume that activation of KATP currents by Mg-nucleotides binding at the NBDs of SUR1 or by PIP2 binding to Kir6.2 are independent processes, the effects of these ligands on nucleotide inhibition can be incorporated implicitly through their effects on . Mg-nucleotide activation of KATP channel currents is well described by assuming independence from nucleotide inhibition; i.e. there is no evidence to suggest that there is a direct interaction between binding of Mg-nucleotides to SUR1 and the ability of nucleotides to bind to Kir6.2 (Peter Proks, Wet, and Ashcroft 2010; Vedovato, Ashcroft, and Puljung 2015). However, there is some evidence to suggest that there is a direct interaction between the nucleotide and PIP2 binding sites (Fan and Makielski 1999; MacGregor et al. 2002; Peter Proks and Ashcroft 2009; Haider et al. 2007). The existence of a direct interaction, either by competition for an overlapping binding site or through allosteric rearrangements of the two binding sites, may make it difficult to incorporate regulation by PIP2 implicitly as an effect on . We investigated how the existence of direct interaction between the two ligand binding sites may manifest in our observations by simulating data from three progressively expanded MWC-like schemes (Figure [4.3](#ch4fig:mwc_model_diagrams)).

If we consider introducing a second ligand which binds to a distinct site on the same monomer and does not directly interact with ligand , we introduce the states shown in Scheme II of Figure [4.3](#ch4fig:mwc_model_diagrams). Each ligand has its own microscopic association constant ( or ) and its transduction factor ( or ). Importantly, there is no interaction term between ligand and ligand ; the only way the binding of the ligands can impact each other is through effects on . Scheme II is therefore a restricted form of scheme III, which explicity introduces a term for direct interaction () between binding sites for ligands and on the same monomer. When is unity, Scheme III becomes Scheme II. When , binding of one ligand reduces the ability of the other ligand to bind on the same monomer. When , binding of one ligand enhances the ability of the other ligand to bind on the same monomer.

Under Scheme II, in which there is no direct interaction between ligands, changes in the parameters describing ligand (perturbations of PIP2 regulation) should manifest in the data in the same way as if there was a change in in Scheme I (Rubin and Changeux 1966). It is unclear whether under Scheme III, with the introduction of the direct interaction , the same assumption is true - and if not, how much it would affect channel behaviour. To determine whether this approximation is appropriate, we generated data using each of the three schemes in Figure [4.3](#ch4fig:mwc_model_diagrams) as the underlying model of channel function and then fit the generated observations to Scheme I (Figure [4.4](#ch4fig:mwc_scheme_1_fits), [4.5](#ch4fig:mwc_scheme_2_fits), [4.6](#ch4fig:mwc_scheme_3_fits)). Ten individual sets of observations were generated using the inputs shown above each figure panel as the centre of a lognormal distribution with a standard deviation of 0.25. These observations were then fit to Scheme I and the values of the three free parameters (, and ) were estimated (Figure [4.7](#ch4fig:mwc_params_1)).

We can show that when Scheme II or Scheme III are the underlying data generating model, with ligand representing PIP2, we are still able to extract the true values of and by fitting the generated data to Scheme I (Figure [[ch4fig:mwc\_models]](#ch4fig:mwc_models)). Parameter choices for Scheme II and III are such that the open probability of the channel at 0 ATP is still 50%, equivalent to in Scheme I.

We can also show that when Scheme I is the underlying data generating model, changes in any of the three parameters are easily identified and retrieved by fitting the observed data to Scheme I (Figure [[ch4fig:scheme\_1\_shifts]](#ch4fig:scheme_1_shifts)). This suggests that introducing mutations or perturbing nucleotide inhibition in any other way which directly affects any of the three parameters of this model would be easily identifiable if Scheme I was the true underlying model.

What if Scheme II or III were the underlying model? We would still expect changes in the three parameters which exist in Scheme I to be identifiable (, and ), although would not represent the true unliganded open/closed equilibrium as we would be estimating an modified by the resting PIP2 concentration, , and - in this case, the estimated parameter in fact represents the ATP-unbound open/closed equilibrium.

