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Excess folic acid as a potential competitor of glutamate may interfere with neural development

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EXCESS FOLIC ACID AS A POTENTIAL COMPETITOR OF GLUTAMATE MAY INTERFERE WITH NEURAL DEVELOPMENT

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Abstract

Folic acid, Vitamin B9, is strongly advised as a supplement taken by pregnant woman to maintain the health of the embryo, and deficiency increases the risk of neural tube defects. However, a safe upper limit of folate to consume has not been established, and an excess of dietary folate may interfere with neurodevelopmental metabolism, increasing the risk of adverse outcomes, including autism spectrum disorder (ASD). It has been suggested that folate affects connectivity among neurons as the brain develops. Glutamate is important in the regulation of neural tissue development, as it is a common excitatory neurotransmitter that binds to synaptic membranes. Because it is so structurally similar to folic acid, it may compete for binding sites on neurons within developing tissues, affecting connectivity of neurons during embryonic brain development. This experiment tested the effects of adding excess folate, glutamate or both to cultures of developing neural tissue to determine whether embryonic neuronal behavior is altered. An inverted phase microscope with a heated stage was used to collect time-lapse images of a region of extending neurites with growth cones. After 30 images (one per minute), a known concentration of folate, glutamate or both was added to the dish and 30 more images were collected. Images were analyzed using *ImageJ* software, and alterations in exploratory behavior of the neurites with growth cones were recorded. The data suggests that excess glutamate can overcome an inhibition of area change per minute by excess folate, which authenticates a mechanism of inhibition of neural development by excess folate. This supports the hypothesis of underconnectivity during brain development leading to ASD.

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INTRODUCTION

Purpose

Folic acid, also known as folate or Vitamin B9, is strongly advised as a supplement taken by pregnant woman to maintain the health of a developing child. A safe upper limit of folate to consume has not been considered, and an excess in dietary folate may increase the risk of the child to develop autism spectrum disorder. Glutamate is a molecule very similar in structure to folic acid, and the two may be in direct competition for binding sites on neurons within developing neural tissue. Glutamate is important in the regulation of neural tissue development, as it is a common excitatory neurotransmitter. The purpose of this experiment was to test the effects of the addition of folate and glutamate to cultures of neural tissue, and draw a conclusion on these effects as well as the competition between folate and glutamate. Excess folic acid has been shown to decrease neural activity leading to underconnectivity among neurons, but addition of glutamate may overcome this inhibition. Data that supports this authenticates the mechanism of inhibition of neural development by excess folate. All of this taken together supports the hypothesis that underconnectivity in developing neurons can lead to an increased risk in developing autism spectrum disorder (ASD). Discovering potential causes of ASD now will help prevent it in the future.

Dorsal Root Ganglia, Neurites, Growth Cones, and Synapses

It is necessary to understand the basics of neural tissue to understand this experiment and how exactly it works. In living organisms, ganglia are defined as clusters of neuron cell bodies in the peripheral nervous system. These neural cell bodies have long dendrites on one end that receive information, and an axon on the other end that transmits information. Dorsal root ganglia (DRG) are an example of ganglia that exist in the nervous system. They bring information from

the periphery, to the spinal cord as displayed in figure 1. In developing neural tissue such as DRGs, this complex organization must be formed. The developing neuron cell bodies produce long thin processes, called neurites, for exploration. At the end of these neurites are oval shaped growth cones. These growth cones have many microspikes that protrude to explore the area, and look for sites to innervate. The neurite that innervates that correct spot becomes the axon of the neuron. These properties allow for observations to be made on the effects of certain substances on the developing neural tissue. The experiments described here utilized DRGs for this reason.

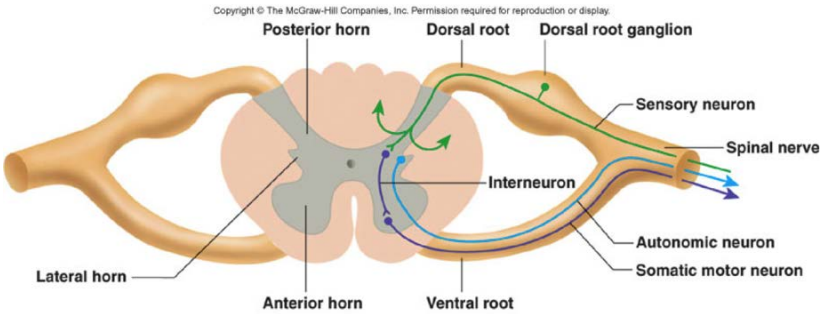

Figure 1 – Dorsal Root Ganglia and Spinal Cord

The neurite that innervates the proper target forms a synapse. This is shown in figure 2. At the synapse, there is a pre-synaptic membrane of the neurite, and a post-synaptic membrane of the target cell. Additionally, there is a space between the two. This is called the synaptic cleft. It is in this cleft that the signal is transmitted to the post synaptic membrane by neurotransmitters, or chemical signals. The neurotransmitter travels from the pre-synaptic membrane to the post-synaptic membrane where it binds to receptors that cause a change in the target cell. This is where the addition of folate and glutamate to the cultures of DRGs took effect. The neurites that extended outward from the DRG created synapses in culture which were sensitive to an inhibitory influence of folate (Wiens et al. 2016).

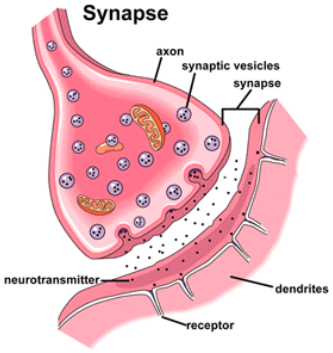

Figure 2- A Common Synapse

LITERATURE REVIEW

Folic Acid Supplementation Trials

Many experiments involving folic acid supplementation for pregnant woman have been conducted in the past 40 years. The substance has been shown to reduce neural tube defects in the developing fetus. As summarized by Czeizel et al. (2013), the brain and spinal cord both develop from the neural tube in a developing embryo. The neural tube is simply the infolding of the neural plate, making a hollow tube. A defect in this process leads to either anencephaly, or spina bifida. Anencephaly occurs when the neural tube fails to close in the cranial region, and spina bifida is a result of the neural tube failure to close elsewhere along the back (Czeizel et al., 2013). The medical research council in the United Kingdom first tested the effects of folic acid on developing embryos. They found that a simple pharmacological dose of folic acid reduced neural tube defect occurrence by 71%. Subsequent trials all showed that defects in neural tube closure can be prevented by folic acid supplementation (Smithells et al., 1983).

Smithells et al. (1981) also performed a study on folic acid supplementation and its effects on neural tube defects. Their study included 550 mothers who had previous children with neural tube defects. They each took periconceptional (period from before conception, to early pregnancy) vitamin supplements, or acted as control meaning no supplements were taken. Two hundred mothers were fully supplemented, 50 were partially supplemented, and 300 were un-supplemented. It was found that neural tube defect defects occurred in 0.5% of the mothers that were fully supplemented, none in mothers that were partially supplemented, and 4% in mothers that were un-supplemented.

