Biochemical Markers of Neuron Growth

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1 Introduction

Alison Barth is a professor in the Carnegie Mellon Biology Department who is interested in the electrophysiology of the plasticity, or learning, found among neurons in vertebrates, that is, she deals with synaptic properties involved in neuronal growth, or decay, at the molecular level. Of particular interest are glutamate and GABBA receptors. These receptors are vesicles found all along the edge of a neuron's multiple dendrites, which receive excitatory and inhibitory neurotransmitters respectively. Neurotransmitters are chemicals that are used to communicate between cells, which are released by one cell and received at the appropriate receptor of another. Under the currently accepted schema, there are many subtypes of these receptors and research of this nature is attempting to learn more about their physical properties. It is generally understood that sensory deprivation will affect the connection between neurons. Much to the same effect as when someone loses their sight, they become more attuned to their auditory senses. However, we will also explore the relationship between sensory deprivation and the expression of a particular gene, known as fosGFP. Our analysis will include the differences between three levels of sensory deprivation: control, spared, and deprived, along with two levels of expression: fosGFP+ and fosGFP-. We perform this analysis by examining measurements of characteristics of the action potentials, otherwise known as a synaptic event, which are measured at the body of a cell. An action potential leads to the transmission of an electrical impulse from a stimulated cell to an adjacent cell via the release of a neurotransmitter through a space between two dendrites, known as a synapse. The study includes numerous cells found in the neocortex of mice and our goal is to determine if there are significant differences between these levels in order to further develop hypotheses regarding synaptic structure of connecting neurons.

2 Data

In order to investigate the structural properties of the synaptic connections between cells we will be working with three levels of sensory deprivation in mice: control, spared and deprived. The mice are categorized by the number of their primary sensory organs (whiskers) remaining along with which neurons the measurements were recorded from: the control group had

no whiskers removed and the neurons that were examined are from the area of the brain that has been directly linked to a designated whisker, the spared group had all but one whisker removed and measurements were taken from the region of the brain that is mapped to this specific whisker, and the deprived group also had all but one whisker removed, but the recordings are from neurons that are mapped to a whisker that has been removed. The mouse is allowed to live for a period of time in order to experience outside stimuli after the whiskers were removed. The targeted brain tissue is then harvested in a manner that allows it to survive for many hours after the animal is sacrificed. Therefore, for all intents and purposes, the brain is still alive and functioning while the cells are communicating and so these synaptic connections are essentially forming in vivo.

Along with the level of sensory deprivation, we also have an indicator variable for the expression of fosGFP. GFP stands for green fluorescent protein, which reflects the color of the fluorescence that is observed under a microscope when the fosGFP gene is expressed. The mice used in the experiment are genetically altered so that every neuron synthesizes this protein, but it is typically only expressed in neurons that display highly active synaptic event structures. Therefore, we will explore the differences between cells that have expressed the gene (fosGFP+) and those that have not (fosGFP-), along with their possible interaction with sensory deprivation level.

The data for this project was given to us in the form of two Excel spreadsheets, one for excitatory post-synaptic currents (EPSC) and one for inhibitory post-synaptic currents (IPSC). This data has been recorded from a machine that registers the minute electrical impulses that occur when neurons communicate. Our main variables of interest are rise time, decay time, and amplitude. Rise time is defined as the amount of time (ms) that the impulse takes from its onset to reach its action potential. The decay time is the amount of time (ms) that the impulse takes to reset back from its peak to its resting voltage. Amplitude is defined as the distance from the synaptic event's baseline to its peak (picoamps). These three variables are collectively known as the cell's kinetics. For the rest of the paper we will be using the rise times as a proxy for the distance from the location on the dendrite where the synaptic event took place and the cell body. This is because regardless of the treatment level and/or fosGFP expression level, rise time is less affected by the structural composition of the receptor than decay time and amplitude. It is important that this distance be taken into effect because it is to be expected that the further an impulse travels, the less intensity it will have

when it is registered. This is similar to dropping a pebble in to a pool of water. The closer you are to the source, the more intense the ripples will be and vice versa.

Below are two tables that display the distribution of the neurons for each grouping.