However, it is unclear how changes in parameters which are not explicitly modelled in Scheme I will affect the generated data and the parameter estimates obtained by fitting the data to Scheme I. Figure [[ch4fig:scheme\_2\_3\_shifts]](#ch4fig:scheme_2_3_shifts) shows the results of increasing by tenfold on data generated from Scheme II (Figure [4.12](#ch4fig:scheme_2_kb_shift)) or Scheme III (Figure [4.13](#ch4fig:scheme_3_kb_shift)). The first observation of note is that the generated data closely resemble those generated from Scheme I when is increased (Figure [4.10](#ch4fig:scheme_1_l_shift)), and indeed when the parameter estimates for a tenfold shift in in Scheme II/III and tenfold shift in for Scheme I are compared (Figure [4.14](#ch4fig:mwc_params_3), right panel) are compared they appear to be similar. So far so good, as an observed increase in when fit with Scheme I would lead us to draw the correct inferences about changes in the underlying model (i.e. the open probability of the channel has indeed increased).

However, changes in under Scheme III are not perfectly captured by changes in when fit to scheme I. Notably, if a direct interaction exists between the nucleotide and PIP2 binding site - if Scheme III is the true underlying model - then fitting the observed data to Scheme I would lead us to estimate an incorrect value for (Figure [4.12](#ch4fig:scheme_2_kb_shift)). Thus, if there is a direct interaction between the sites, then a mutation which induces an increase in the binding affinity for PIP2 would not just increase our estimate of (which would lead to a correct inference) but it would also decrease our estimate of by a not-insignificant amount. This could lead to the incorrect inference that a mutation is causing a direct change in nucleotide binding when it is in fact causing a direct change in PIP2 binding, which is influencing our estimates of through a direct interaction with the inhibitory nucleotide binding site.

### Determining open probability

As represents the fraction of channels in the open state, it is directly measurable by determining the channel open probability. Ideally then, to fit an MWC model to our data we would like to establish the open proability of the channels in our experiments. Measuring the open probability of an ion channel is most accurately accomplished by single-channel electrophysiological recordings, which allows direct measurement of the time a channel spends in an open state. Measuring open probability directly is not possible in macroscopic patches, which consist of hundreds or thousands of individual channels. Thus it would not be possible to determine single channel open probability simultaneously with nucleotide binding, as the fluorescence signal from a small number of channels would be impossible to resolve.

Another approach is noise analysis of currents from large populations of channels (Heinemann and Conti 1992; Alvarez, Gonzalez, and Latorre 2002). The ’noise’ in noise analysis refers to current fluctuations which occur when recording from a population of ion channels due to the stochastic channel gating of individual channels. If there are a constant number of channels () which are gated independently from each other and share a homogenous open probability () and a single open conductance level (), the observed macroscopic current level can be described by equation [[eq:inpo]](#eq:inpo):

and the observed variance of the macroscopic current can be described by the variance of the binomial distribution, equation [[eq:bin\_1]](#eq:bin_1):

where the single channel current is essentially a scaling factor. If we assume that in a given recording and remain constant, and it is which changes in response to any given stimuli, then we can combine equations [[eq:inpo]](#eq:inpo) and [[eq:bin\_1]](#eq:bin_1) to yield equation [[eq:bin\_2]](#eq:bin_2):

This equation yields a parabola from to . Intuitively, there can be no variance when is exactly 0 or 1, as there will be no opening or closing events which can give rise to current fluctuations. Once and have been determined for a given experiment, the observed current magnitude can be converted into the for the population of channels by rearranging equation [[eq:inpo]](#eq:inpo) as follows:

Equation [[eq:bin\_2]](#eq:bin_2) can be fit to experimental data by calculating the variance of observed current at different current magnitudes. This calculation is not exactly trivial, and has been accomplished a number of different ways for different purposes. For channels with fast inactivation such as the NaV family, non-stationary noise analysis involves repeating a stimulus multiple times and measuring variance as the squared sum of deviations from the mean of the current magnitude calculated at the same time point across multiple stimuli, referred to in the literature as an ’isochrone’ (Sigworth 1980). For channels which do not inactivate, stationary noise analysis is possible, and variance can be measured as the squared sum of deviations from the mean current magnitude over a period of time for which is ’stationary’(Figure [4.15](#ch4fig:noise_example_1), [4.16](#ch4fig:noise_example_2), [4.17](#ch4fig:noise_example_3)).