In a similar experiment, Schorah et al. (1982) tested vitamin (including folic acid) blood concentration levels in woman that were taking supplements. The women were either at a high or

low risk for bearing children with neural tube defects. They also tested the effects of this vitamin supplementation on the probability of neural tube defects recurring in successive children. The results of the study showed a neural tube defect recurrence in 13 of 308 fetuses in the unsupplemented high-risk group, and only one recurrence in 196 of the fully supplemented high-risk group. Additionally, the mothers taking supplements had elevated levels of blood vitamins meaning the mothers were indeed absorbing the supplements and they could affect the developing fetuses.

Yet another study conducted by Milunsky et al. (1989) revealed similar results. Information from 22,776 pregnant women about multivitamin use was obtained. They found that neural tube defects occurred in 3.5 fetuses per 1,000 mothers that never used multivitamins before or after conception, or that used multivitamins solely before conception. In contrast, neural tube defects occurred in 0.9 fetuses per 1,000 mothers that took folic acid containing multivitamins during the first 6 weeks of pregnancy. It is clear that folic acid is important early in pregnancy, as early events in development are affected by the presence or absence of folate. These early events are heavily dependent on proper cell division, which is aided by folate.

Metabolic Importance of Folic Acid

Folate has multiple important roles in nucleotide synthesis and repair. It plays a role in epigenetic control of gene expression as it is involved in methylation of cytosine bases at specific areas. It also provides methylation groups for the synthesis of thymidine, which is necessary for DNA construction. These functions are necessary for proper cell division, and closure of the neural tube may depend on adequate cell division, timed adhesion, or cell shape changes.

Beginning in 1998, the United States government mandated a folic acid fortification program as summarized by Choi et al. (2014). This program quickly spread to other countries

such as Canada, Chile, and Australia. Fortification of food with folic acid was preferable over supplementation as it is low risk. Cereals and flour are the two main food components targeted for fortification as they are consumed regularly by woman of childbearing age.

Folic acid and Autism Spectrum Disorder

Interestingly, maternal plasma levels of folate may be related to the risk of developing autism spectrum disorder (ASD) in children. Surén et al. (2013), performed a study focusing on the effects of folic acid on developing children. Their study included 85,176 mothers who participated by taking supplements of folic acid from four weeks before, to eight weeks after pregnancy. It was found that in children whose mothers took folic acid, 0.10% had ASD. In children with mothers unexposed to folic acid, 0.21% had ASD.

However, in contrast to these findings, DeSoto and Hitlan (2012) report that mothers taking folic acid supplements during pregnancy were more likely to have children with ASD. As stated above, folic acid could be involved in the epigenetic control of the functioning of certain genes by providing methyl groups for methylation and thereby altering expression. Because getting enough dietary folic acid is heavily stressed, and foods are fortified with the substance, mothers typically circulate high levels of unmetabolized folic acid in their system. This excess can actually negatively impact the functioning of hundreds of genes, therefore raising questions about the safety of folic acid supplements. Moreover, Barua et al. (2014) have shown that folic acid also can alter brain structure and function through epigenetics. This means that there could be a definite consequence to high amounts of dietary folic acid for pregnant mothers.

Ebert and Greenberg (2013) have noted that ASD may be a result of the “dysregulation of synaptic function in general, impairment of neurotransmission, and defects in earlier steps in

nervous system development." The human brain is capable of synaptic plasticity, which is the ability to remodel itself as certain synapses are more active than others (activity dependent signaling). Synapses strengthen and weaken over time in response to varying activity, correlating with learning, memory, mental states and development. Ebert and Greenberg (2013) summarize this saying "neuronal activity triggers local changes at the synapse altering the composition, shape and strength of the synapse." It is clear then that dysregulation of this process can result in ASD. Activity dependent signaling and activity dependent synapse development and maturation can be disrupted by alterations in neurotransmission. These alterations can come from mutations of natural physiology, or in this case, the addition of folate and glutamate to neural tissue. It is also true that many of the proteins associated with autism spectrum disorder are regulated by neuronal activity. It makes sense then that disruption of these molecular programs can negatively influence synaptic functioning, theoretically leading to an increased risk of developing ASD.

Folic Acid and Glutamate Binding

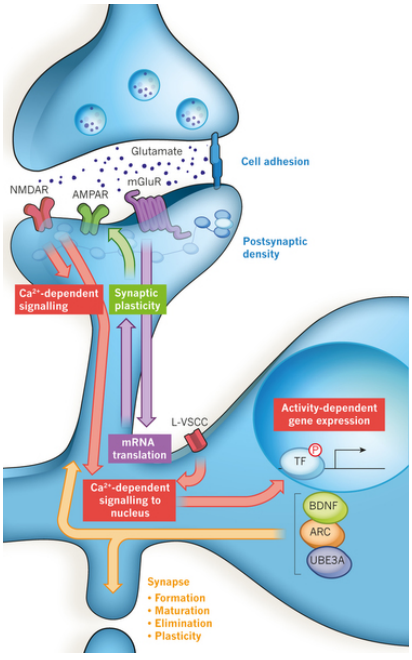

Figure 3 – NMDA and AMPA Activation by Glutamate (Ebert and Greenberg, 2013)

The receptors N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxaxolepropionic acid (AMPA) are abundant on the post-synaptic membrane in developing synapses. Glutamate binds to both of these receptors, resulting in depolarization of the membrane. This is an excitatory response. NMDA activation by glutamate also fluxes calcium, which activates a series of intracellular signaling pathways to stimulate gene transcription in the nucleus. This can cause the synapse to become more sensitive to future stimulation. This is how neural tissue is able to respond to different signals, and it is an example of synaptic

plasticity. This is shown in figure 3. Developing neurites and growth cones have the same receptors as do fully formed synapse as described above. Clearly then, alterations in the levels of glutamate and folate, and binding competition between them could affect connectivity development in the embryonic brain.

Previous Lab Work

Previously in this lab, high levels of folate added to neural cultures resulted in a reduction in vital parameters of the neurite and growth cone behavior that establishes neural connectivity (Wiens et al., 2016). Growth cone dynamic area change, synapse formation, and net advance distance were all reduced with the addition of folate in cultured developing neurons. The experiment described in this paper is an extension of these findings, as well as a test of the effects of competition between the binding of folate and glutamate. Additionally, Wiens et al. (2016) discovered the minimal concentration of folate that would have an effect on the neurons. A series of concentration from 0.25 uM to 20 uM was used to establish 5 uM as the minimum concentration that still had an effect on the dynamic growth cone area change and net advance distance of the neurite itself. This was the concentration utilized in the experiments described in this study.

HYPOTHESIS

It is well known that folate is a long molecule with glutamate, a common excitatory neurotransmitter, at one end. This led to the hypothesis that folate may compete with glutamate for binding to the glutamate receptor(s) at the postsynaptic membrane, and exert its effects on neural connectivity through the receptor in developing synapses. The effects of glutamate and folate on neurite outgrowth and growth cone behavior will indicate whether folate competes with glutamate, and provide information about how successful innervation and synaptic function will be. It may provide insight into the possible involvement of excess folate in the development of ASD. It is also hypothesized that glutamate can overcome the inhibitory effects of folic acid.

