Appendix C: Simulation of M_2AChR Dose-Response Relationship Data Using an Asymmetric Current Change Model

C.1 Introduction

To try to understand the source of the variability in suppressed M_2AChR cEC_{50} data, we performed an experiment in which we repeated the same concentration of ACh (0.3 μM) at various intervals throughout a dose-response experiment (see Figure 3.22). Current responses to the same dose of agonist increased on average throughout the course of the experiments. More importantly, this change was not constant: current changes appeared to be low at the early doses in the series, but increased at the half-way point of the series.

What effect does asymmetric current change have on a dose-response relationship? Figure C.1 provides an illustration of the effect. Before we fit our data to the Hill equation, we normalize $I_{K,ACh}$ measurements (Figure C.1a) to the maximum value in the dose series. This maximum response is often found in the last two doses of the series. Our interpretation of the current change data suggests that this maximum response is higher than what it would be absent any current change mechanism. Therefore, the other $I_{K,ACh}$ values would appear to be smaller relative to this maximum dose than they would without the current changes. In other words, the normalized responses for the current change data would be smaller at each dose, effectively shifting the cEC_{50} to higher values (Figure C1b).

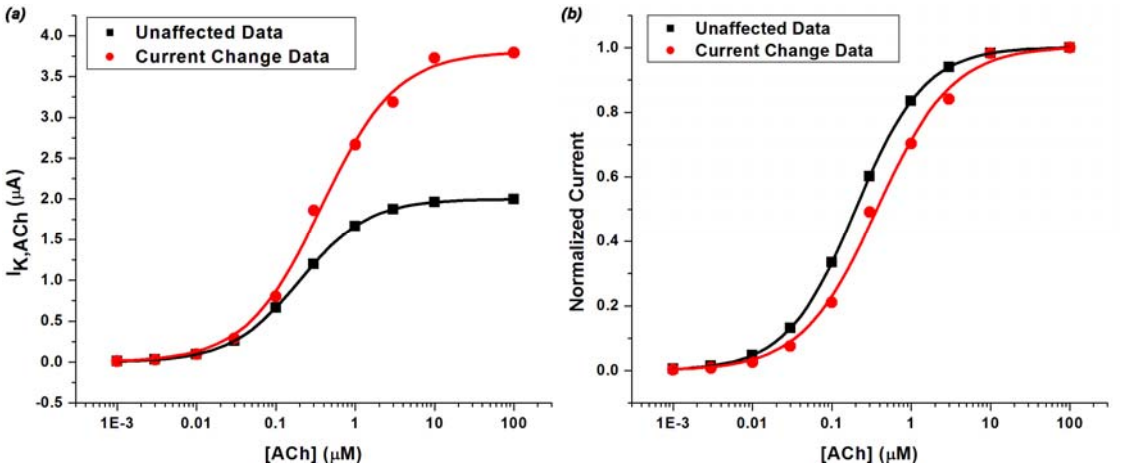

Figure C.1. Example of how asymmetric current changes can affect dose-response relationships. ***(a)*** $I_{K,ACh}$ data for an unaffected cell and a cell that experiences asymmetric current changes. The affected data were derived from the unaffected data by applying a set of current change values (α) used in the data simulation exercises. ***(b)*** Both data sets from ***(a)*** are normalized and fit to the Hill equation. The unaffected data has a cEC_{50} value of 200 nM, while the current change cEC_{50} value is 370 nM.

Once we realized the effect these current changes could produce in our cEC_{50} data, we wanted to determine if we could use the current change data we had collected to simulate dose-response data. Would this simulated cEC_{50} data exhibit variability similar to the actual data we had collected? This appendix describes the method we used to simulate 10.10.0 W7.40Trp data and how this generated data set matched real data.

C.2 Methodology

We decided to try to simulate 10.10.0 W7.40Trp data because it employed the experimental conditions that we had used to generate the current change data and was the largest suppression data set (N = 42). Our goal was to generate five different sets of N = 42 cell dose-response relationships and then compare each set to the actual data for differences in mean (*t*-test) and variance (F-test). The actual 10.10.0 W7.40Trp data had

a mean of 230 nM and a standard deviation of 120 nM (CV = 0.52; the ln transformed data set had a mean of 5.3 and a standard deviation of 0.5).

C.2.1 Mathematical Model for Simulating M_2AChR Data

Let $Y_n = \{Y_1, Y_2, \dots, Y_{10}\}$ be an ideal set of dose-response data, where Y_n refers to the $I_{K,ACh}$ measurement at dose n of the dose-response series.