Stationary noise analysis has been performed for KATP channels before by a number of different researchers (S.-L. Shyng, Ferrigni, and Nichols 1997; C. A. Cukras, Jeliazkova, and Nichols 2002; Andrey P. Babenko and Bryan 2002; Paolo Tammaro and Ashcroft 2007; Emily B. Pratt et al. 2009, 2012; Peter Proks, Wet, and Ashcroft 2010). Unfortunately, in most of the published research the exact procedure for extracting the parameters in equation [[eq:bin\_1]](#eq:bin_1) is described in the methods section, but the quality of the fits and the value of the fitted parameters besides the final calculated is not discussed. A notable exception to this rule is in reference (Paolo Tammaro and Ashcroft 2007), in which two findings are discussed. Firstly, fitting equation [[eq:bin\_2]](#eq:bin_2) to the mean and variance of 200 ms sections of macroscopic currents from wild-type Kir6.2+SUR2A resulted in a systematic underestimation of the single channel current . From single channel experiments, the single channel current was determined to be 4 pA, while the value obtained from fitting macroscopic currents was only 2 pA. In the case of WT-GFP+SUR1, we see a similar understimation of single channel current (Figure [4.18](#ch4fig:noise_example_fits_1), [4.19](#ch4fig:noise_example_fits_2), [4.20](#ch4fig:noise_example_fits_3)), with fits yielding estimates of 1.66 pA–2.64 pA, while measured single channel currents are at least 4 pA at a holding potential of −60 mV (S.-L. Shyng, Ferrigni, and Nichols 1997; Peter Proks et al. 2001). This underestimate of is most likely due to a reduction in observed channel current variance when compared to the predictions of equation [[eq:bin\_2]](#eq:bin_2).

There are two possible explanations for this reduction. Firstly, the process of filtering and digitising channel currents can lead to underestimates of variance depending on the relationship between the open time of the measured channel and the cut-off frequency of the filter used. It is unlikely that this phenomenon is responsible for our findings, as the KATP mean open time duration is close to 1 ms and therefore filtering at 5 kHz would lead to less than a 5 % underestimation of . Even if the mean open time of WT-GFP+SUR1 was closer to 0.1 ms, we would expect a 20 % reduction rather than the 50 % we actually observe. Empirically, we can use the frequency power spectrum of our measured current fluctuations to determine whether there may be high frequency channel openings we are missing (Figure [4.21](#ch4fig:spectra_converge)). For WT-GFP+SUR1, we observe that at frequencies approaching our filter cut-off at 5 kHz there is very little observed amplitude in active channels when compared to fully inhibited channels, suggesting we are not missing high frequency current fluctuations.

Secondly, an underestimation of could occur due to violations in the underlying assumptions of the binomial distribution. The first two assumptions are that and are constant throughout a recording. We know that is unaffected by nucleotide inhibition of KATP channels, nor is it affected by PIP2 or channel rundown. Given that we are recording from excised patches, it is unlikely that there will be any change in the number of channels present in the membrane () during the short time course of a recording. The third assumption in using equation [[eq:bin\_2]](#eq:bin_2) is that the channels in a patch share a homogenous , which can be perturbed to a similar extent by a stimulus (in our case, application of nucleotide). This assumption is far harder to justify for our experimental condition, in which channel rundown due to loss of PIP2 results in a complicated mixture of channel populations with different s, which respond differently to nucleotide inhibition.