METHODOLOGY

Dissection

Dorsal root ganglia were the source of neural tissue for testing the effects of glutamate and folate. Dorsal root ganglia (DRGs), were dissected from 8-day-old chicks incubated at 38 degrees centigrade. The dissected chicks were bathed in Earle's Balanced Salt Solution (EBSS). DRGs are found along the spinal cord of the developing embryo. This is displayed in figure 4. The head and limbs were removed first, and then the remaining tissue covering the spinal cord was removed. The collected DRGs were placed in a separate dish containing fresh EBSS. They were then divided up into 35 mm Falcon Primaria tissue culture dishes with four or five in each dish bathed in 2 mL of medium 199 (Sigma-Aldrich Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (CELLect, Gold, MP Biomedicals, LLC) and 1% antibiotics/anitmycotics (BD Biochemicals). They were then incubated at 38 degrees centigrade for one to two days before being treated with folate and/or glutamate (Sigma-Aldrich Chemical Co., St. Louis, MO).

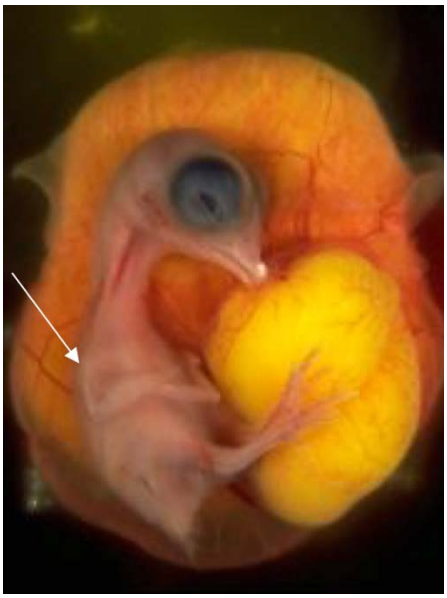

Figure 4 – 8 Day Old Chick Embryo (Hamburger and Hamilton, 1951)

Addition of Experimental Substances

As the DRGs attached to the bottom of the dishes, they started to extend neurites to explore the area. A clearly visible neurite with a growth cone on the end was found among the cluster of cells to analyze. A series of 60 images was captured using image Pro Premier software (Media Cybernetics, Inc., Lexington, MA) over the course of an hour using a Leica inverted phase/fluorescence microscope with a heated stage. The first 30 images served as a control to

view the general activity of the culture. After this, 2 μL of a stock solution of 2 mM folic acid (Sigma-Aldrich, St. Louis, MO) was added to the dish. The next 30 images were taken. This procedure was completed again on another dish containing a visible neurite with a growth cone. After the first 30 control images, 2 μL of a 2 mM solution of glutamic acid and 2 μL of the folate solution were added to the culture. The next 30 images were then taken.

Analysis

The series of images was analyzed using Image J software (Schneider et al. 2012). For each image, the area of the growth cone and the length of the neurite was found. This produced a set of results from which the change in area and change in length could be determined between successive pictures. The control images and experimental images were then compared. These calculations provided the data used to analyze the effects of glutamate and folate on neurite and growth cone development and behavior.

RESULTS

Wiens et al. (2016) showed that excess folate has an inhibitory effect on the outgrowth of neurites as well as dynamic growth cone area. These authors proposed the hypothesis that folate might do this by competing with the excitatory neurotransmitter glutamate for binding to developmentally important receptors such as the NMDA receptor (Wiens et al., 2016). To test this hypothesis, excess folate was combined in a culture dish with equivalent excess glutamate to see if it would override the inhibitory effects of the folate. This would result in either little change in the measured parameters of the neural tissue, or a possible excitatory effect. Similarly, addition of only excess folate to the culture dishes should result in an inhibitory effect, meaning dynamic growth cone behavior and neurite length should show a net decrease confirming (or not) the previous finding.

Addition of Both Folate and Glutamate

A total of 12 experiments were conducted, 8 of which were analyzable and provided results. The rest were either contaminated, or the DRG did not attach, providing no neurites or growth cones to work with. When folate and glutamate were added to the culture dish containing the DRG, it was found that there was not a significant change to the dynamic area change of the growth cone, nor to dynamic neurite length change. Table 1 contains the medians of the area change of the growth cone per minute as well as the length change of the neurite per minute for each experiment. The medians give a truer picture of the results than averages as the data was not normally distributed, and outliers significantly raise the average. (The empty boxes signify that no data available in that experiment.)

Table 1 – Medians of growth cone area change per minute and neurite length change per minute for 8 experiments

Experiment Number	Median Growth Cone Area Change (μm²/min) before (control) and after addition		% Increase (+) or Decrease (-) Following Treatment	Neurite Dynamic Length Change (μm/min)		% Increase (+) or Decrease (-) Following Treatment
	Control	Folate/ Glutamate		Control	Folate/ Glutamate	
1	1.69	0.72	-57%	2.85	1.47	-48%
2				2.48	3.21	+29%
3	0.43	0.48	+12	0.86	0.56	-35%
4	0.30	0.32	+7%	0.77	0.78	+1.3%
5	0.21	0.19	-10%	1.16	1.01	-13%
6	1.10	1.95	+77%	1.42	1.59	+12%
7	0.40	0.46	+15%	0.42	0.69	+64%
8	0.69	0.71	+3%	0.41	0.48	+17%
Overall			+47%			+27%

The data revealed that neither the median dynamic area change nor the median dynamic neurite length change was consistently altered by the addition of folate with glutamate. Table 1 shows that the results were variable. There were increases or decreases in growth cone area change activity after addition of folate with glutamate, depending on the experiment number (growth cone) analyzed. An increase of 77% in experiment 6 was contrasted with a decrease of 57% in experiment 1. Additionally, there were two large increases (29% and 64%), and two large decreases (49% and 44%) in neurite dynamic length change after the addition. If the amount of

absolute change (independent of whether it was increased or decreased) is calculated, it averages 23% for growth area change and 27% for length change on a per minute basis.

This data is represented graphically in a box plot combining all experiments shown below in figures 5 and 6. Figure 5 represents the growth cone area change per minute, and figure 6 represents neurite length change per minute.

