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Does folic acid compete with glutamic acid at the postsynaptic membrane NMDA receptor?

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DOES FOLIC ACID COMPETE WITH GLUTAMIC ACID AT THE POSTSYNAPTIC MEMBRANE NMDA RECEPTOR?

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Date Dr. Darrell Wiens, Honors Thesis Advisor

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Date Dr. Jessica Moon, Director, University Honors Program

Abstract

During the first month of a human pregnancy folic acid (FA) is vital to the closing of the neural tube. However, overconsumption of FA has been linked to the rise of Autism Spectrum Disorder (ASD), although this linkage is still under debate and testing. It has been hypothesized that the glutamate (GA) portion of FA may compete for binding to the N-methyl-d-aspartate receptor (NMDA-R) with the neurotransmitter glutamate, causing inhibited growth cone activity. In order to test this hypothesis, we cultured eight-day chick dorsal root ganglia (DRGs) and assessed parameters of neural development in the presence of FA, GA, or both and compared these to controls. We found that neurite number was initially inhibited by both FA and GA, though the GA was no longer inhibitory in the more advanced DRGs. Furthermore, when the two were combined the GA partly overcame the FA's inhibition. We found no consistent effects on neurite length, or on dynamic activity of neurites and growth cones. We found that both agents inhibited synaptogenesis. Additionally, we found that synaptogenic area was increased as the DRGs advanced.

Acknowledgements

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Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects an individual's ability to interact socially (Mayo Clinic, 2018). In the United States approximately one in 88 children is affected by ASD. More males are affected by ASD with one in 54 males and one in 252 females being diagnosed with ASD (Centers for Disease Control and Prevention, 2012). The average cost of needs throughout the entire life of an individual affected by ASD is $2.4 million (Buescher et al., 2014). The high cost can place a strain on families so, in order to minimize costs, researchers are looking for effective therapies for the individuals who may need them. In order to find effective therapies, it would be beneficial to understand what factors may lead to ASD, which have led to many studies such as this one investigating a potential cause for ASD.

ASD is considered a spectrum due to the range of differing effects and the severity of these effects. Some differences in individuals affected by ASD can include difficulty interacting with others, difficulty communicating, sensitivity to light or sound, fixations on different objects, and repetitive behaviors. Children affected by ASD may not exhibit these behaviors initially, but most individuals show symptoms by the time they are two years old (Mayo Clinic, 2018). The reason that some children are born with ASD is not fully understood and many supplements and genetic factors are being tested in order to determine potential causality. One supplement being investigated is folic acid (FA).

FA is a B9 vitamin utilized for many DNA processes including methylation, repair, and synthesis (Shane, 2010). It has been found to be important in preventing major birth defects during the first month of pregnancy. The current recommendation is for women to take FA

during pregnancy and while she is trying to conceive (Folic Acid, 2017). One portion of FA is glutamate, which is a form of glutamic acid that contains one less hydrogen.

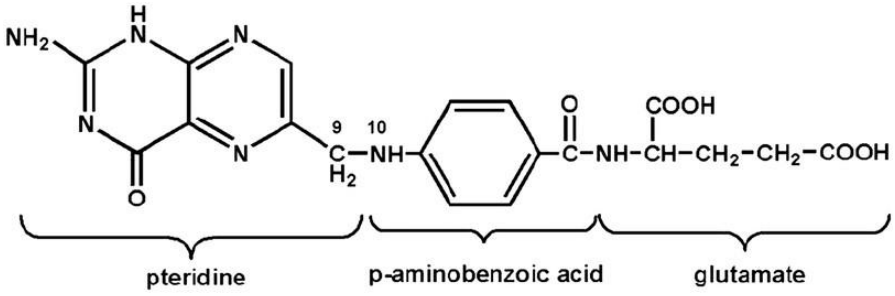

Figure 1. Folic acid with glutamic acid section labeled.

Glutamic acid (GA) is a commonly found neurotransmitter and binds to the N-methyl-D-aspartate receptor (NMDA-R) during development *in utero* (Ebert and Greenburg, 2013). The NMDA-R controls the channels of ions such as Na^+, K^+, and Ca^{2+} (Ebert and Greenburg, 2013). When GA binds to the NMDA-R the concentrations of these ions change on either side of the channel. Changes in Ca^{2+}concentrations are particularly important as they affect the formation of connections in the brain (Gill et al., 2015). Different connections may change how the brain functions, potentially leading to conditions such as ASD. This means that a decreased or increased occurrence of binding to the NMDA-R may change how the brain develops. Furthermore, if FA is competing with GA then the amount of binding may change, potentially leading to the aforementioned connectivity differences.

A model system for neural development is required to test for differences in connectivity. Chicken embryos have been previously used as such a model (Cohen et al., 1954; Levi-Montalcini, 1964; Letourneau, 1975), and so chicken dorsal root ganglion (DRGs) were selected as the model for this study. DRGs are found along the spinal column and eight-day old chicken

embryos provide a viable source of these. The DRGs can be cultured and the process of synaptogenesis can be observed as growth cones lead the neurites to extend and retract. Typically, after the DRG innervates the spinal cord to connect it to the peripheral receptors it begins the aforementioned process of neurogenesis in order to establish synaptic networks (Wiens et al., 2016).

Purpose

The overconsumption of an important maternal supplement, FA, has been potentially linked to a rise in the occurrence of ASD (Beard et al, 2011; Barua et al, 2015). FA is known to be structurally similar to the neurotransmitter glutamic acid, which is important to brain development. It has been suggested that this structural similarity may cause competition to bind to a receptor in the brain, which would change concentrations of chemicals during brain development, potentially causing ASD (Wiens, 2016). The competition between FA and the structurally similar neurotransmitter, glutamic acid, will be investigated in this study.

Literature Review

Folic Acid Supplementation

Nervous system formation begins with the thickening of the ectoderm of an embryo. The thickened ectoderm will become the neural plate, which will fold up, forming the neural groove. The groove will extend down length of the embryo and close, forming the neural tube. The neural tube will become the brain and spinal cord as development progresses. Without closure of the neural tube, the brain and spinal cord will not correctly develop (see Figure 2), leading to a variety of birth defects such as spina bifida and anencephaly (Greene & Copp, 2009).

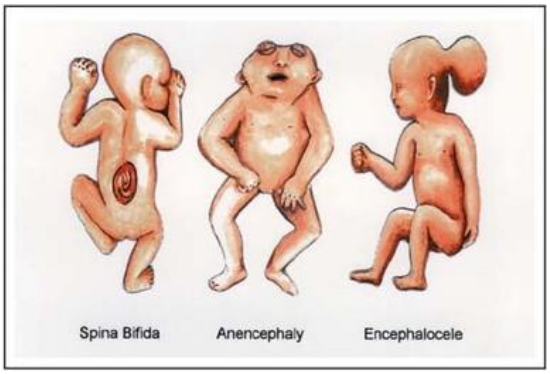

Figure 2. Neural tube closure defects

During the first month of a human pregnancy a multivitamin supplement including FA is vital to the closing of the neural tube (Källén, 2017). While the reason for the necessity of FA in this process is not yet completely understood, it is known that the intake of multivitamin supplements that include FA during the first month of pregnancy decreases the likelihood of birth defects such as spina bifida and anencephaly. Spina bifida is due to a failure of a caudal portion of the neural tube to close and can lead to issues such as partial paralysis (Mayo Clinic, 2018). Anencephaly is caused by the failure of a cranial portion of the neural tube to close which leads to incomplete skull and brain development, usually resulting in the infant's death (Centers

for Disease Control and Prevention, 2017). While vitamin supplements that include FA are necessary in preventing these defects and can be found in many foods such as leafy green vegetables, meats, nuts and dairy products, many people are unable to afford or access some of these food items (Wiens et al., 2016). FA is additionally important in the human body's metabolic methylation process. Methylation is a process where a methyl group is added on to a strand of DNA, where the methyl group then helps with gene regulation and protection of the DNA molecule. FA provides the necessary methyl groups for this process. It can also add methyl groups to homocysteine to synthesize methionine, an amino acid that is important for growth and development and will prevent the accumulation of homocysteine, which can cause malformations in embryos and lead to vascular disease in adults (Rosenquist, 2013). In 1998, the government decided to add FA to many grain and cereal products in order to supplement the diets of those unable to purchase foods rich in FA (US Food and Drug Administration, 1996).