An extreme case in which channels transition between two states, one where and one where can be approximated by equation [[eq:bin\_1]](#eq:bin_1), with a channel transitioning to the state essentially considered to be no longer available to open, reducing . Thus, fitting the observed current-variance data with [[eq:bin\_1]](#eq:bin_1) would yield a straight line where the slope of the line is equal to . This formulation of equation [[eq:bin\_1]](#eq:bin_1) has been used successfully in the analysis of currents from CRAC channels (Prakriya and Lewis 2006), VSOA channels (P. S. Jackson and Strange 1995; Paul S. Jackson and Strange 1996), and in the analysis of a specific cardiac KATP channel mutation (Paolo Tammaro and Ashcroft 2007). Unfortunately, in our case channel rundown does not render the KATP channel completely unable to open, with fully rundown channels still displaying openings. Instead of each current measurement coming from a single binomial distribution, we are instead observing a mixture of binomial distributions with different . We can demonstrate how this could lead to an underestimation of by simulating a simple case where there are two populations of channels, and , with a shared single channel conductance but one with a tenfold lower than the other:

where population consists of channels with an open probability , and population consists of channels with an open probability , constrained to be tenfold lower than .

Comparing the mean current/variance relationship of simulated currents from a single binomial (Figure [4.22](#ch4fig:simulated_noise_1), [4.23](#ch4fig:simulated_noise_2)) to that of simulated currents from the mixture of binomials in equation [[eq:bibi\_sim]](#eq:bibi_sim) (Figure [4.24](#ch4fig:simulated_noise_3)) reveals that equation [[eq:bin\_1]](#eq:bin_1) is no longer able to retrieve the true values of and when the data generating process is not a single binomial distribution. In fact, the underestimation of from fitting to data simulated in this way is very similar to the underestimation of we see when fitting to our measured data (Figure [[ch4fig:noise\_manual]](#ch4fig:noise_manual)).

We considered whether the underestimation of and the poor fits to equation [[eq:bin\_2]](#eq:bin_2) when was fixed to 4.32 pA (Figure [[ch4fig:noise\_manual]](#ch4fig:noise_manual)) may be due to the low number of data points when selecting segments of current manually. We took our full current records from each excised patch from cells expressing WT-GFP+SUR1 or W311\*-GFP+SUR1, divided them into 1 second segments, and plotted the mean current/variance relationship for each segment (Figure [4.25](#ch4fig:noise_fits_1)). We fit the data to equation [[eq:bin\_2]](#eq:bin_2) either with allowed to vary freely, or with fixed to 4.32 pA. Our estimates for when it was allowed to vary freely were similar to our estimates from Figure [[ch4fig:noise\_manual]](#ch4fig:noise_manual), with no patch yielding a value above 3 pA (Figure [4.26](#ch4fig:noise_fits_2)). The fits with fixed to 4.32 pA clearly fit the data less well, and the resulting estimate for the open probability on patch excision exceeded 1 for nearly every patch, which is of course not possible.

Given these results, we chose not to use noise analysis to calculate the directly for each patch. In addition, C. A. Cukras, Jeliazkova, and Nichols (2002) compared the calculated from noise analysis and the calculated by application of saturating concentrations of PIP2 to a variety of KATP channel mutants, and found only a weak correlation between the two methods (C. A. Cukras, Jeliazkova, and Nichols 2002).

### Comparing models

We expanded Scheme I from Figure [4.3](#ch4fig:mwc_model_diagrams) to account for the four inhibitory nucleotide binding sites of KATP (Figure [4.27](#ch4fig:model_expansion_1)). In addition, we considered an alternate model in which only the first nucleotide binding event contributes towards closure of the channel, and thus there is no cooperativity between subunits (Figure [4.28](#ch4fig:model_expansion_2)). We then fit our observed TNP-ATP binding and current inhibition data from excised patches expressing W311\*-GFP+SUR1 to equations [[eq:mwc\_binding]](#eq:mwc_binding) and [[eq:normalised\_po]](#eq:normalised_po) respectively.