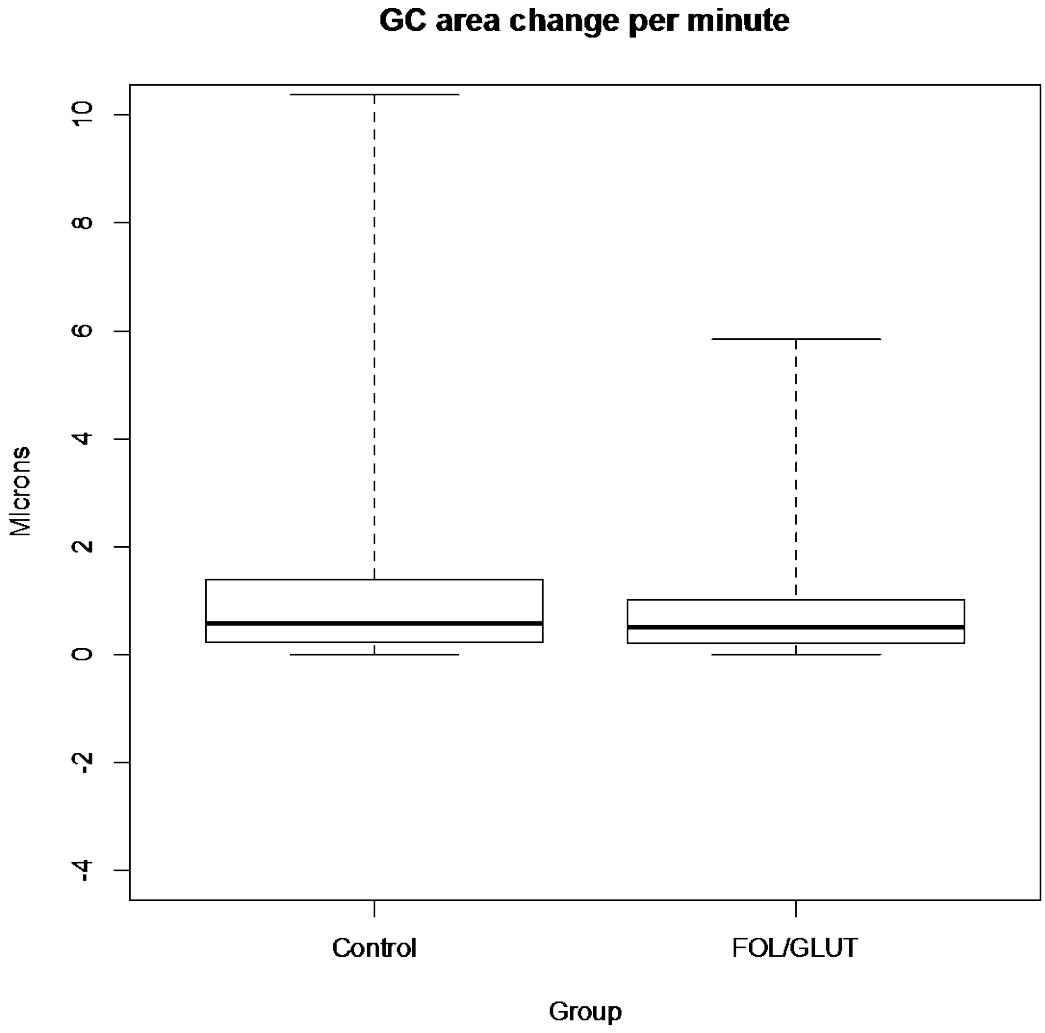

Figure 5 – Box plot of growth cone area change per minute before and after addition of folate and glutamate. All 8 experiments are represented. P-Value: 0.10

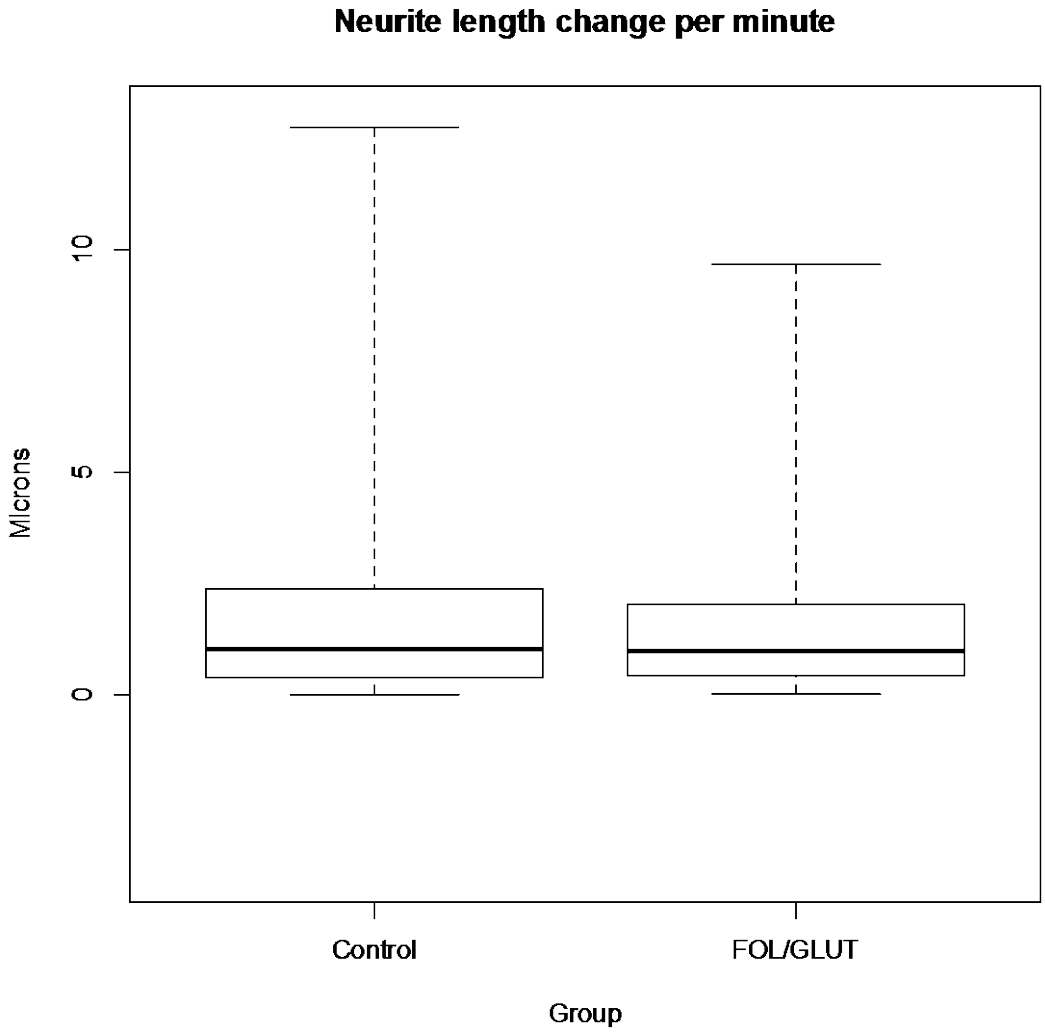

Figure 6 - Box plot of neurite length change per minute before and after addition of folate and glutamate. All 8 experiments are represented. P-Value: 0.64

Figure 7 parts A and B displays a neurite and its growth cone both before and after addition of folate and glutamate respectively. It is not possible to view the area change per minute with a single picture, but it can be seen that there is not much difference in growth cone size or neurite length before and after addition. This was consistent among all 8 experiments.