The ample access to foods containing FA, the physician-recommended vitamin supplementation, as well as the often-repeated knowledge of the importance of FA to the diet of pregnant women has led to an overconsumption of FA. The overconsumption of FA has been noted due to its excessive presence in maternal plasma (Sweeney et al., 2006) and even umbilical cord blood (Obeid *et al*., 2010). Excessive FA has been linked to the rise of ASD (Beard et al. 2011), although this linkage is still under debate and testing (Choi *et al*., 2014).

Potential Competition at NMDA-R

It has been found by Wiens et al. (2016) that FA inhibits growth cone activity, synapse formation, and neurite extension, and the effects increase with increased concentration of FA, (Wiens et al., 2016). It is not yet known how FA causes these changes, but it is suspected to be related to the glutamate structure located at one end (Wiens, 2016). GA is one of the most

common excitatory neurotransmitters and is associated with the receptor NMDA-R. The NMDA receptor can be found in presynaptic neurons and it serves to regulate neurotransmitters. The NMDA-R is involved in synaptogenesis, or connectivity in the brain, though the extent of this is not yet fully understood. Additionally, the NMDA-R may regulate cortical brain development by affecting some of the changes of brain-derived neurotrophic factor (BDNF) (Gill et al. 2015).

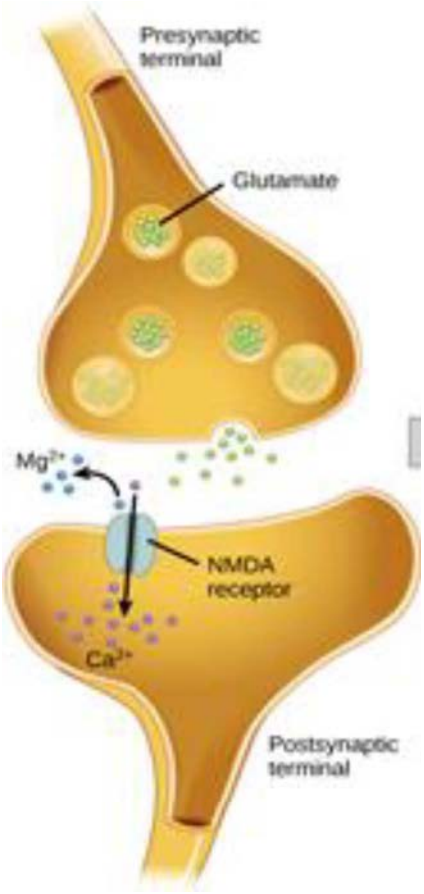

Figure 3. N-methyl-d-aspartate receptor

As seen in Figure 3, GA is released by the presynaptic terminal and enters the synapse, which lies between the pre and post synaptic terminals. Some of the GA in the synapse binds to the NMDA-R, which then allows Ca^{2+} to flow into the postsynaptic terminal and Mg^{2+} to flow out of the postsynaptic terminal. When the concentrations of Ca^{2+} and Mg^{2+} change then a signal is sent along the neuron, triggering the next presynaptic terminal to initiate the same reaction.

NMDA-R is furthermore important because it controls the channels which allow changes in concentrations of Na^{+}, K^{+}, and Ca^{2+} (Ebert & Greenburg, 2013). When GA binds to the receptor, the channels allow these ions to flow through, changing the concentrations on either side of the channel. The effects on the change of Ca^{2+} levels are of particular interest in this study because the concentrations of Ca^{2+} affect the formation of synapses. It is known that Ca affects the synaptogenesis and therefore connectivity of the neural networks in the brain (Gill et al., 2015).

If the glutamate portion of FA competes with the GA already found in the developing brain, it could change the concentration of Ca^{2+} available to affect synapse formation. The different concentration would change how the synapses form and alter brain development. One difference in brain development that may occur is inhibition of connectivity in the brain. If underconnectivity is occurring, it would be consistent with previous findings of Wiens et. al (2016) that inhibited growth cone activity and synaptogenesis are present in FA treated DRGs.

Underconnectivity

Research done by Wiens et al. (2016) reported that FA *in vitro* to cause inhibition of growth cone activity and synaptogenesis. The inhibition is consistent with the idea that underconnectivity in the brain is linked to an individual being affected by ASD. It has been shown that children affected by ASD have larger brains than those of their peers. However, there has also been a large amount of evidence found supporting the idea that certain areas of the brain important to social interaction and language may be underconnected (Lewis et al. 2012). Furthermore, those affected by ASD may have less activation in Wernike's and Broca's areas, which are related to language and speech. The overall underconnectivity, especially in these areas, could explain some social difficulties experienced by those affected by ASD (Just et al. 2004).

Hypotheses to be Tested

FA may be competing with GA to bind to a receptor that is important to brain development. It is hypothesized that the FA will be competing with GA at the NMDA-R (Wiens, 2016). Competition would be seen when treated DRGs will be compared to DRGs that simply contain control levels of FA and GA from the culture medium and fetal calf serum supplement.

The DRGs cultured in excess FA supplemented dishes are expected to show an inhibition of synaptogenesis while dishes that are treated with both FA and GA should overcome this inhibition.

Methods and Materials

In order to test the hypothesis, I dissected eight-day old chicken embryos (as seen in Figure 4) to obtain DRGs. These DRGs were cultured and their neuronal development was assessed microscopically as neurites extend, led by growth cones. For each experiment, I obtained five to six DRGs, placed them in culture dishes containing two-milliliters of culture medium, and waited 48 hours for them to attach and then extend outward in all directions. After 48 hours I captured images and used image analysis software to record the dynamic behavior of a single neuron's growth cone. The growth cones were recorded using ImagePro™ software in conjunction with a Leica DMIRE-2 inverted microscope and Q-Imaging CCD camera. The growth cone activity was recorded by capturing digital images at the rate of one image per minute for half an hour. Then a stock solution containing either 6µl of 5mM FA, 6µl of 5mM GA, or 6µl of both FA and GA was added and the growth cone behavior was recorded for another half an hour as described. *ImagePro Premier*™ software was used on the time-lapse videos to compare the area change per minute and net extension/retraction of several treated DRG control neurons to the same parameters of growth cone behavior before treatment (controls).

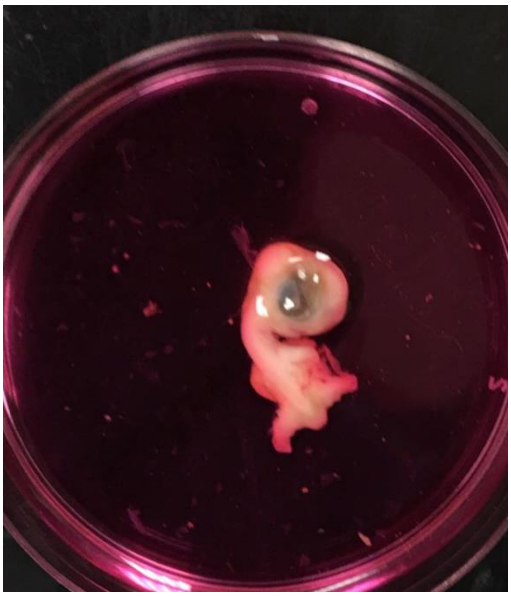

Figure 4. eight-day old chicken embryo

To another set of dishes had medium containing the excess FA, GA, or combined FA and GA was added to them before they were placed in the incubator. Once the DRGs had been thus incubated 48 hours, they were fixed and taken through a process of immunostaining to produce red color wherever synaptogenic areas had developed (as seen in Figure 5). A monoclonal

antibody that specifically recognizes and binds synaptic vesicles (SV2, obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA) was employed for this staining together with a biotinylated secondary antibody and a streptavidin-peroxidase conjugate (Vector Laboratories, Inc., Burlingame, CA). Development of a red-brown color was achieved through incubation in 1% 3-amino-9-ethylcarbazole in 1 M acetate buffer. Multiple high-resolution images of the DRGs were then taken and tiled together (as seen in Figure 5) using *Image-Pro Premier* into one high resolution aggregate image. This software was then used to determine the number of neurites and their lengths. This was done by drawing lines through each image (as can be seen in Figure 6). Any neurites which crossed the lines were measured and counted in order to obtain a random sample size of each DRG. Another program, *ImageJ* (free software available through the NIH), was used to quantitatively access the synaptogenic (stained) area of each DRG. This was measured in square microns after calibration, and using the color thresholding feature in the program. The treated DRGs could then be compared to controls.