Both models fit our data reasonably well (Figure [[ch4fig:w311\_model\_comparison]](#ch4fig:w311_model_comparison)), although the posterior distributions of the fits to the MWC model (Figure [4.29](#ch4fig:w311_mwc_fit_1), [4.30](#ch4fig:w311_mwc_fit_2)) are narrower than those for the single binding model (Figure [4.31](#ch4fig:w311_single_fit_1), [4.32](#ch4fig:w311_single_fit_2)). Examining the posterior distributions for the three parameters, both models yield similar estimates, with much narrow distributions for than for and (Figure [4.33](#ch4fig:w311_mwc_fit_3)). The cross-correlation plots for the parameter estimates indicate that the model is identifiable given the data, with well bounded ellipses clearly visible (Figure [8.1](#apxfig:inhib_cc_1)). We compared the ability of the two models to explain the data with two complimentary methods. First, we used bridge sampling to calculate a Bayes factor of 1.1 × 104 in favor of the MWC model over the single binding model (Gronau, Singmann, and Wagenmakers 2020). The Bayes factor can be interpreted as the weight of evidence in favour of one model over another (Wagenmakers 2007). Specifically, the observed data are 1.1 × 104 more likely to have occured under the MWC model than they are under the single binding model. In addition, we performed leave-one-out cross-validation (LOO-CV), which approximates the out-of-sample predictive accuracy of each of the fitted models (Vehtari, Gelman, and Gabry 2017). The MWC model fit has a higher expected predictive accuracy than the single binding model (ELPD increase of 27.3 (63)). Together, the Bayes factor and LOO-CV scores favour a concerted MWC binding model.

The 95 % credible intervals for are 9 × 103 /–1.7 × 104 / for the MWC model, corresponding to a of 56 μ–110 μ. The 95 % credible intervals for are .007–.254, which is equivalent to an unliganded of <0.01–0.2. This is a broad range of predicted , which may reflect the variability of PIP2 concentrations in the excised patches. The 95 % credible intervals for are .002–.096. This low range for indicates very strong coupling between TNP-ATP binding to Kir6.2 and KATP channel closure. Based on the fits to the data, we can illustrate how these models couple nucleotide binding to the functional state of the channel by predicting the occupancy of the different states of the KATP channel defined in Figure [[ch4fig:model\_compare]](#ch4fig:model_compare). Figure [[ch4fig:w311\_model\_comparison]](#ch4fig:w311_model_comparison) shows the weighted occupancy of each of the ten states across a range of TNP-ATP concentrations as predicted by our fits to an MWC or single binding model. Qualitatively, the models make similar predictions; as nucleotide binding is so tightly coupled to pore closure, there is a vanishingly small proportion of KATP channels which exist in the open state with two or more TNP-ATP molecules bound to Kir6.2 subunits under the MWC model. Crucially however, for the single binding model to explain the data in Figure [[ch4fig:model\_compare]](#ch4fig:model_compare), it predicts that even at saturating nucleotide concentrations a reasonable fraction of KATP channels (up to 1 %) will be open even with all four Kir6.2 subunits bound to nucleotide.

### Discussion

Measuring binding of TNP-ATP to KATP channels concurrently with measuring inhibition of channel currents has allowed us to test the predictions of prior studies about the stochiometry of nucleotide binding, and the suitability of an MWC model to describe inhibition of the channel. We have established that a simple MWC model is capable of describing both inhibition of KATP channel currents as well as the binding of nucleotides to the Kir6.2 subunit. There has been some debate over the stochiometry of nucleotide inhibition of the KATP channel, with some research indicating that a single Kir6.2 subunit binding to ATP is sufficient to close the channel, while other studies have suggested that further subunit binding events contribute additively to pore closure. Our data suggest that these findings can be reconciled with the observation that even within the framework of a concerted MWC model, where each nucleotide binding event is energetically coupled to the pore, the proportion of KATP channels which are in the open state with more than one TNP-ATP molecule bound to a Kir6.2 subunit is essentially nil. This is of course caveated by our assumption that TNP-ATP, while exhibiting a higher affinity for the channel than by ATP, does not alter transduction of binding to inhibition.

However, a model capable of describing KATP channel function should also be able to explain how mutations or other perturbations disrupt the regulation of channel function by nucleotide inhibition. In the following chapters, we will explore whether this method and model are capable of discerning between alterations of nucleotide binding, KATP channel gating, and transduction of nucleotide binding to the pore. In addition, these experiments should allow us to test the ability of a simple MWC model to explain the variety of functional changes we observe.