The Effects of Periconceptual Folic Acid Supplementation in Excess on Rats in the VPA Model of Autism

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April 1, 2012

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Submitted to the Neuroscience Department, Allegheny College

In partial fulfillment of the requirements for the degree of

BACHELOR OF SCIENCE

April 1, 2012

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A Senior Comprehensive Project

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Acknowledgements

Dr. Hollerman: Thank you for pushing me to pursue a topic I was interested in, even though it was outside of your expertise too. Thank you for agreeing to be my first reader and for the suggestions at the start of this project. I would have never gotten to this point if you hadn't been so willing to work on it, way back in junior sem. Dr. Hollerman, your suggestions were greatly appreciated.

Nicole, Emily and Anna: Woohoo!!! I made it, I truly wouldn't have done it without you two helping me. Thank you for everything throughout this project. Without the laughter and jokes, many of the days would have been miserable. I can't think of anyone else I would rather spend time in the lab with, even though I know you were only there for the babies, especially the runt. Thanks again, it's been a great ride!

Claire: Thank you. Thank you for putting up with me for four years. Thank you for making the TA job more fun. Thank you for listening to me rant about equipment and protocols and things that don't work. Thank you for driving me to McDonalds at 2 in the morning when I'd lost my inspiration. I'm going to miss you next year.

Family: I would like to thank my Mom and Dad for never actually asking me to explain my project beyond, "I'm working with rats." It was a welcome change, thank you for keeping me up to date on life outside of college.

Chelsea: Thank you for keeping me distracted through piles of wonderful things. Be warned your chomp time is coming soon too and turnabout is fairplay!

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Abstract

Autism is a complex developmental disorder diagnosed using three core criteria: qualitative impairment in social recognition, qualitative impairments in communication or language, and the presence of restricted, repetitive, and stereotyped behaviors. Folic acid has been consumed during pregnancy as a possible preventative measure for neural tube defects. In the Valproic acid model of autism rats were given VPA or VPA and folic acid during pregnancy. The auditory pathway was examined using the Auditory Brainstem Response (ABR), behavioral testing used the Y maze to test for the preference for familiarity. It was hypothesized that folic acid consumed in excess of the recommended intake would have a protective effect against damage caused by the injection of valproic acid injection. Behavioral testing using the Y maze found no significant main effect and a very weak interaction ($\eta^2 = 0.04$). Testing of the ABR was unsuccessful, further research is needed to explore the relationship between folic acid and VPA.

Introduction

Autism Background

Autism is a developmental disorder that, according to the American Psychiatric Association, is characterized by reduced social interactions, language deficits, and restricted and repetitive behaviors (American Psychiatric Association, 2000). Approximately 1.3 people per 1000 in North America alone fit the criteria for autism set by the APA in 1997, and prevalence rates continue to rise; in 2007 the prevalence of autism was as high as 1 in150 people (Rodier, Ingram, Tisdale, & Croog, 1997; Courchesne, Pierce, Schumann, Redcay, Buckwalter, Kennedy, & Morgan, 2007). Autism is diagnosed approximately four times more often in boys than in girls (Steyaert & De La Marche, 2008).

Leo Kanner first described the symptoms of autism in 1943. In the 1990's autism gained a broader classification as "autistic spectrum disorders" (ASD), in order to encompass individuals with a wide range of symptom severity in the three core domains of autism including, for example, Asperger's syndrome (Steyaert & De La Marche 2008). In the original diagnoses, Kanner proposed that a single biological problem or deficit was behind the development of autism (Kanner, 1943). However, further research has indicated that while autism has a strong biological basis, it is not the result of a single abnormality.

Research has shown various abnormalities in the neuro-anatomy and brain function of autistic individuals. As the severity of core autism symptoms display a broad range, so too does the structural abnormality of the autistic brain. Abnormalities reported include hypoplasia of the superior olive, posterior cerebellum and of the brainstem (Courchesne et al., 2007) of autistic individuals. Hyperserotenemia is also prevalent in autism; this increase in blood serotonin levels is considered to be one of the most commonly observed physiological differences between

autistic and non-autistic individuals, yet this difference is present in only 30% of autism cases. Conclusive physiological indications of autism do not exist.

Genetic Factors of Autism

The inheritance of the disorder has strong implications for the prevalence of autism. The disorder runs in families, yet this genetic link is not a direct one. The siblings of an autistic individual have a three to eight percent chance of being diagnosed with autism (Rodier, 2000). This chance is higher than the 0.16 percent risk of development seen in the general populace, but it does not have the fifty percent chance that characterizes a disorder caused by a single dominant gene mutation. This percentage is also smaller than the 25 percent chance that would account for a disorder caused by a single recessive gene mutation. There is a thirty six percent concordance rate amongst monozygotic twins, twins that share all of the same genes. This concordance rate is much larger than random chance but it does not match the concordance of a one hundred percent heritable disorder (Rodier et al., 1997). In an earlier study, Folstein and Rutter (1977) studied eleven pairs of monozygotic twins and ten pairs of dizygotic twins, where at least one of the twins was autistic. In these pairs, four of the eleven monozygotic twins were concordant while none of the dizygotic twins were concordant. Folstein and Rutter (1977) also determined that those monozygotic twins that were not autistic did display severe deficits in language and cognition, but did not meet the requirements for a diagnosis of autism. Further evidence for the heritability of autism is that couples with an autistic child have a 2-8 percent chance of having a subsequent child with the disorder (Steyaert & De La Marche, 2008).