Let $X_n = \{X_1, X_2, \dots, X_{10}\}$ be the normalized Y_n data set such that $Y_n = \beta X_n$, where β is the maximum current of Y_n or max(Y_n).

Let $Z_n = \{Z_1, Z_2, \dots, Z_{10}\}$ be the set of current data that has been modified through asymmetric current changes such that $Z_n = Y_n(1 + \alpha_n)$, where $\alpha_n = \{\alpha_1, \alpha_2, \dots, \alpha_{10}\}$ is the set of percent changes in current relative to dose $n = 1$ of the dose-response series ($\alpha_1 = 0$).

Let $W_n = \{W_1, W_2, \dots, W_{10}\}$ be the normalized Z_n data such that $W_n = \frac{Z_n}{\max(Z_n)}$.

As $Y_n = \beta X_n$ and $Z_n = Y_n(1 + \alpha_n)$, $Z_n = \beta X_n(1 + \alpha_n)$.

Therefore, $W_n = \frac{Z_n}{\max(Z_n)} = \frac{\beta X_n(1+\alpha_n)}{\max(\beta X_n(1+\alpha_n))}$.

Because β is a positive coefficient, $\max(\beta X_n(1 + \alpha_n)) = \beta \max(X_n(1 + \alpha_n))$.

In the end, $W_n = \frac{X_n(1+\alpha_n)}{\max(X_n(1+\alpha_n))}$.

C.2.2 Implementation of the Mathematical Model

The above derivation provided us with an equation for normalized simulated data (W_n) expressed in terms of ideal normalized data (X_n) and a set of current changes at each dose in a dose-response series (α_n). Ideal normalized data were generated by evaluating the Hill equation at the ten doses used in our dose-response experiments (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 100 μM) using a seed EC_{50} and n_H.

To generate sets of α values, we utilized the current change data set we had obtained from the 10.10.0 W7.40Trp experiments. In those experiments, we measured the change in current of a test dose of ACh at three positions within the dose-response series, thus obtaining sets of α values. (For the purpose of these simulations, we numbered the test doses as follows: the test dose between 0.01 μM and 0.03 μM was numbered as n = 3.5, the test dose at 0.3 μM as n = 6, and the test dose between 3 μM and 10 μM as n = 8.5. This numbering system allowed us to refer to the ten doses within the dose-response series as n = 1 through n = 10.) Our proposed current change model consisted of linear progressions connecting the three measured α values (Figure C.2). At dose n =1, α = 0 and α values increased linearly through dose n = 2 and n = 3 towards the α value measured for n = 3.5. Similar progressions were created to connect n = 3.5 with n = 6 and n = 6 with n = 8.5. At doses n = 9 and n = 10, we set α at the value measured for n = 8.5.

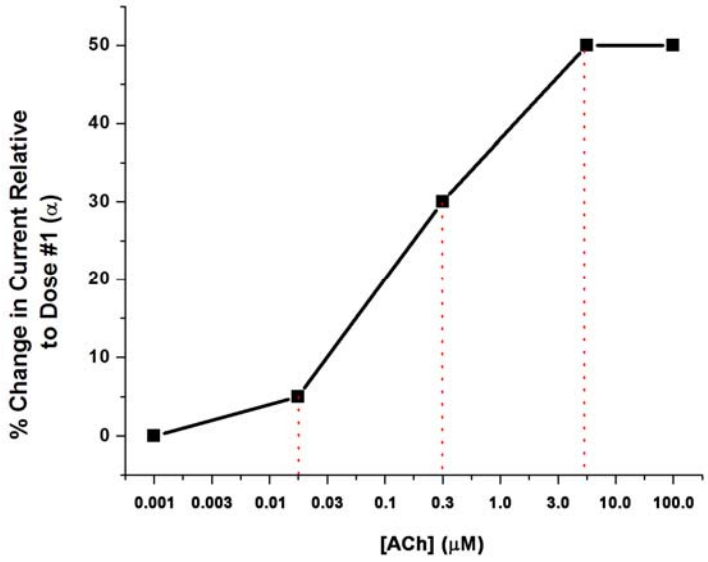

Figure C.2. Model for progression of α values throughout a dose-response relationship series. Dotted red lines denote the data points for α values measured during the current change experiments described in Section 3.2.6.3 and above. We refer to these data points as doses n = 3.5, n = 6, and n = 8.5, where the 0.001 μM dose is n = 1 and the 100 μM dose is n = 10.