DRGs used varied in size and each DRG contained a large amount of synaptogenic area. Therefore, the difference in size of DRGs could alter any conclusions based upon the results. In order to avoid this issue, the DRGs were removed (as can be seen in Figure 6). This allowed the results to be strictly related to the synaptogenic area of the outgrowth for each DRG.

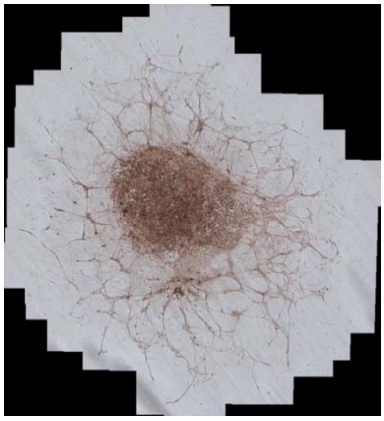

Figure 5. Tiled image of a control DRG with the DRG present

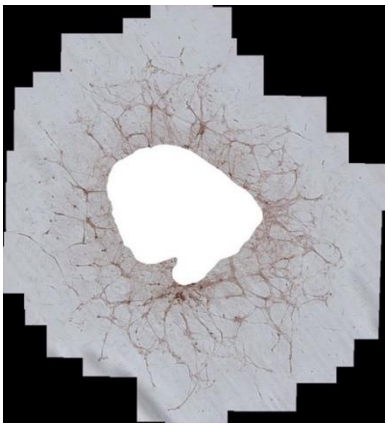

Figure 6. Tiled image of a control DRG with the DRG removed

Results

The DRGs in culture would begin to adhere to the dishes within a few hours (as can be seen in section A of Figure 7 below). Some of the DRGs would not attach to the dishes and were therefore not used in this study. As time went on, flat migratory cells were found to extend out from the edge of the 3-D DRG explants (as can be seen beginning in section A of Figure 7 below). Eventually, a layer of flat fibroblastic cells would form around the DRGs with fewer cells found the farther away from the DRG. Some of the cells developed rounded cell bodies and extending out thin neurites (as shown in section C of Figure 7).

As research was conducted, it was observed that as the DRGs were incubated, the neurites of some of the DRGs began to bundle together to form nerves. It was determined that the DRGs with nerves could be more developmentally advanced, therefore changing results if the developmental stage was not taken into account. This led to the dividing of the data, based on the developmental stage of the DRG. When the data was collected for the immunostained DRGs, each one was carefully looked at and it was determined whether nerves were present. This led to the collection of two separate data sets for each test type done on each treatment type. Figure 7 shows the progressive change in appearance of a DRD at successive hours of culture.

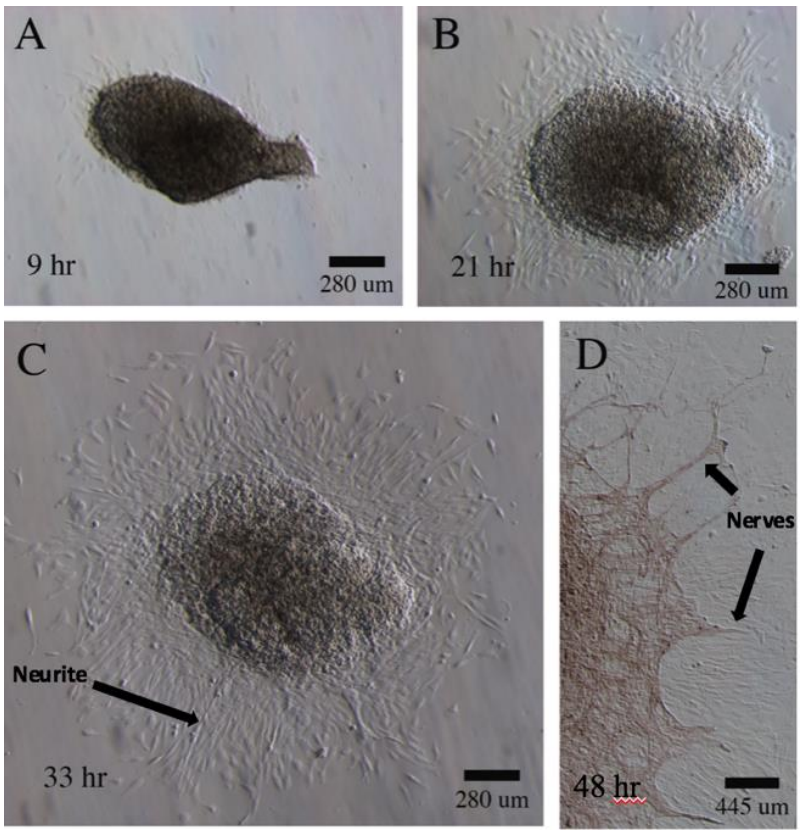

Figure 7. DRGs in culture at different times A. At 9 hours in culture, the DRG has attached and cells began to migrate outward. B. At 21 hours in culture, more cells have migrated outward, creating an epithelial pavement and clusters. C. At 33 hours in culture the epithelium had expanded outward in all directions, and neurites were more visible and numerous. An example of a neurite is indicated by an arrow. D. Edge of a 48-hour DRG after it had been immunostained with TAG-1, an antibody that stained neural and glial cells red. A lower magnification was used for this image. Many neurites bundled into nerves were positively stained and these extended out on top of a flat substrait of non-neural epithelial and other cells which remained unstained by TAG-1. DRGs which exhibited nerves were considered to be more advanced than DRGs with only neurites.

Immunostained Neurite Length

As a recognizable parameter of neurogenesis, the lengths of neurites and the thicker bundled nerves present were measured and averaged for each DRG and averaged for each DRG. Then, the average lengths of each treatment type were averaged together but were categorized as either more developmentally advanced or less developmentally advanced DRGs, depending on the presence of nerves. Next, the average lengths of both neurites and nerves in the developmentally advanced DRGs of each type were compared to using a t-test. The average lengths of neurites in the less developmentally DRGs were compared in the same way. For both sets of data an $\alpha=0.05$ was used. No significant difference in neurite lengths was found in these tests.