	Control	Spared	Deprived	Total
fosGFP+	13	3	1	17
fosGFP-	7	7	13	27
Total	20	10	14	44

Table 1: number of neurons for each group for IPSC data

	Control	Spared	Deprived	Total
fosGFP+	8	2	1	11
fosGFP-	6	9	9	24
Total	14	11	10	35

Table 2: number of neurons for each group for EPSC data

In both Table 1 and Table 2, we see that there is a very small proportion of fosGFP+ neurons for the spared and deprived levels. This is because neurons were randomly chosen between fosGFP+ and fosGFP- for these two levels, but was not for control. The distribution of fosGFP+ and fosGFP- in control was purposefully chosen so that there would be more fosGFP+ neurons than the other two.

3 Questions

3.1

Our ultimate goal for this project is to use post-synaptic kinetics to get a better understanding of the neuronal connection process. We will examine if there any significant differences in the means of our three variables between the three levels of sensory deprivation. For instance, we expect to see a change in the amount of activity for the cells in each of the two non-control levels due to a lack of synaptic events from neighboring neurons, since we

have removed the sensory organs that map to these cells. This should result in a change in either the structure or function of the neuronal connections. Regardless of this change in post-synaptic activity, we do expect there to be an increase in the strength of connection between neurons within spared and deprived. This strength of connection can be measured by the event's amplitude.

It has also been hypothesized that there is a relationship between the kinetics of the three levels of sensory deprivation and fosGFP expression. Since every neuron in these mice synthesizes the fosGFP protein, but only some actually express the gene, it seems very reasonable that even within depravation levels, fosGFP+ cells may display different kinetics than fosGFP- cells. This is especially due to the fact that fosGFP expression is very much related to the cell's synaptic activity level. These questions can be answered using the standard statistical technique of two-way analysis of variance with interaction. We will use a fixed effects model with sensory deprivation level as one factor and fosGFP expression level as another.

3.2

It has also been hypothesized that the absolute length of the dendrites found in fosGFP+ cells are generally longer than the length of the dendrites found in fosGFP- cells. To test this hypothesis, we will examine the amplitude and decay times of the events that took place within the first quartile of the rise times for fosGFP+ cells and compare these to the amplitude and decay times found in the first quartile of rise times for fosGFP-. We will perform a similar analysis for the amplitudes and decay times for the final quartile of rise times. The reason for using rise time as our independent variable is because it is more of a function of proximity than structural composition. If it were to be the case that fosGFP+ dendrites are longer than fosGFP- dendrites then we would expect the following behavior. The means of both amplitude and decay time associated with the first quartile of rise times to be similar between fosGFP+ and fosGFP-, but at the same time, we expect the means of both amplitude and decay time found in the final quartile of rise times to be different between the two gene expression levels. We expect the decay times to be slower and the amplitudes to be smaller on average for the fosGFP+, simply because the impulse has farther to travel along the longer dendrite.

3.3

So far we have used only ANOVA to analyze the difference between the 6 unique subsets of the two factors. While an insignificant difference in means would point to no difference between two subsets of our data, it is possible that the data in the subsets has come from different distributions that happen to have the same means. To test this theory, a naive bayes classifier will be employed to categorize a cell as being in a particular subset of the data. If we observe a high success rate of classification in tandem with an insignificant difference in means we could conclude that the analysis of variance by itself is not enough to conclude such differences.

their rise times: fast and slow. Based on the graphs data analysis confirmed Alison's intuition to an extent. It is interesting to note that the control group IPSC's, but each of the treatment levels displayed multiple modes for the We are currently developing an EM algorithm that will populations within the groups of neurons, each sharing similar rise times. Once identified, we would then If this approach proves to be a successful, then we can use this the rise time. In other words, we may be able to use to better understand the true effect of amplitude. dendrites and because of this, witnessing a small amplitude distance that the impulse had to travel.

4 Results

4.1 Two-Way ANOVA

Before we begin implementing the two-Way ANOVA, it is fruitful to take a quick look at a table of the means of the kinetic measures for each of the factor levels.

fosGFP+	Control	Spared	Deprived		fosGFP-	Control	Spared	Deprived
Rise	2.38	2.05	1.80		Rise	2.32	1.87	2.00
Decay	5.14	5.44	4.36		Decay	4.97	4.88	5.40
Amplitude	7.87	8.57	7.77		Amplitude	7.87	8.59	8.56

Table 3: Means table for EPSC data. Left part is for fosGFP+ cells and right part is for fosGFP- cells. Rise and Decay are both in units of ms and Amplitude is in units of picoamps.

fosGFP+	Control	Spared	Deprived		fosGFP-	Control	Spared	Deprived
Rise	2.65	2.88	2.99		Rise	2.76	2.71	2.83
Decay	12.33	12.65	9.66		Decay	11.28	11.16	11.83
Amplitude	13.85	13.38	10.27		Amplitude	13.77	12.79	11.49

Table 4: Means table for IPSC data. Left part is for fosGFP+ cells and right part is for fosGFP- cells. Rise and Decay are both in units of ms and Amplitude is in units of picoamps.