With this model of current change, we then randomly generated α values for n = 3.5, n = 6, and n = 8.5. To generate these values (#1, #2, and #3) we randomly sampled from three data sets using the MiniTab software package: #1 was generated from the set of n = 3.5 current change percentages, #2 was generated from the set of differences between n = 6 and n = 3.5 percentages, and #3 was generated from the set of differences between n = 8.5 and n = 6 percentages. α values for n = 6 and n = 8.5 were then produced by adding the randomly generated #2 to #1 and adding randomly generated #3 to the previous sum (#1 + #2), respectively. This method for randomly generating values at n = 6 and n = 8.5 was used to provide the context of current change observed in the cells to our generated numbers.

Once n = 3.5, n = 6, and n = 8.5 α values were obtained, the rest of the α values were determined by linear progressions between them as described above (Figure C.2). An Excel (Microsoft) spreadsheet was used to program the equations that described the

lines connecting n = 1 with n = 3.5, n = 3.5 with n = 6, and n = 6 with n = 8.5. Figure C.3 shows the mean of generated α values at each dose for the five sets of 42 dose-response relationships compared with the actual measurements at n = 3.5, n = 6, and n = 8.5.

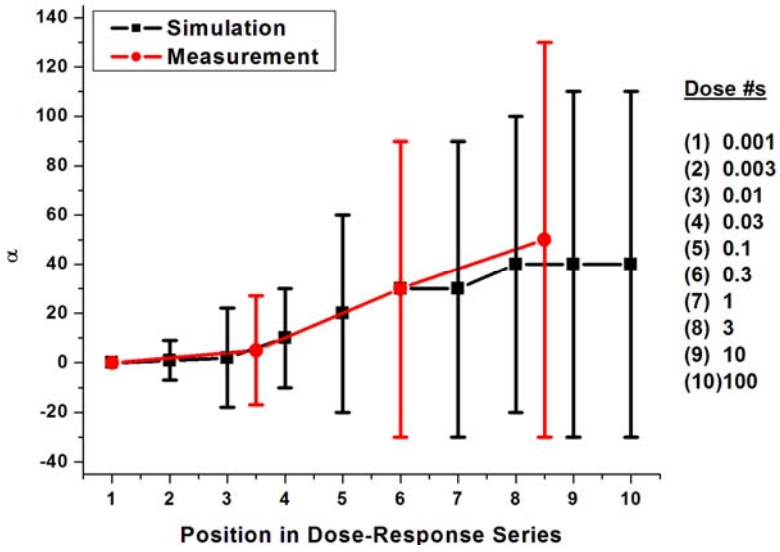

Figure C.3. Comparison of measured and randomly generated α values. The measured α values (red) were only determined at doses n = 3.5, n = 6, and n = 8.5. Randomly generated α values (black) were used in the five sets of 42 simulated dose-response relationships; these values were generated from the measured data as described in the text. *Right*: The ACh concentrations for each numbered dose

C.2.3. Seed EC_{50} Value and Dose-Response Relationship Data Generation

After five sets of 42 α value progressions were created, we took one set, Rand1, and used it to determine the appropriate seed EC_{50} value. We sought a seed value that generated dose-response data with a mean cEC_{50} value similar to that of the actual data. We tested seed values of 200, 190, 170, 160, 150, 140, and 135 nM and plotted the mean of the resulting cEC_{50}s versus the seed value (Figure C.4a). Because cEC_{50} data is log-normally distributed, we made a similar plot for the mean cEC_{50} of the ln transformed simulated data (Figure C.4b). Using the linear equations from both plots, we solved for

the seed value that would produce the mean cEC_{50} of the actual data (230 nM or 5.3, for the ln transformed data). Both equations yielded 140 nM.