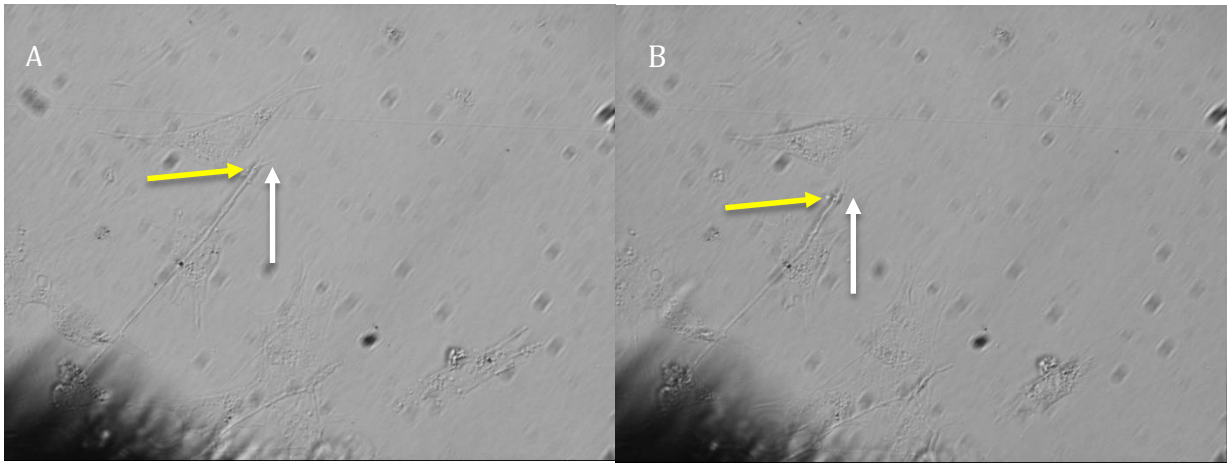

Figure 7- Growth cone and neurite before (A) and after (B) addition of folate and glutamate. Horizontal arrows point to the growth cone. Vertical arrows point to microspikes on the growth cone. Stability of the growth cone and neurite is displayed.

Addition of Folate

In addition to these eight experiments, four were also carried out to test the effects of only folate, in order to verify (or not) the inhibitory effects reported by Wiens et al. (2016). Table 2 displays results for each experiment.

Table 2- Medians of growth cone area change per minute and neurite length change per minute for 4 experiments

Experiment Number	Median Growth Cone Area Change (μm^2/min)		% Increase (+) or Decrease (-) Following Treatment	Neurite Length Change (μm/min)		% Increase (+) or Decrease (-) Following Treatment
	Control	Folate		Control	Folate	
1	1.12	0.96	-14%	0.77	0.96	+25%
2	0.73	0.85	+16%	1.03	1.20	+17%
3	0.59	0.42	-29%	0.81	0.85	+5%
4	0.28	0.14	-50%			
Overall			-77%			+47%

One large decrease in growth cone area change per minute of 50% was observed, along with two more moderate inhibitions. The remaining growth cone showed an unexpected increase. However overall, there was a combined 77% decrease for the four experiments following folate treatment. The neurite length changes per minute showed moderate increases in length changing activity. Perhaps folate does indeed have an inhibitory effect on growth cone area change per minute, but more tests would need to be conducted to confirm this.

Figures 8 and 9 display these results graphically as box plots. Figure 8 represents the data for growth cone area change per minute while figure 9 represents the data for neurite length change per minute.

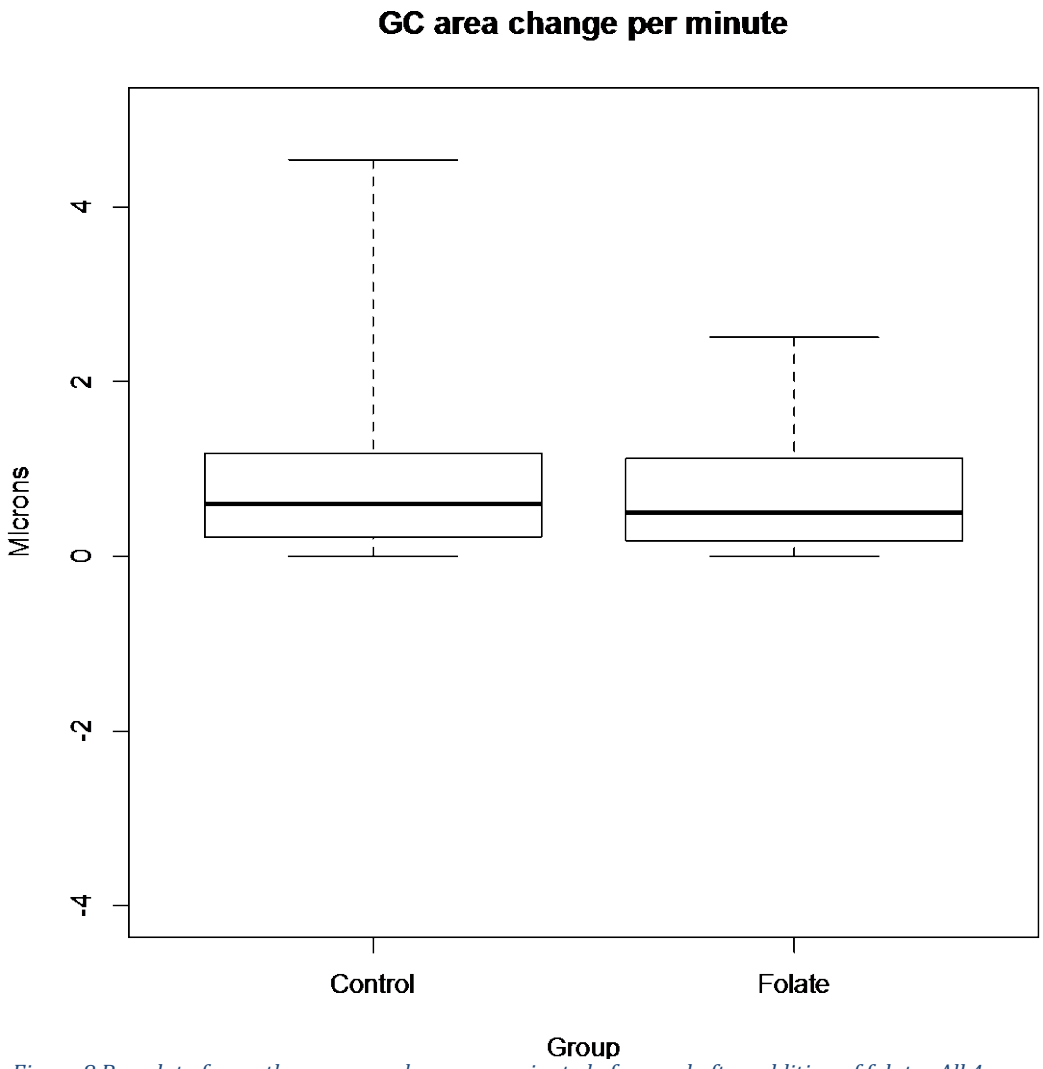

Figure 8 Box plot of growth cone area change per minute before and after addition of folate. All 4 experiments are represented. P-Value: 0.40