The next step in the natural progression of analyzing such data is to run a two-way fixed effects ANOVA model in order to determine if there is possible interaction between the two factors and to determine if there are any significant differences between these means.

EPSC	Rise	Decay	Amplitude
Sensory Level	.00	.94	.05
fosGFP Level	.56	.57	.69
Interaction	.39	.25	.67

Table 5: P-values from two-way fixed effects ANOVA model for EPSC data. Sensory Level is to see if there are significant differences between the three levels of sensory deprivation. fosGFP Level is to see if there are differences between fosGFP+ and fosGFP- neurons. Interaction tests whether there is significant interaction between the two factors. Significance level is $\alpha = .05$

IPSC	Rise	Decay	Amplitude
Sensory Level	.13	.82	.002
fosGFP Level	.99	.22	.70
Interaction	.24	.21	.79

Table 6: P-values from two-way fixed effects ANOVA model for IPSC data. Sensory Level is to see if there are significant differences between the three levels of sensory deprivation. fosGFP Level is to see if there are differences between fosGFP+ and fosGFP- neurons. Interaction tests whether there is significant interaction between the two factors. Significance level is $\alpha = .05$

Based on the two-Way ANOVAs that are summarized in Table 5 for EPSC data and Table 6 for IPSC data, we can arrive at numerous conclusions. First,

the interaction effect is not significant for any of the variables in either of the two sets of data. Therefore, we must look at the main effects (Sensory Level and fosGFP Level) of each separately. Also, for each of the two tables, there is a lack of significance for the main effect fosGFP expression. Regardless of sensory deprivation level, there is no significant difference in the means of fosGFP+ and fosGFP- for each of the variables. There are, however, significant differences within the means of sensory deprivation levels for the variables rise and amplitude for EPSC data, along with amplitude for the IPSC data. Because our null hypothesis for this model is that all of the means are the same and we have rejected that possibility in a few instances at the $\alpha = .05$ level, we must now explore as to which of the means are actually different from one another using t-test's.

EPSC	Control-Spared	Control-Spared	Spared-Deprived
Amplitude	.01	.08	.78
Rise	.00	.00	.24

Table 7: P-values for EPSC data from t-test comparing the means of the two sensory deprivation levels provided in the first row. For instance, there is a significant difference between the means of the amplitudes between control and spared because the value in the cell is $\leq \alpha = .05$.

IPSC	Control-Spared	Control-Spared	Spared-Deprived
Amplitude	.50	.00	.06

Table 8: P-values for IPSC data from t-test comparing the means of the two sensory deprivation levels provided in the first row. For example, there is a significant difference between the means of the amplitudes between control and deprived, but not between control and spared. Significance level is $\alpha = .05$

Hence, we can conclude that for the EPSC data, the mean of amplitude for the spared level is significantly higher than the mean of amplitude for the control level, but not significantly different than the deprived level. Similarly, we can also conclude that the mean of the rise time for control is significantly less than the mean of rise time for both the spared and deprived levels, but spared and deprived themselves were not significantly different.

For the IPSC data, we can conclude that the mean of the amplitude for control was significantly higher than the mean of the amplitude for spared. No other significant differences were found in this data.

4.2 Quantile Analysis

In order to test the hypothesis that the dendrites found in fosGFP+ neurons are longer than the dendrites in fosGFP- neurons we will examine the amplitude and decay times of the events that took place within the first quartile of the rise times for fosGFP+ cells and compare these to the amplitude and decay times found in the first quartile of rise times for fosGFP-, and similarly for the final quartile. Referring to Table 1, we can see that control is the only depravation level that has a nearly balanced mix between fosGFP+ cells and fosGFP- cells. Because of this, we have decided to only include an analysis on these neurons, with the hopes that in future, more data can be collected for the other two levels. Also, we will not include an analysis of IPSC data because there was a large problem with the decay data. The lab technician who collected the data calibrated the machine incorrectly, which led to only times up to 20 (ms) being recorded, anything longer was truncated down to 20. Over seventy percent of the IPSC data was affected by this problem and so any analysis using this data unreliable.