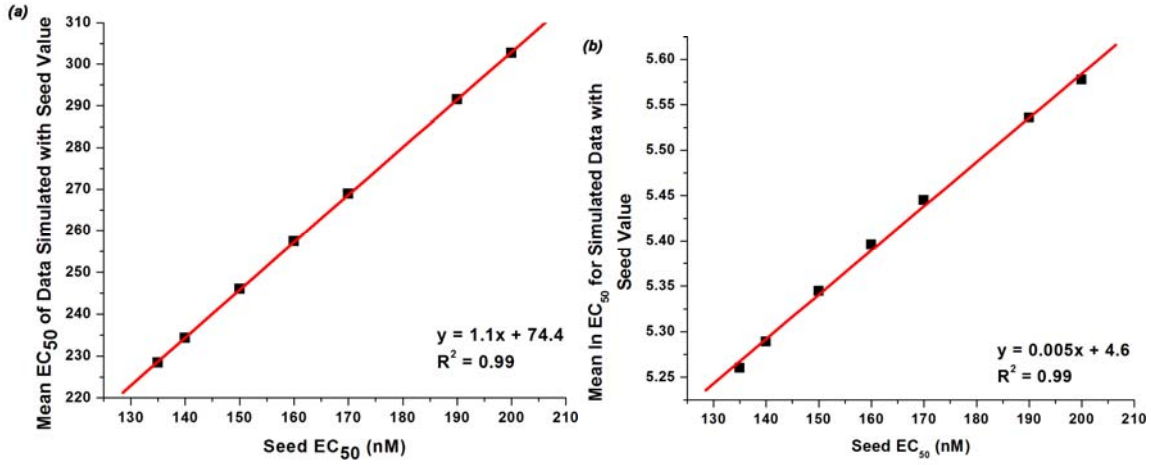

Figure C.4. Determination of simulated data EC_{50} seed value. ***(a)*** Mean of generated cEC_{50}s plotted versus the seed value for that data set. ***(b)*** Mean of the ln transformed data set plotted versus the seed value. Both plots show the equation of the line that fits the data. These equations were used to determine the appropriate seed value as discussed in the text.

The five sets of 42 simulated dose-response relationships, referred to as Rand1 through Rand5, were then created using the randomly generated α values and the 140 nM EC_{50} seed value (the n_H seed value was set at 1, because only one ligand binds to each GPCR). To create these data sets, we programmed the W_n equation derived above into an Excel spreadsheet and fit the normalized data to the Hill equation ($I_{Norm} = \frac{1}{(1+\left(\frac{EC_{50}}{A}\right)^{n_H})}$, where A is the concentration of drug) with the Origin software package (Origin Lab, Northhampton, MA). Each cEC_{50} data set's mean and variance were compared to those of the 10.10.0 W7.40Trp data set through *t*- and F-tests, resepectively.

C.2.4. "Correcting" Actual Data with the Asymmetric Current Change Model

After generating random M_2AChR-like data, we attempted to use this model of asymmetric current changes to "correct" our measured dose-response relationships. By "correct" we mean remove any effect current change had on the dose-response relationship and leave what should be a more accurate cEC_{50} value.

To perform this "correction", we used the measured n = 3.5, n = 6, and n = 8.5 α values for each cell to generate current change progressions as illustrated in Figures C.2 and C.3. With α values for doses n = 1 through n = 10, the actual measured response for each dose was divided by the quantity $1 + \alpha_n$ to produce "corrected" current responses. After normalizing these "corrected" responses, the data were fit to the Hill equation to obtain "corrected" cEC_{50} values. These values were compared to the "uncorrected" data through *t*- and F-tests.

C.3. Results and Discussion

C.3.1. Comparing Simulated Data with Actual Data

As described in Section 3.2.6.3, Figure 3.23, and Table 3.3, the five simulated data sets had similar means and variances to the 10.10.0 W7.40Trp data set. The *p*-values for the Rand4 *t*-test and the Rand5 F-test were only slightly above 0.05. Visual inspection of the distribution of these two data sets confirms these slight deviations from the actual data (Figure C.5a).