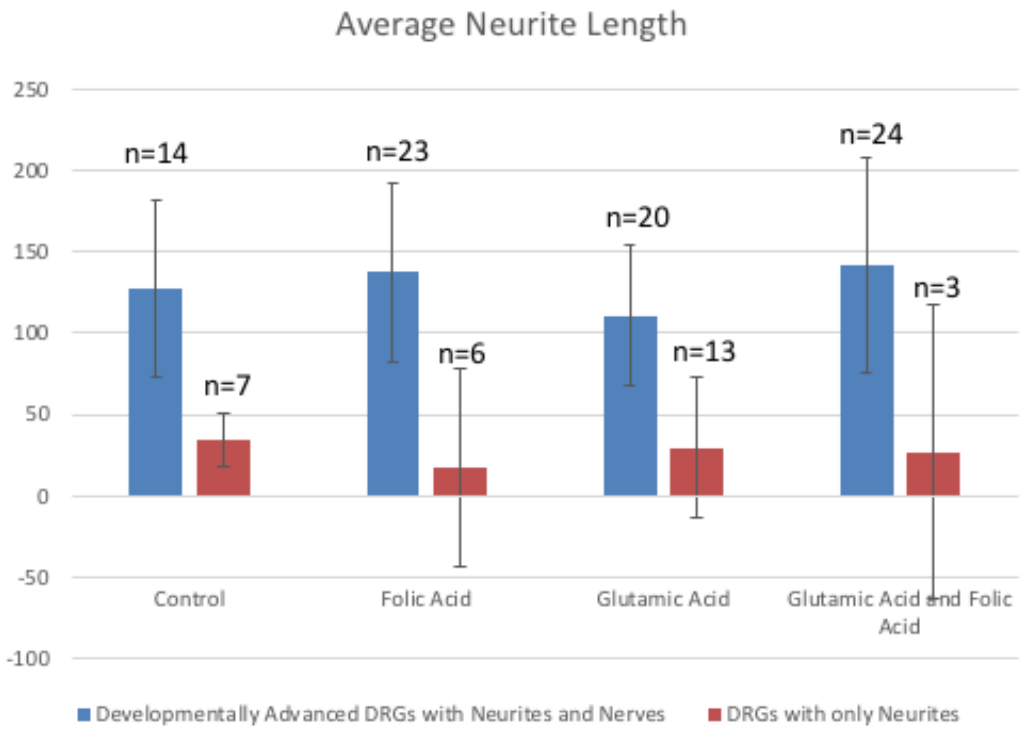

Figure 8. Average Neurite Length. The lengths of more developmentally advanced DRGs are shown in blue and less developmentally advanced DRGs are shown in red. The sample size of each DRG type is equal to the n value shown above that bar. Error bars were calculated and

DRGs were compared to other DRGs of the same developmental advancement. A value of α=0.05 was used to determine significance. The number of samples are indicated by "n".

Immunostained Neurite Number

As another fundamental parameter of neurogenesis, neurites and nerves were counted in each DRG, and the average number of neurites were found for each treatment, keeping in consideration the presence of nerves. DRG treatments of the same developmental stage were compared using t-tests with 0.05 as the chosen α value. When treatments of each developmental stage were compared, it was found that both FA and GA initially inhibited the number of neurites. However, in the more advanced DRGs, the GA no longer inhibited the number, of neurites formed whereas FA did still inhibit the number of neurites that formed. When the FA and GA were combined in more advanced DRGs, GA overcame the FA inhibitory effect.

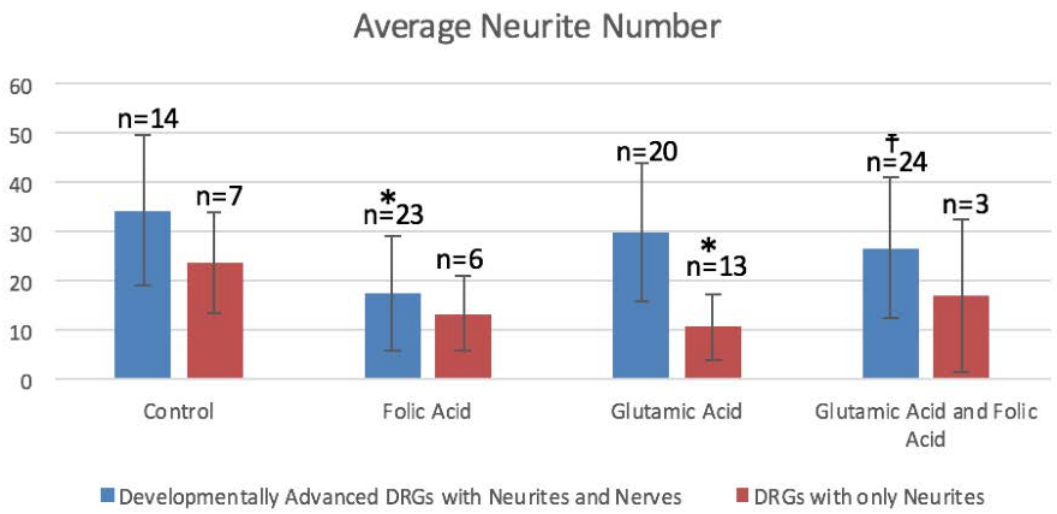

Figure 9. Average Neurite Number. The lengths of more developmentally advanced DRGs are shown in blue and less developmentally advanced DRGs are shown in red. The sample size of each DRG type is equal to the n value shown above that bar. Error bars were calculated and DRGs were compared to other DRGs of the same developmental advancement. A value of α=0.05 was used to determine significance. The number of samples are indicated my "n". *Significantly less than control, p=0.02 for folic acid and p=0.02 for glutamic acid. †Significantly less than folic acid treatment DRGs, a value of p=0.02 was used.

Immunostained Synaptogenic Area

Synapses are the spaces between neurons through which signals can be sent. In order to form more connections to share information and signals, neurons search for other neural growth so they can form more synapses, a process called synaptogenesis. When the DRGs were immunostained, any areas where synaptogenesis was occurring were stained red. Using *ImageJ* the amount of stained areas were found, giving a total synaptogenic area as seen in Tables 1 and 2 below. The median and mean synaptogenic areas were found using a measurement of thousands of square microns and compared across the different treatments.

In less advanced DRGs, the control group of 8 DRGs showed an unusually low median and a great range of synaptogenesis, making comparisons difficult. However, there is shown to be an inhibitory effect occurring in GA.

Synaptogenic Area of Only Less Advanced DRGs without Developed Nerves				
Synaptic Area (thousands of square microns)	Control (n=8)	Folic Acid (n=7)	Glutamic Acid (n=15)	Glutamic Acid and Folic Acid (n=3)
Median	0.23	2.5	0.23*	4.6
Mean	2.5	4.3	0.38	6.9

Table 1. Synaptogenic Area of Only Less Advanced DRGs without Developed Nerves. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n". *Significantly less than area of more advanced DRGs

In more advanced DRGs that had bundled nerves, FA and GA inhibited synaptogenic area development, especially GA, and when FA and GA were combined the FA appeared to

moderate the strong effect of GA. The data were not normally distributed.

Syntaptogenic Area of Only Advanced DRGs with Developed Nerves				
Synaptic Area (thousands of square microns)	Control (n=14)	Folic Acid (n=24)	Glutamic Acid (n=20)	Glutamic Acid and Folic Acid (n=25)
Median	16	12	0.6*	9.4
Mean	19	18	1.1	24

Table 2. Synaptogenic Area of Only Advanced DRGs with Developed Nerves. Compared utilizing t-tests with an α=0.05 used to determine significance. The number of samples are indicated by "n". *Significantly less than control

Videos Data of Neurite Length Change

As neurites search to make connections with other neural growth they extend and retract. An inhibition of this exploratory behavior would be represented by more retraction or less extension occurring. In order to test whether an inhibitory effect was occurring with any treatment the lengths of neurites were measured were measured during 40-minute time lapse image videos. The first 20 minutes of the video were of an untreated (control) neurite. Then, the DRG was treated with FA and recorded for another 20 minutes. Average change was found for the advancement, retraction, net change, and median length change. A t-test with a α=0.05 was done comparing the length changes of FA-treated neurites to those observed prior to adding FA to the dish. As seen in Table 3, FA at this concentration did not have any significant effect on the dynamic neurite length change.