We analyzed the means of the three kinetic variables of interest found that the quartiles of the rise time for the EPSC data within the control sensory deprivation level for fosGFP+ and fosGFP- neurons using a standard t-test.

EPSC	1st quartile	4th quartile
Rise	.59	.83
Decay	.99	.87
Amplitude	.31	.48

Table 9: P-values for EPSC data from t-test comparing the means of the respective kinetic variables for fosGFP+ and fosGFP-,using the quartiles of rise time.

Referring to the results of the fixed effects one-way anova results in table 9, it can quickly be seen that there exists no significant differences. Due to the lack of any conclusive differences, the hypothesis that the fosGFP+ cells have longer dendrites could not be confirmed.

5 Conclusions/Discussion

Our analysis of the kinetic post-synaptic data has been aimed at determining if there are any significant differences between sensory depravation levels and fosGFP expressions levels in order to learn about the neuronal connection process. From our analysis, we have concluded that there is not a significant interaction effect between depravation level and gene expression level. Hence, we looked at each of the main effects separately. From this analysis, we determined that the level of fosGFP expression does not significantly impact the means of our kinetic variables. We have also found no evidence that supports the hypothesis that dendrites found on fosGFP+ cells are significantly longer than the dendrites found on fosGFP- neurons in the EPSC control depravation level. For the main effect of depravation level found in the EPSC data, we confirmed one of our hypotheses: that the amplitude is significantly higher for the spared level than the control level. This is likely due to plasticity, or strengthening of a neuron's synaptic connections that results from a loss in connection with neighboring cells. We are unsure as to the biological implications of the other significant results and further research should be conducted on the topic.

If we were to continue working on this project, it would be necessary to perform two more analyses, in particular: a power analysis and mixed effect models. There is a very good chance that the negative results that we got from the analysis of fosGFP+ and fosGFP- neurons where there were only one or two cells in each group is due to a lack of power. In the future, we would find out what the power of our tests are so that we can properly reject when we are supposed to reject the null hypothesis. The other future analysis that we would perform would be a mixed-effect model for our analysis of variance. This is because we have assumed that the individual neuron's differ only in their mean, and not in their distribution and so we have pooled them together. We feel that this is not necessarily the case, and a mixed-effect model, treating the cell's themselves as random variables, would be more appropriate.

So far we have used only ANOVA to analyze the difference between the 6 unique subsets of the two factors. While an insignificant difference in means would point to no difference between two subsets of our data, it is possible that the data in the subsets has come from different distributions that happen to have the same means. To test this theory, a naive bayes classifier could be employed to categorize a cell as being in a particular subset of the data. If we

observe a high success rate of classification in tandem with an insignificant difference in means we could conclude that the analysis of variance by itself is not enough to conclude such differences.

6 Appendix A

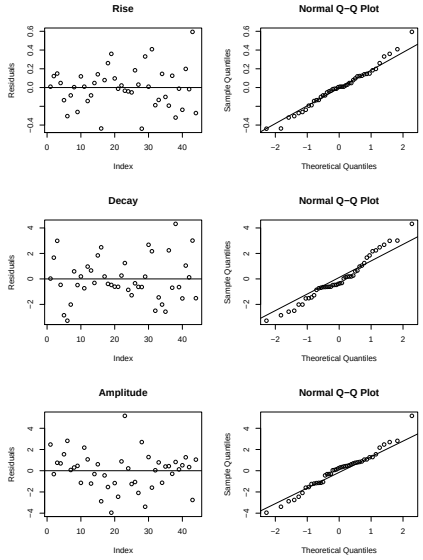

Figure 1: Residual Analysis for IPSC

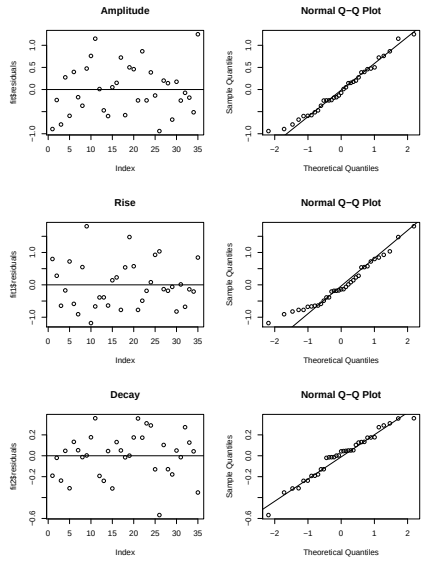

Figure 2: Residual Analysis for EPSC