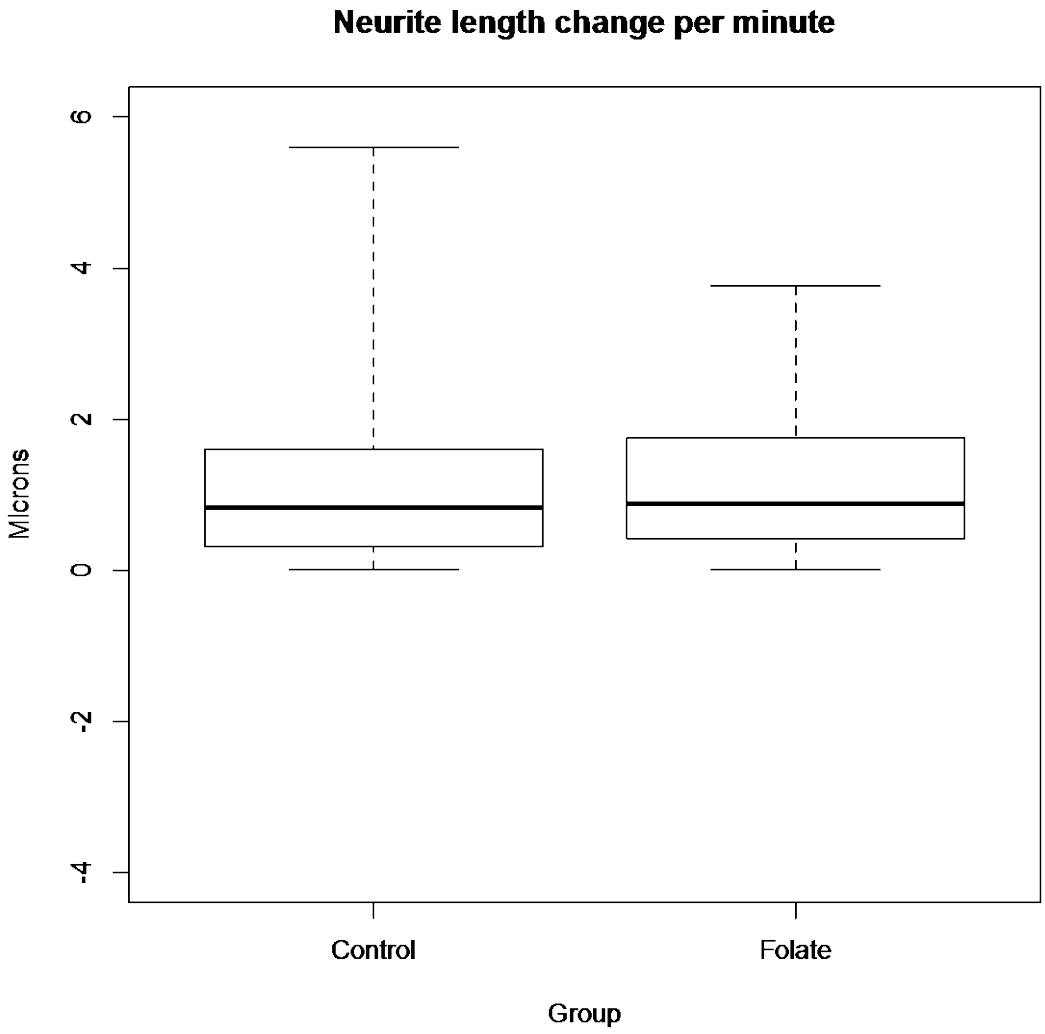

Figure 9 - Box plot of neurite length change per minute before and after addition of folate. All 4 experiments are represented. P-Value: 0.51

Figure 10 parts A and B shows examples of a growth cone at the end of a neurite before and after addition of folate respectively. Three of the four experiments were consistent with this response, but the last experiment showed drastic change in the growth cone and neurite after addition of folate. Before and after addition for this experiment is shown in figure 11 parts A and B respectively. The horizontal arrows point to the growth cone, while the vertical arrows point to microspikes on the growth cone.

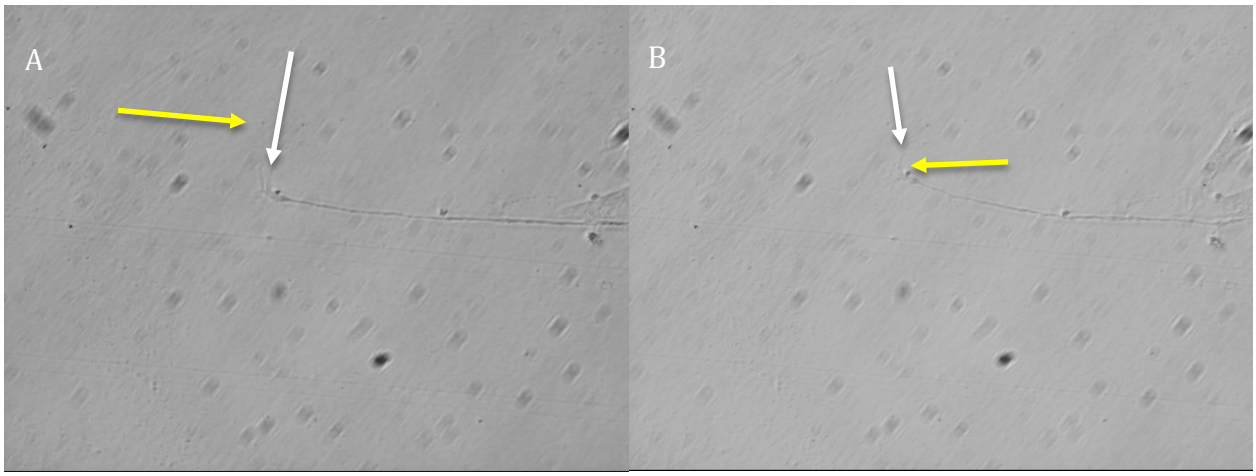

Figure 10 – Growth cone and neurite before (A) and after (B) addition of folate. Horizontal arrows point to the growth cone. Vertical arrows point to microspikes on the growth cone.

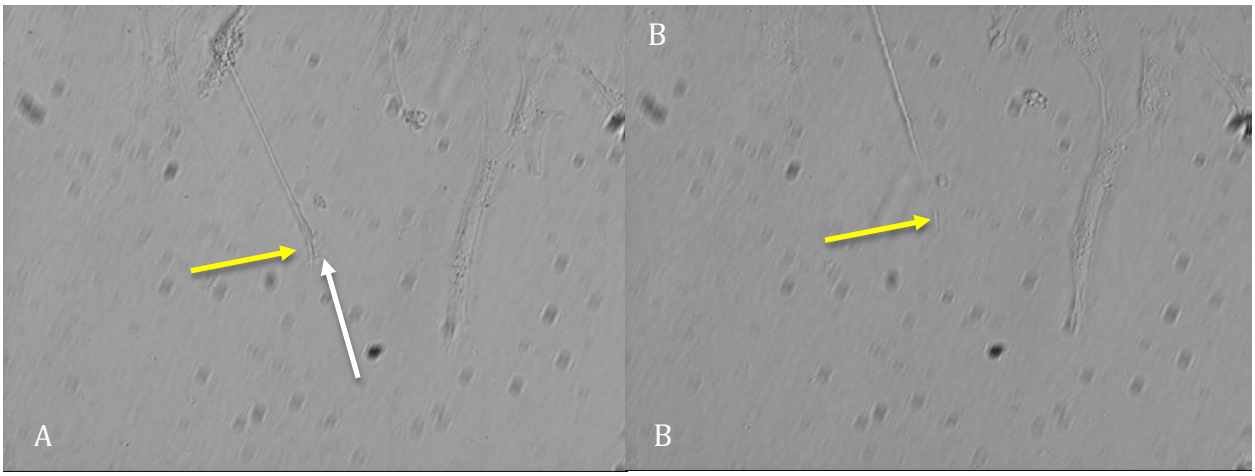

Figure 11 – Drastic change in growth cone and neurite after addition (B) compared with before addition (A) of folate. Horizontal arrows point to the growth cone. Vertical arrows point to microspikes on the growth cone.