Neurite Length Change for Folic Acid Treated DRGs in microns per minute		
	Control (n=5)	Folic Acid (n=5)
Average of Net Advancement	11	12
Average of Net Retraction	-15	-20
Average of Net Change	-4	-7
Average of Median Length Change	-0.3	-0.09

Table 3. Neurite Length Change for Folic Acid Treated DRGs in microns per minute. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n".

The process was completed in the same way with GA treated DRGs. As seen in Table 4, GA at this concentration did not have any significant effect on the dynamic neurite length change.

Neurite Length Change for Glutamic Acid Treated DRGs in microns per minute		
	Control (n=6)	Glutamic Acid (n=6)
Average of Net Advancement	17	15
Average of Net Retraction	-17	-22
Average of Net Change	0.7	-7
Average of Median Length Change	0.2	0.01

Table 4. Neurite Length Change for Glutamic Acid Treated DRGs in microns per minute. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n".

The process was completed once more with DRGs that were treated with both FA and GA. As seen in Table 5, when combined, GA and FA at this concentration did not have any significant effect.

Neurite Length Change for Glutamic Acid and Folic Acid Treated DRGs in microns per minute		
	Control (n=8)	Glutamic Acid and Folic Acid (n=8)
Average of Net Advancement	19	20
Average of Net Retraction	-17	-16
Average of Net Change	4	-1
Average of Median Length Change	0.1	-0.4

Table 5. Neurite Length Change for Glutamic Acid and Folic Acid Treated DRGs in microns per minute. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n".

Video Data of Growth Cone Area Change

Neurites utilize growth cones to search for other neural growth. The growth cones change area due to the extension and retraction of their microspikes. The growth cone area change can indicate the potential for connectivity. The area of each growth cones was measured throughout the 40-minute videos. The first 20 minutes of the video were of the dynamic change of an untreated DRG while the final 20 minutes were of a FA treated DRG. The data found for all of the DRGs was not normally distributed so minimum, maximum, and median lengths of treated DRGs were compared to the controls for each type of growth cone. As seen in Table 6, FA

increased the median area change, however this difference did not show significance when tested.

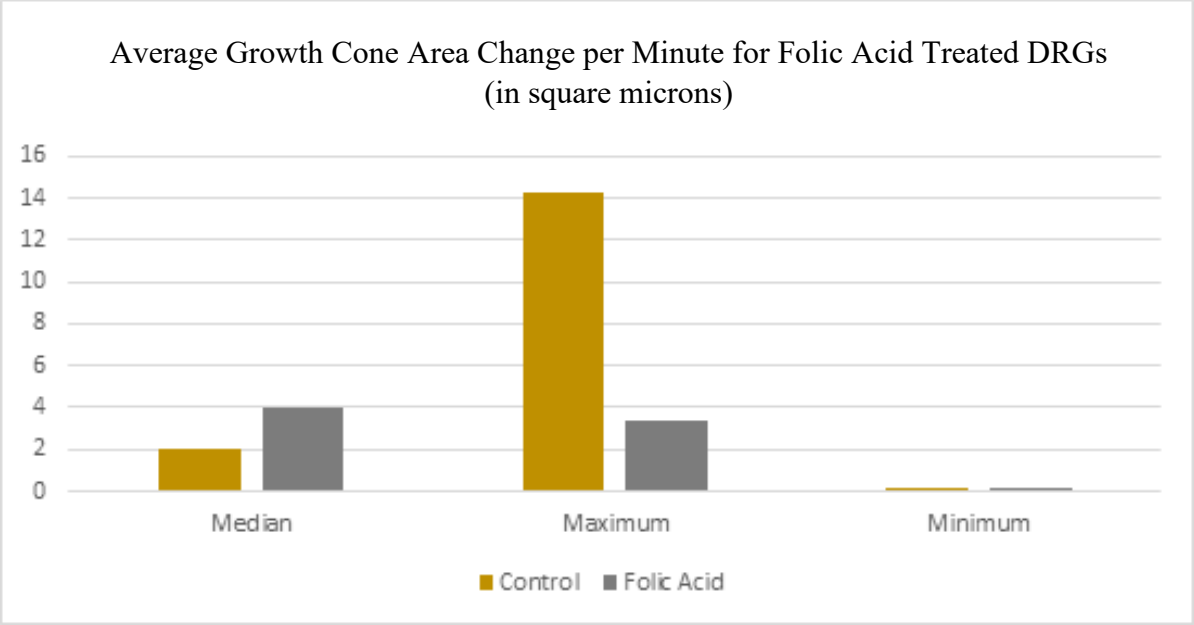

Table 6. Average Growth Cone Area Change per Minute for Folic Acid Treated DRGs. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n".

The same tests were run with GA treated DRGs. Table 7 shows that GA did not affect dynamic area change of the growth cones.

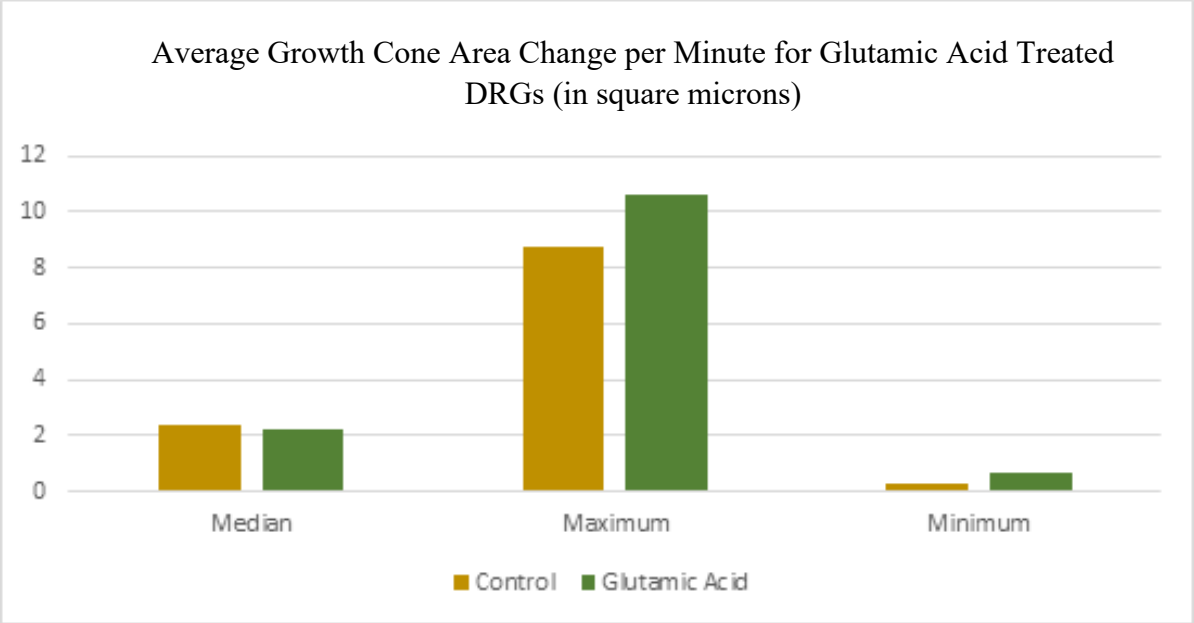

Table 7. Average Growth Cone Area Change per Minute for Glutamic Acid Treated DRGs. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n".

The same tests were run once more for the combined FA and GA treated DRGs. Table 8 illustrates the fact that, when combined, GA and FA did not affect dynamic area change of the growth cones.