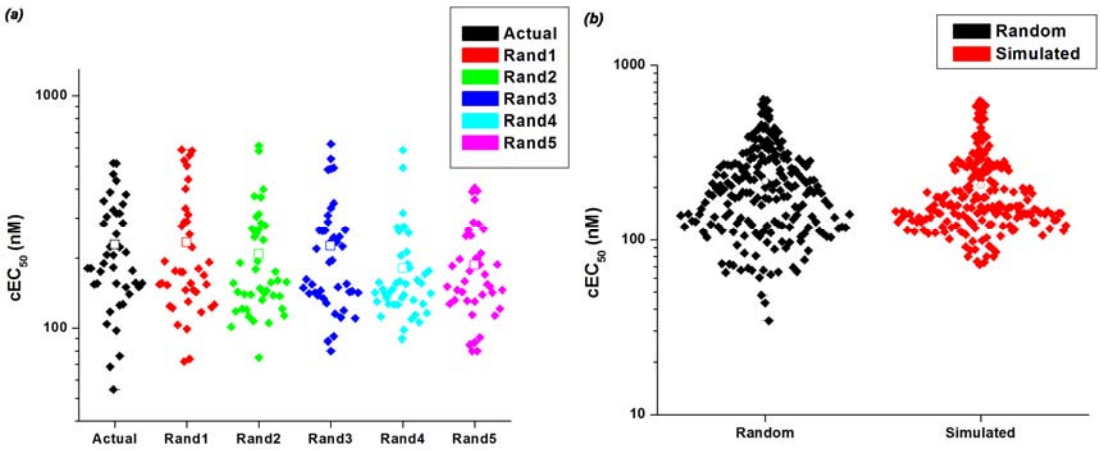

Figure C.5. Simulated and randomly generated 10.10.0 W7.40Trp data. ***(a)*** Distribution of actual 10.10.0 W7.40Trp data and simulated data sets Rand1 through Rand5. Open squares denote the cEC_{50} mean. ***(b)*** Distributions of 204 cEC_{50} values randomly generated from a log-normal distribution with the shape parameters $\mu = 5.3$ and $\sigma = 0.537$ and the 204 simulated cEC_{50} values shown in ***(a)***. Comparisons of groups in both ***(a)*** and ***(b)*** described in the text

To further confirm that the simulated data fit a distribution similar to that of the actual data, we randomly generated 204 cEC_{50} values from a log-normal distribution with shape parameters $\mu = 5.3$ and $\sigma = 0.537$ and compared them to the 204 simulated cEC_{50} values (Figure C.5b). The mean and variance of the two sets of numbers were not significantly different (*t*-test $p = 0.4$ and F-test $p = 0.8$).

We therefore conclude that our model for generating M_2AChR data is capable of reproducing the means and variability of data that we observe in the laboratory. More specifically, this exercise suggests that asymmetric current changes during the course of a dose-response relationship experiment introduce both variability and a general upward shift in cEC_{50}. The fact that the seed value that best replicates the mean cEC_{50} of our actual data was 140 nM, almost 40% lower than the measured mean cEC_{50}, suggests the actual ACh EC_{50} for M_2AChR may be lower than 230 nM. If a more direct readout of receptor activation were used, we predict that the dose-response relationship would be shifted to lower cEC_{50} values.

C.3.2. Data Not "Corrected" Through α Values

Given the success of the data simulation exercises, we predicted that the "corrected" 10.10.0 W7.40Trp data would have a lower mean cEC_{50} value and a lower standard deviation. Both predictions were incorrect. As shown in Figure C.6, the original and "corrected" data are indistinguishable; the means are 230 nM and 290 nM, while the standard deviations are 140 nM and 260 nM for the original and "corrected" data sets, respectively. In fact, the two variances are significantly different as determined by the F-test ($p = 0.01$).

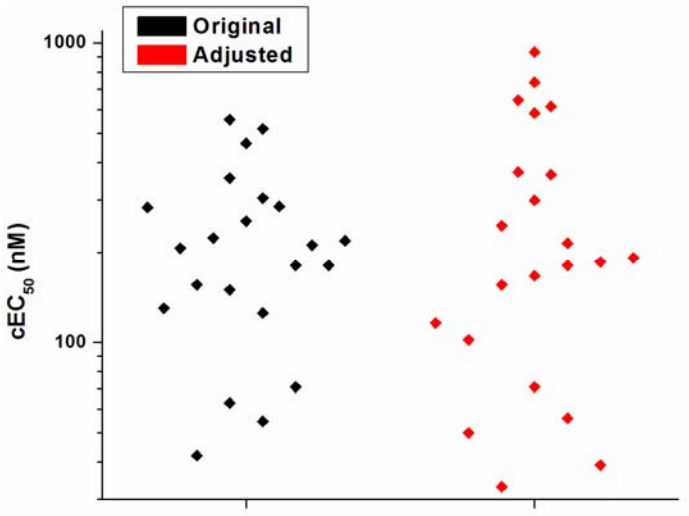

Figure C.6. Original and "corrected" 10.10.0 W7.40Trp data. 22 cells of data were "corrected" through the use of current change data collected during the dose-response relationship experiment. Comparisons of the two data sets are discussed in text.

Why did this "correction" fail to lower data variability or change the population mean? One possibility is that the less variable 10.10.0 injection conditions may have mitigated much of the current change-induced variability and that the remaining variability emanates from other sources within the cell. Although if this were true, the

data simulation exercise should not have been as successful at replicating data variability. We believe that the asymmetric current changes are not the sole source of variability in the M_2AChR-GIRK 1/4 signaling system. If another data set with higher cEC_{50} variability were "corrected", it is possible that more of its variability would come from current changes and would thus show a greater degree of "correction". Despite the failure of the "correction" methodology with this data set, this procedure should not be abandoned; future data sets may show improvement through this methodology and produce less variable data from this complex signaling system.