Statistical Significance of Results

In addition to the box plots, a Wilcoxon rank sum test was conducted on each data set to determine if there was a significant difference between the data set before addition of folate and glutamate and after addition. A t-test was not performed as the data was not normally distributed. The Wilcoxon rank sum test accounts for this abnormal distribution. The p-values for each data set including growth cone area change per minute before and after addition of folate and glutamate, neurite length change per minute before and after addition of folate and glutamate, growth cone area change per minute before and after addition of folate, and neurite length change per minute before and after addition of folate are displayed in the box plot legends. It is clear that no values were statistically significant as the values are greater than 0.05. This is unexpected for addition of folate, but expected for addition of both folate and glutamate.

Complete Analysis

Tables 4 and 5 represent a complete analysis of all experiments. Table 4 displays the total averages of area change per minute of the growth cone, the average of the medians and standard deviation. Table 5 displays the total averages of length change per minute of the neurite, the average of the medians, standard deviation, average net extension before and after addition of folate, and average net retraction before and after addition of folate. The standard deviation in this case refers to the neurite length change per minute data set.

Table 3 - Growth cone area change averages

Growth cone area change per minute (microns)

	Folate + Glutamate		Folate	
	Control	**Folate+Glutamate**	**Control**	**Folate**
Average area change per min	1.06	0.82	0.83	0.68
Average of the medians	0.53	0.69	0.68	0.59
Standard Deviation	1.63	0.98	0.85	0.58

Table 4 – Neurite length change averages

Neurite length change (microns)

	Folate + Glutamate		**Folate**	
	Control	**Folate+Glutamate**	**Control**	**Folate**
Avg length change per min	1.63	1.43	1.13	1.16
Avg of the medians	0.84	0.85	0.87	1.00
Avg net extension	21.23	22.78	18.68	21.06
Avg net retraction	41.66	26.49	14.70	14.20
Standard Deviation	2.03	1.43	1.05	0.91

It is important to note that a complete control was performed over the entire 60 minutes with no addition of either folate or glutamate. There was no significant change in the neurite length change per minute or growth cone area change per minute as the t-test showed no significant differences ($P > 0.05$). It can therefore be concluded that any observed changes in these parameters is not due to the extended period of time in which the cultures are left out of the incubator. Raw data for this experiment can be found in the appendix.

DISCUSSION

Evidence for Competition of Folate with Glutamate

As laid out by the hypothesis for this study, folate may be competing with glutamate for binding sites to receptors at the postsynaptic membrane therefore exerting its effects on developing neural tissue. By adding both folate and glutamate to the cultures of neurons, the competition between the two molecules was observed. The results from those experiments in which glutamate was added to the cultures along with presumably inhibitory folate, in order to reveal putative competition, were variable in all cases. In experiments where both folate and glutamate were added, there were a few noticeable differences before and after the addition. Out of the eight experiments analyzing growth cone area change per minute, there was one where the median displayed a large increase after addition, and one that displayed a large decrease after addition. This was similar in the results of the neurite length change per minute as the medians displayed two big increases after addition and two big decreases after addition. This demonstrates the variability of the results. As noted in the results section, the averages for overall growth cone area change per minute and neurite length change per minute independent of whether they were increases or decreases were 23% and 27% respectively. This suggests greater activity after addition, and it may indicate that glutamate may have been in excess or that it was simply able to out-compete folate for binding. This possibility requires testing through an experiment with glutamate addition alone.

The box plots display the median for all eight experiments combined as well as the minimum and maximum values. The maximum value was significantly decreased after addition of both folate and glutamate. Perhaps this suggests the growth cone and neurite make incrementally smaller changes in area or length change per minute after addition of the

substances. This could point toward a less active growth cone and neurite due to the addition of folate. The median values of area change per minute or length change per minute however remained unchanged. Similarly, pictures of the growth cone before and after addition of the substances are very similar. Although the dynamic area change per minute cannot be determined from the single pictures, it is clear that there is no visible difference in area size after addition showing its relative stability throughout the experiment. Overall, the addition of folate together with glutamate had neither excitatory nor inhibitory effects on neurite extension and growth cone activity.

Putative Inhibition of Neural Activity by Folate

In the four experiments in which only folate was added to the cultures, the median values were very similar to each other before and after addition. The similarity of the growth cone area changes per minute and neurite length change per minute after addition was somewhat surprising, because previous work in lab (Wiens et al., 2016) indicated a significant inhibition of neurite length change per minute after addition of folate. However, there was one noticeable difference in the growth cone area change per minute after addition. The median value decreased by 50 percent. It is apparent in this experiment that the growth cone area change per minute significantly decreased after addition of folate. This is displayed in figure 11 as the growth cone is much smaller in size, whereas figure 10 is representative of the other three experiments in which the growth cone and neurite did not visibly change after addition of folate.

The box plots are similar to those that represented growth cone area change and neurite length change per minute after addition of glutamate together with folate. The maximum values decreased significantly after addition of folate for both growth cone area change per minute and neurite length change per minute. As mentioned above, this could suggest a less active growth

cone and neurite due to presence of folate. The median value for growth cone area change per minute was slightly less after addition of folate, whereas the median value for neurite length change per minute was slightly more after addition of folate. This indicates the variability of the results and challenges the previous work in lab (Wiens et al., 2016) which reported that folate mostly affects neurite length change per minute, whereas results from the present experiments displayed that folate mostly had an effect on the growth cone area change per minute. Overall, addition of only folate did not appear to excite or inhibit the activity of the growth cone or neurite, which is in contrast with the experiments conducted previously in lab (Wiens et al., 2016). There was one exception noted in the present study in which the growth cone area change per minute was drastically reduced.

Statistical Significance of Results

Despite the few changes in growth cone area change per minute and neurite length change per minute discussed above, the p-values collected for all experiments combined show that the results are not significant ($P > 0.05$). In previous work (Wiens et al., 2016), the addition of folate caused a significant decrease in neurite length change per minute. This was not the case in the present experiments; however, only four experiments were conducted. Wiens et al (2016) found a significant inhibition of neurite dynamic length change, and reported a reliable result, but the present study has revealed no significant inhibition in this parameter. There are no significant increases or decreases shown in tables 4 or 5 in growth cone or neurite length change per minute after addition of folate. The decrease in growth cone area change per minute from 0.83 to 0.68 after addition of folate appears to be significant; however, as mentioned above, the p-value was greater than 0.05 meaning no conclusion can be drawn about the effects of folate.

My present finding that adding glutamate along with folate during neurogenesis resulted in no significant changes in growth cone and neurite behavior is key. It affirms the expectation that glutamate can overcome the inhibitory effects of folate (assuming they exist). This is displayed in tables 4 and 5, which show the averages for all experiments. There were no big increases or decreases after addition of glutamate with folate at the 30-minute point. The only large decrease present is the average net retraction after addition. Because the neurite would be retracting, retraction being an inhibitory response, the implication is that there is less inhibition because of the augmented glutamate level. This supports the proposition above as glutamate could indeed be overcoming the inhibition of folate. Assuming folate does produce inhibitory effects on the neural tissue, absence of significant change after addition of both folate and glutamate shows that glutamate can overcome this. This finding would support the hypothesis that the mechanism of folate's action, when present at elevated concentrations, is able to compete with glutamate for binding sites at the postsynaptic membrane. Moreover, if further work can confirm this mechanism, continuing investigation and caution with regard to dietary folate during pregnancy is prudent.