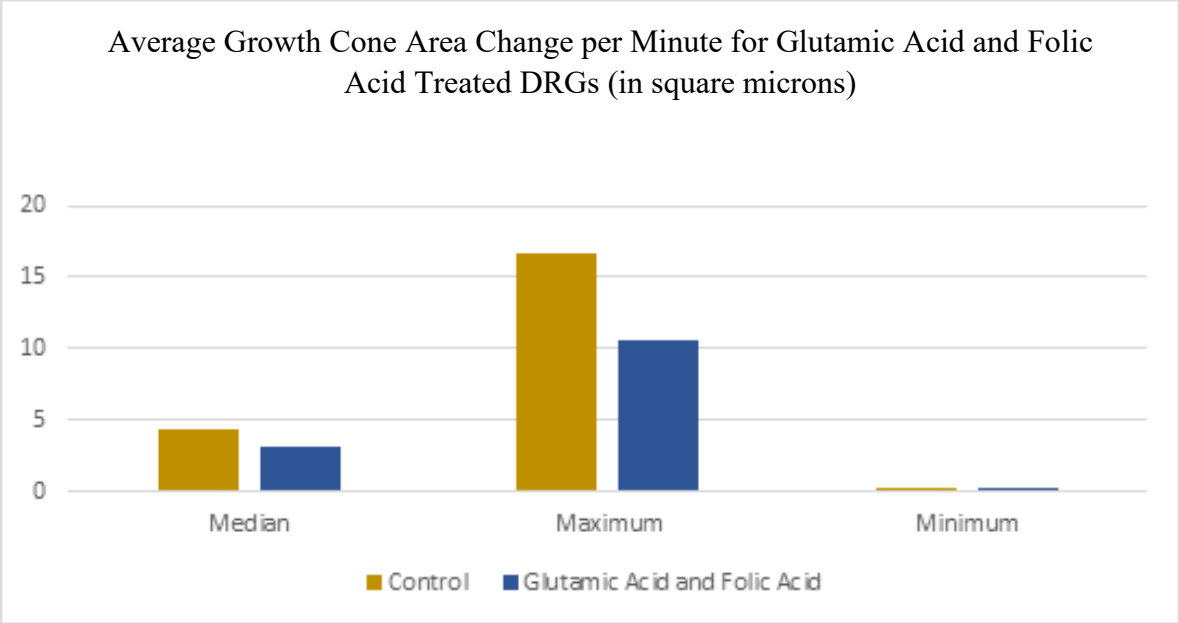

Table 8. Average Growth Cone Area Change per Minute for Glutamic Acid and Folic Acid Treated DRGs. Compared utilizing t-tests with an $\alpha=0.05$ used to determine significance. The number of samples are indicated by "n".

Discussion

This study of neurogenesis, has described a developmental process *in vitro* encompassing initiation and outgrowth of neurites, and differentiation of synaptic networks as described previously (Wiens et al., 2016). In addition, the assembly of neurites and glial cells into nerve bundles has been described. This last event occurs between 36 and 48 hours. Results for the effect of FA on neurite length, dynamic length activity, and growth cone dynamic activity did not show the same inhibitory effect found by Wiens et al. (2016). There was additionally found to be an inhibitory effect of both FA and GA on the number of neurites formed by the DRG neurons, whereas Wiens et al. (2016) did not report this effect. This is likely because the DRGs are no longer forming many new neurites, but instead bundling neurites into nerves which are growing longer.

It was found that that FA inhibited the development of synaptogenic areas, confirming some of the findings of Wiens et al. (2016), but this effect was only seen on the developmentally more advanced DRGs and the data was not reliable and robust enough to allow assessment in the less advanced DRGs. The contradictions were unexpected.

A likely explanation is that the DRG cultures in the previous study were less advanced. Inhibition due to FA was found when the measurements of developmental events were made after 36 hours in culture, while this study used DRGs that were in culture for 48 hours. Thus, sensitivity to FA may be high early as neurites are becoming established, but then diminishes as DRGs become more advanced. We also found differing effects of GA. Again, age and culture may have been important factors.

Concentrations of the supplements and in the medium were slightly different than in the previous study done by Wiens et al. (2016) which could have caused an unforeseen difference in results. First of all, the fetal bovine serum supplementation used in the medium was 10% rather than the 12% that we used. Another difference was that higher concentrations of both FA and GA were used than the amounts used in previous studies. The different concentrations could have affected the NMDA-R differently. Future studies will have to take all of these factors into account.

Future studies should be done to determine the effect of FA on DRGs of different culture times or stages of development. DRGs could be cultured for differing periods of time and separated depending upon the appearance of nerves. This would serve to determine specific effects of FA at different stages of development.

Additional research was done utilizing human M-17 neuroblastoma cells in culture. They were tested in similar ways to those stated previously. The cells were placed in culture and had retinoic acid added to begin neurite growth. Control dishes and dishes containing neurons treated with 5 μM FA were cultured for 48 hours. Then, the dishes were immunostained and neurite numbers, neurite lengths, and synaptogenic areas were calculated for each dish. It was found that while synaptogenic area and neurite lengths were unchanged, the average number of neurites increased by a significant amount. This differs from Wiens et al.'s previous findings with chick embryo DRGs. The difference may be due to the glial cells and fibroblasts that are present in the chick embryo DRGs but lacking in the human neuron cultures. Additionally, the chick DRGs have a more 3-dimensional morphology that the human neuron cultures do not. Overall, the M-17 data shows that over growth, or too many neural connections may be occurring. This contradicts previous findings by Wiens et al. (2016) as well as the results from the rest of this

study. However, it still supports the idea that excessive FA supplementation may lead to autism. A difference in the connectivity in the brain, be it too many connections or too few, still suggests that the transmission of signals will be different. Any type of abnormal connectivity in the brain may still lead to ASD in that individual.

Conclusion

Autism spectrum disorder has a higher prevalence in society today and despite research being conducted, the specific differences in the brain as well as the underlying causes are still not fully known. One potential cause for ASD is underconnectivity in the brain. This study set out to investigate the potential inhibitory effects of FA on neural growth and connectivity, which could lead to underconnectivity. Competition between FA with already present GA at the NMDA-R was the suggested mechanism for FA's inhibitory effects. This study did not reveal evidence of competition between FA and GA. Furthermore, this study did not find the inhibitory effects of FA that were previously found, except in a few cases. The unexpected disparity between this study and the previous study done by Wiens et al. (2016) was that the DRGs were found to be affected by FA and GA differently, depending upon the presence of nerves, which indicate further developmental advancement. This difference could be a reason why studies have found divergent effects of FA. While many studies may find inhibition, some find no effect, and others find promotion of growth. Future studies could look into the variable effects of FA that may depend upon the developmental advancement of the neural growth. These studies should work to determine what amount of FA is needed to change DRGs and how FA effects DRGs at successive developmental stages.

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Appendix

Immunostained neurite length raw data

	Bundled Control Neurite Lengths	
	Average Length	Count
	63.76232441	49
	99.62322202	24
	143.210275	34
	114.0322487	53
	141.2416954	21
	56.53765587	43
	55.13801278	36
	87.65188803	34
	123.2803868	72
	183.9807514	28
	239.9909748	24
	193.2587611	27
	156.2176484	20
	128.6505875	18
Average	**127.6126023**	**34.5**
	n=14	**n=14**

	Unbundled Control Neurite Lengths	
	Average Length	Count
	89.93110365	37
	94.88011308	21
	59.68297774	26
	88.85632225	13
	96.31457431	18
	72.83011583	37
	109.3020194	13
Average	**87.39960375**	**23.57142857**
	n=7	**n=7**

	Bundled FA Neurite Lengths	
	Average Length	Count
	164.130334	12
	72.96669759	5
	111.4047619	12
	138.1496036	11
	113.0966659	17
	108.0332042	17
	91.98811902	13
	148.1769282	35
	232.4153424	23
	53.30291231	38
	88.60980983	36
	153.5029232	43
	245.2808442	8
	130.3446025	27
	94.42594844	29
	60.76758307	11
	104.1294726	14
	176.4650278	10
	168.4117579	8
	174.9375205	17
	226.105102	5
	200.0412801	3
	102.01921	12
Average	137.3350283	17.652174
	n=23	n=23

	Unbundled FA Neurite Lengths	
	Average Length	Count
	80.51366166	22
	68.89169759	10
	73.25742115	6
	77.06794991	24
	102.1979489	9
	227.6660998	9
Average	104.9324632	13.33333333
	n=6	n=6

	Bundled GA Neurite Length	
	Average Length	Count
	120.2466441	34
	68.22776747	15
	119.6716379	39
	101.6289867	21
	78.03980435	11
	77.79649532	41
	43.2436705	66
	51.53016775	24
	54.55125232	22
	62.30888939	29
	126.8815598	14
	103.9255688	19
	155.3432282	36
	142.966141	25
	177.6769149	14
	166.8889147	32
	136.9984569	52
	176.2863459	47
	155.9827226	24
	96.68445617	32
Average	**110.8439812**	**29.85**
	n=20	**n=20**

	Unbundled	
	Average Leng	Count
	78.53293135	12
	162.0025332	13
	70.42709077	26
	73.51049924	11
	116.0017007	9
	83.0403525	4
	62.38218924	6
	95.7648423	2
	79.93904055	7
	70.19423596	22
	186.7067486	8
	160.7733844	12
	56.76188544	8
Average	**99.69518725**	**10.7692**
	n=13	**n=13**

	Bundled	
	Average Length	Count
	68.71474954	17
	80.93039104	26
	80.97642239	18
	59.93380813	31
	70.19925088	53
	82.89298966	42
	133.9562152	10
	112.2236076	51
	161.7334943	29
	142.0369821	30
	104.4699907	10
	103.4156153	30
	71.19697494	59
	207.0323129	33
	142.1873712	36
	81.73593776	22
	140.1378031	29
	208.0563212	7
	286.5645292	32
	243.8563312	20
	238.0310761	11
	178.8377694	13
	244.1343186	22
	157.9908501	11
Average	**141.7185464**	**26.75**
	n=24	**n=24**

	Unbundled	
	Average Length	Count
	36.80556313	34
	49.05951192	13
	198.9437616	4
Average	**94.93627888**	**17**
	n=3	**n=3**

Immunostaining Synaptogenic Area Raw Data

Control DRG with Neurites	Control DRG with Nerves and Neurites
201.9692752	3137.223563
211.1823844	28239.50765
204.4177053	15637.60013
1508.218616	6355.060055
1777.850343	9447.351686
253.232738	1057.355292
81.74714287	8365.406019
16072.03007	15826.43465
	57131.72027
	37113.79053
	18618.52931
	14939.98645
	28129.75892

Folic Acid Treated DRGS with Neurites	Folic Acid Treated DRGs with Nerves and Neurites
669.7533068	9875.758753
14380.31473	281.6841886
98.48247187	82.21883733
2499.47873	5588.342538
8527.877961	14398.07157
528.981879	54097.7912
3128.8073	29987.99967
	2412.661681
	3902.733482
	14856.58332
	6237.013704
	67982.35142
	22962.62872
	17324.7284
	43820.46623
	9938.61479
	11239.59118
	29410.34581
	13181.45222
	9716.158888
	7534.786946
	38308.20104
	16755.46268
	2443.264887

Glutamic Acid Treated with Neurites	Glutamic Acid Treated with Neurites and Nerves
3.836516092	15.28694366
6.281415682	1694.498053
12.29972078	34.06453284
68.06161674	36.53631673
12.14215851	487.05472
234.0773325	297.8600973
45.00269737	123.0539087
156.1616685	43.29704836
559.1587413	712.2078483
273.4291611	194.0565375
2579.540161	709.6914747
364.1158035	772.4480714
706.0071507	172.9008885
396.5746219	282.0047089
255.3931002	963.169488
	5546.009171
	2721.604136
	731.3416549
	933.6153552
	4764.564214
	598.3730974

Folic Acid and Glutamic Acid Treated DRGs with Neurites	Folic Acid and Glutamic Acid Treated DRGs with Nerves and Neurites
4579.553569	3463.250469
1891.590145	5237.653801
14093.15075	15907.55981
	13057.65161
	5825.848485
	8744.485205
	1345.160347
	2037.238223
	2183.398393
	50894.06577
	3310.110513
	9387.600429
	33444.54508
	40048.19877
	8389.820998
	7351.774949
	10843.92633
	12442.57662
	4119.549228
	6071.672467
	13279.56223
	156671.8705
	41461.55764
	11078.28866
	129807.4629

Video Raw Data

Folic Acid Control						
EXP 1 (5-26)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	12.756	3.794	25.743	-0.182	1.217
	Standard Deviation	3.729	3.033	1.694	1.511	0.89
	Median	12.279	3.652	25.733	-0.457	0.935
	Maximum	23.13	14.45	28.513	2.944	2.995
	Minimum	7.357	0	21.116	-2.995	0.022
EXP2 (6-16 1)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	2.417	0.605	26.983	-0.671	1.218
	Standard Deviation	1.385	0.581	5.114	1.74	1.394
	Median	1.957	0.346	29.13	-0.64	0.778
	Maximum	5.765	1.744	34.868	1.692	6.403
	Minimum	0.799	0.013	18.877	-6.403	0.049
EXP3 (6-16 2)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	205.508	0.336	29.037	0.11	0.742
	Standard Deviation	3.284	0.446	1.657	0.918	0.527
	Median	205.333	0.146	29.296	0.144	0.768
	Maximum	212.667	1.624	32.331	1.596	1.797
	Minimum	198.333	0	26.216	-1.797	0.052

Folic Acid Treated					
EXP 1 (5-26)		ABS GC area diff	NL	NL diff	ABS NL diff
		3.314	21.584	-0.157	1.629
	Average	3.058	1.99	2.146	1.372
	Standard Deviation	2.462	22.067	0.222	1.352
	Median	11.539	24.096	4.601	5.53
	Maximum	0.265	15.504	-5.53	0.203
	Net Change				
EXP2 (6-16 1)		ABS GC area diff	NL	NL diff	ABS NL diff
		0.383	14.75	-0.706	1.323
	Average	0.327	3.807	1.874	1.48
	Standard Deviation	0.293	14.598	-0.295	0.834
	Median	0.945	21.109	1.827	5.746
	Maximum	0.04	7.345	-5.746	0.009
	Net Change				
EXP3 (6-16 2)		ABS GC area diff	NL	NL diff	ABS NL diff
		0.226	31.835	-0.014	1.271
	Average	0.189	1.398	1.667	1.035
	Standard Deviation	0.213	31.789	0.011	1.1
	Median	0.785	36.276	2.765	3.834
	Maximum	0.013	29.782	-3.834	0.011

Glutamic Acid Control						
EXP 1 (6-16)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	163.862	0.655	64.495	-0.476	1.206
	Standard Deviation	5.973	0.558	2.486	1.487	0.960
	Median	164.073	0.612	63.822	-0.545	0.915
	Maximum	174.000	2.449	71.162	1.841	4.161
	Minimum	154.667	0.054	61.346	-4.161	0.095
EXP2 (6-21)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	16.733	5.608	189.694	0.273	2.220
	Standard Deviation	6.622	4.569	2.429	3.262	2.354
	Median	15.508	4.975	189.6	0.359	1.278
	Maximum	32.445	14.344	194.173	8.428	8.428
	Minimum	8.204	0.529	185.738	-6.608	0.005
EXP3 (6-29)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	8.777	2.143	61.723	0.232	0.860
	Standard Deviation	3.563	1.486	1.294	0.962	0.451
	Median	8.305	1.554	61.921	0.490	0.790
	Maximum	16.985	4.447	63.340	1.601	1.601
	Minimum	4.126	0.267	58.783	-1.591	0.088
EXP 4 (7-5 1)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.101	3.897	79.127	-0.020	2.612
	Standard Deviation	3.522	3.694	3.723	3.170	1.693
	Median	12.015	2.699	78.954	0.895	2.458