CONCLUSION

The purpose of this experiment was to test the effects of the addition of glutamate with folate to cultures of neural tissue, and to draw a conclusion on these effects as well as the possibility of the competition between folate and glutamate. Growth cone area change per minute and neurite length change per minute after addition of just folate were shown to be unaffected, and therefore confirmation of the inhibitory effects of folate reported by Wiens et al. (2016) was not possible; however, it seems likely that additional experiments would do so. Because of this, no conclusions can be drawn specifically about the effects of folate on the developing neural tissue. In contrast, evidence from a large number of experiments supported a mechanism of folate competition exerting its effects on neural connectivity through the glutamate receptor in developing synapses, although this depends on the assumption that folate is inhibitory.

Limitations and Future Studies

It is evident that further experiments testing the effects of folate only on growth cone area change per minute and neurite length change per minute must be conducted to confirm the previous work in lab (Wiens et al., 2016). It is clear that the way in which neural tissue responds to the addition of folate is not necessarily concrete. Additionally, experiments testing the effects of only excess glutamate on neural tissue need to be performed to confirm that it has an excitatory effect on the growth cone and neurite. Lastly, dorsal root ganglia from other species of animals could be used to examine if the effects of folate and/or glutamate are similar.

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9. Appendix

Raw data for addition of folate and glutamate

Folate and Glutamate								
Exp 1								
	Folate/Glut							
	C GrowthCo	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	2.28123729	1.01791525	3.82042373	2.14771186	39.268	-186.137	51.412	-75.303
ST DEV	2.04400659	0.97021493	3.60110513	1.87371127				
MEDIAN	1.694	0.723	2.858	1.469				
MAX	10.374	5.839	11.934	9.669				
MIN	0	0	0	0.009				
TTEST	4.8303E-05		0.00213516					
Exp 2								
	C GrowthCo	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE			3.4924	3.47184	50.169	-54.603	53.23	-36.668
ST DEV			3.3953156	1.82648261				
MEDIAN			2.4775	3.21				
MAX			12.747	7.829				
MIN			0.193	0.122				
TTEST			0.97732774					
Exp 3								
	Folate/Glut							
	C GrowthCo	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.78036667	0.62713333	0.8818	0.75472414	12.313	-14.141	5.622	-16.265
ST DEV	0.8240663	0.70344868	0.64868989	0.67582589				
MEDIAN	0.433	0.48	0.861	0.563				
MAX	2.771	3.474	2.832	3.154				
MIN	0.014	0.014	0.018	0.178				
TTEST	0.44178278		0.46451019					
Exp 4								
	Folate/Glut							
	C GrowthCo	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.42173333	0.47433333	0.92190323	1.01743333	16.59	-11.219	17.158	-16.103
ST DEV	0.36650031	0.42773351	0.8128526	0.76739657				
MEDIAN	0.3035	0.3235	0.77	0.7795				
MAX	1.24	1.564	2.748	3.237				
MIN	0	0.013	0.057	0.101				
TTEST	0.61100441		0.44500892					

Exp 5								
	Folic Glut							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.34876667	0.23166667	1.25186667	1.0873	8.888	-28.668	8.165	-24.454
ST DEV	0.33540513	0.18026099	0.86471118	0.77968818				
MEDIAN	0.211	0.191	1.1585	1.013				
MAX	1.197	0.68	3.071	3.053				
MIN	0	0.013	0.016	0.021				
TTEST	0.09911094		0.44201413					
Exp 6								
	Folic Glut							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	2.24856667	1.9406	1.63286667	1.70803333	29.734	-19.252	29.361	-21.88
ST DEV	2.43562044	1.54736699	1.20412511	1.320628				
MEDIAN	1.1055	1.9555	1.421	1.597				
MAX	8.73	5.442	4.774	5.651				
MIN	0	0	0.035	0.093				
TTEST	0.56151707		0.81862413					
Exp 7								
	Folic Glut							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.43456667	0.4875	0.50113333	0.6643	4.111	-10.923	8.649	-11.28
ST DEV	0.32614791	0.37520411	0.40026782	0.41562128				
MEDIAN	0.408	0.4625	0.4195	0.6875				
MAX	1.334	1.306	1.814	1.443				
MIN	0.068	0.014	0.008	0.058				
TTEST	0.56207087		0.12687402					
Exp 8								
	Folic Glut							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.86983333	0.96316667	0.56986667	0.62076667	8.778	-8.318	8.639	-9.984
ST DEV	0.813249	0.86606498	0.47033134	0.58169406				
MEDIAN	0.6895	0.71	0.4135	0.4755				
MAX	3.055	3.407	1.783	2.52				
MIN	0.054	0.014	0.03	0.03				
TTEST	0.66858239		0.71079722					

Raw data for addition of folate

Folate								
Exp 1								
	FOLATE ONLY							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	1.2374	0.95243333	0.96473333	1.08626667	16.454	-12.488	14.617	-17.971
ST DEV	0.92482925	0.6035945	0.83142337	0.79153861				
MEDIAN	1.116	0.959	0.7725	0.9605				
MAX	3.156	2.013	3.247	2.714				
MIN	0.041	0.027	0.039	0.009				
TTEST	0.16376901		0.56425273					
Exp 2								
	FOLATE ONLY							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.89862069	0.9862	1.41353333	1.43923333	24.234	-16.877	36.795	-7.677
ST DEV	0.8519488	0.63315724	1.33155708	1.16385061				
MEDIAN	0.729	0.852	1.033	1.1975				
MAX	4.107	2.509	5.604	3.766				
MIN	0	0.086	0.008	0.026				
TTEST	0.6567592		0.93683784					
Exp 3								
	FOLATE ONLY							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.57743333	0.5367	1.0031	0.94596667	15.357	-14.736	11.774	-16.975
ST DEV	0.40373017	0.39132394	0.88018984	0.64388329				
MEDIAN	0.5855	0.4145	0.808	0.8515				
MAX	1.347	1.564	3.203	2.493				
MIN	0.013	0.041	0.02	0.033				
TTEST	0.69296975		0.77526949					
Exp 4								
	FOLATE ONLY							
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.61073333	0.2306						
ST DEV	0.96047441	0.27588423						
MEDIAN	0.284	0.1415						
MAX	4.543	1.163						
MIN	0.013	0						
TTEST	0.04484988							

Raw data for control

Control								
	C GrowthCor	Growth Cone	C Neurite L	Neurite L	C NL EXT	C NL RET	NL EXT	NL RET
AVERAGE	0.63103333	0.5385	0.7846	1.07666667	11.538	-12	16.378	-15.922
ST DEV	0.48396755	0.52235823	0.66297332	0.97605474				
MEDIAN	0.5085	0.4005	0.685	0.784				
MAX	1.845	2.007	2.294	4.214				
MIN	0	0	0.004	0.004				
TTEST	0.47949731		0.1811352					