	Maximum	20.007	12.226	86.390	4.519	5.895
	Minimum	7.780	0.106	72.370	-5.895	0.106
EXP 5 (7-5 2)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.461	3.154	61.000	0.325	1.940
	Standard Deviation	2.718	2.690	4.407	2.893	2.125
	Median	13.232	2.170	59.387	-0.112	1.338
	Maximum	20.430	10.956	72.498	9.584	9.584
	Minimum	9.368	0.635	56.704	-3.423	0.040
EXP 5 (7-5 3)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	9.911	2.586	32.206	-0.103	1.187
	Standard Deviation	3.162	2.007	1.475	2.304	1.959
	Median	9.369	2.223	31.907	0.188	0.494
	Maximum	15.984	8.310	36.892	5.455	7.833
	Minimum	4.711	0.000	29.059	-7.833	0.083

Glutamic Acid Treatment						
EXP 1 (6-16)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	169.306	0.741	60.892	-0.090	0.838
	Standard Deviation	2.638	0.623	1.442	1.072	0.656
	Median	170.210	0.413	60.679	-0.077	0.643
	Maximum	173.623	2.077	63.237	1.730	2.611
	Minimum	164.233	0.027	57.934	-2.611	0.048
EXP2 (6-21)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	10.093	3.339	188.574	-1.676	2.986
	Standard Deviation	4.242	2.086	7.479	4.070	3.154

	Median	9.315	3.017	189.908	-0.429	1.823
	Maximum	18.260	7.251	196.563	3.428	11.120
	Minimum	4.287	0.635	173.461	-11.120	0.036
EXP3 (6-29)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	14.914	2.976	62.651	-0.095	1.086
	Standard Deviation	5.873	2.410	1.318	1.679	1.260
	Median	15.003	2.518	62.446	-0.206	0.712
	Maximum	25.773	9.323	66.563	4.944	4.944
	Minimum	5.412	0.000	60.992	-4.021	0.185
EXP 4 (7-5 1)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	18.644	6.691	68.504	-0.847	2.496
	Standard Deviation	6.851	5.411	8.917	4.090	3.305
	Median	15.773	2.752	62.955	0.247	0.856
	Maximum	32.233	14.132	86.436	6.098	11.801
	Minimum	12.862	2.170	61.324	-11.801	0.185
EXP 5 (7-5 2)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.461	3.154	65.755	-0.164	2.477
	Standard Deviation	2.718	2.690	3.128	3.093	1.771
	Median	13.232	2.170	65.715	0.463	2.057
	Maximum	20.430	10.956	72.256	4.226	6.113
	Minimum	9.368	0.635	59.500	-6.113	0.151
EXP 5 (7-5 3)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	15.395	4.229	32.796	0.135	2.336
	Standard Deviation	7.326	4.663	3.630	3.524	2.586
	Median	13.867	2.673	32.016	0.066	1.171
	Maximum	39.643	19.954	41.983	9.284	9.284
	Minimum	6.828	0.318	28.172	-7.142	0.053

Glutamic Acid and Folic Acid Control						
EXP 1 (6-16)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	163.862	0.655	64.495	-0.476	1.206
	Standard Deviation	5.973	0.558	2.486	1.487	0.960
	Median	164.073	0.612	63.822	-0.545	0.915
	Maximum	174.000	2.449	71.162	1.841	4.161
	Minimum	154.667	0.054	61.346	-4.161	0.095
EXP2 (6-21)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	16.733	5.608	189.694	0.273	2.220
	Standard Deviation	6.622	4.569	2.429	3.262	2.354
	Median	15.508	4.975	189.600	0.359	1.278
	Maximum	32.445	14.344	194.173	8.428	8.428
	Minimum	8.204	0.529	185.738	-6.608	0.005
EXP3 (6-29)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	8.777	2.143	61.723	0.232	0.860
	Standard Deviation	3.563	1.486	1.294	0.962	0.451
	Median	8.305	1.554	61.921	0.490	0.790
	Maximum	16.985	4.447	63.340	1.601	1.601
	Minimum	4.126	0.267	58.783	-1.591	0.088
EXP 4 (7-5 1)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.101	3.897	79.127	-0.020	2.612
	Standard Deviation	3.522	3.694	3.723	3.170	1.693
	Median	12.015	2.699	78.954	0.895	2.458

	Maximum	20.007	12.226	86.390	4.519	5.895
	Minimum	7.780	0.106	72.370	-5.895	0.106
EXP 5 (7-5 2)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.461	3.154	61.000	0.325	1.940
	Standard Deviation	2.718	2.690	4.407	2.893	2.125
	Median	13.232	2.170	59.387	-0.112	1.338
	Maximum	20.430	10.956	72.498	9.584	9.584
	Minimum	9.368	0.635	56.704	-3.423	0.040
EXP 6 (7-5 3)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	9.911	2.586	32.206	-0.103	1.187
	Standard Deviation	3.162	2.007	1.475	2.304	1.959
	Median	9.369	2.223	31.907	0.188	0.494
	Maximum	15.984	8.310	36.892	5.455	7.833
	Minimum	4.711	0.000	29.059	-7.833	0.083
EXP 8 (6-29 2)						
		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.915	2.114	35.312	-0.421	3.102
	Standard Deviation	1.995	1.681	4.366	3.880	2.261
	Median	13.583	1.662	36.377	-0.929	2.230
	Maximum	18.754	5.412	40.688	7.265	8.275
	Minimum	10.234	0.160	27.940	-8.275	0.684

Glutamic Acid and Folic Acid Treatment						
EXP 1 (6-16)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	169.306	0.741	60.892	-0.090	0.838
	Standard Deviation	2.638	0.623	1.442	1.072	0.656
	Median	170.210	0.413	60.679	-0.077	0.643
	Maximum	173.623	2.077	63.237	1.730	2.611
	Minimum	164.233	0.027	57.934	-2.611	0.048
EXP2 (6-21)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	10.093	3.339	188.574	-1.676	2.986
	Standard Deviation	4.242	2.086	7.479	4.070	3.154
	Median	9.315	3.017	189.908	-0.429	1.823
	Maximum	18.260	7.251	196.563	3.428	11.120
	Minimum	4.287	0.635	173.461	-11.120	0.036
EXP3 (6-29)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	14.914	2.976	62.651	-0.095	1.086
	Standard Deviation	5.873	2.410	1.318	1.679	1.260
	Median	15.003	2.518	62.446	-0.206	0.712
	Maximum	25.773	9.323	66.563	4.944	4.944
	Minimum	5.412	0.000	60.992	-4.021	0.185
EXP 4 (7-5 1)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	18.644	6.691	68.504	-0.847	2.496
	Standard Deviation	6.851	5.411	8.917	4.090	3.305
	Median	15.773	2.752	62.955	0.247	0.856
	Maximum	32.233	14.132	86.436	6.098	11.801
	Minimum	12.862	2.170	61.324	-11.801	0.185
EXP 5 (7-5 2)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	13.461	3.154	65.755	-0.164	2.477
	Standard Deviation	2.718	2.690	3.128	3.093	1.771

	Median	13.232	2.170	65.715	0.463	2.057
	Maximum	20.430	10.956	72.256	4.226	6.113
	Minimum	9.368	0.635	59.500	-6.113	0.151
EXP 6 (7-5 3)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	15.395	4.229	32.796	0.135	2.336
	Standard Deviation	7.326	4.663	3.630	3.524	2.586
	Median	13.867	2.673	32.016	0.066	1.171
	Maximum	39.643	19.954	41.983	9.284	9.284
	Minimum	6.828	0.318	28.172	-7.142	0.053
EXP 8 (6-29 2)		GC area	ABS GC area diff	NL	NL diff	ABS NL diff
	Average	9.902	1.827	34.076	0.171	3.928
	Standard Deviation	1.765	1.345	7.245	5.896	4.307
	Median	9.698	1.287	32.069	-0.882	2.023
	Maximum	12.377	5.090	50.308	15.578	15.578
	Minimum	6.751	0.215	25.926	-11.597	0.454