The concordance rates indicate that more than genetic factors are at work. Research on the possible environmental factors associated with the development of autism found that factors

such as exposure to teratogens such as the rubella virus, the varicella virus, valproic acid or thalidomide during pregnancy play a role in the risk of developing autism. A teratogenic agent is a chemical, infectious agent or physical condition that, if exposed prenatally, has the ability to cross the placenta barrier causing changes in fetal morphology and\or causing deficits in function. While autism might be influenced by exposure to teratogens, none are prevalent enough to account for the majority of autism cases.

Autism Diagnosis

The inconclusive mixture of genetic and environmental factors affecting the risk of developing autism combined with the lack of consistent and well defined physiological indicators has caused the diagnosis of autism to depend on behavioral assessments. The formal set of criterion for autism has been changed several times since Kanner first defined them. In 1943, Kanner described autism based on the behaviors of eleven children who demonstrated an impairment of social engagement, a lack of communication and a need for sameness (Kanner, 1943). When first describing the disorder, Kanner outlined two criteria for diagnosis, the "profound lack of affective contact" and "repetitive, ritualistic behavior, which must be of an elaborate kind" (Eisenburg and Kanner, 1956). Today, the DSM- IV has created three domains as criteria for the diagnosis of autism. These domains include: qualitative impairment in social interaction, qualitative impairments in communication or language, and the presence of restricted, repetitive, and stereotyped behaviors (DSM-IV). Within these domains the specific symptoms of autism are widely diverse and are seen in varying degrees of severity. The onset of autistic symptoms generally occurs by thirty months of age (Rodier et al., 1997).

Social Interaction Impairment

The impairments in social interactions in autistic individuals include the inability to form relationships with peers, a lack in social reciprocity, and deficits in using and understanding nonverbal cues such as eye contact, facial expressions and body language. Research has indicated that autistic individuals have impaired eye contact, and impaired gaze may lead to several other deficiencies including problems with joint attention, face processing, recognizing emotions, and the interpretation of what others are thinking which is normally observed through body postures (Kana et al., 2011). These impairments can subsequently affect social interactions as a whole.

Restricted Behaviors

Restricted, repetitive behaviors are defined as a group of maladaptive behaviors including, for example, a very narrow range of restricted interests, intolerance to changes in routine, and the preservation of sameness (Lewis and Kim, 2009). Other behaviors included are repetitive motor mannerisms (hand or arm flapping), persistent occupation with parts of an object (such as the wheels of a toy car), ritualistic conduct, and self-injurious behavior. Many autistic individuals adhere to a strict routine and do not react favorably to changes. In studies of repetitive behavior, autistic individuals do not explore novel stimuli as frequently as non-autistic individuals do (Pierce and Courchesne, 2001). This study also established a possible link between the cerebellum and the predisposition to explore novel objects. This lack of investigative action may play a significant role in other impairments, because a lack of exploration could lead to a deficit in learning, and social opportunities that are outside the individuals' restricted range of interests (Pierce and Courchesne, 2001).

Communication and Language Deficits

Deficits in communication and language are the final category of core symptoms. This includes a delay or total lack of spoken language, the impairment of an individual to initiate or continue a conversation with other people, and echolalia (the repetition of words and sounds heard by the individual) and the absence of varied, spontaneous, imaginative or imitative play that is appropriate for the individuals' developmental level (American Psychiatric Association, 2000).

Linking Communication and Audition

Siegal and Blades (2003) suggested a link between language impairments and the perception of auditory stimuli. In a review, Siegal and Blades indicated that children with autism share characteristics of children with Specific Language Impairment (SLI), where both groups display the same types of grammatical error. They two groups also show an abnormal brain asymmetry in comparison to normally developing children. In normally developing children the language areas in the left hemisphere are larger than those on the right. In autistic and SLI children the asymmetry may be reversed (Siegal and Blades, 2003). Children with autism have displayed problems in extracting the meanings of information when it is received through auditory perception (Tager-Flusberg and Joseph, 2003). If this is the case the difficulties autistic individuals have with communication may stem from a lack of understanding the spoken word. Furthermore, studies have indicated autistic children display abnormalities in hearing. These abnormalities include asymmetrical patterns of activity in the olivocochlear system, which is involved in the detection of speech while background noise is present or in noisy environments (Siegal and Blades, 2003; Russo, Zecker, Trommer, Chen, & Kraus, 2009; Roth et al, 2012).

Such auditory impairments would cause deficits in conversation participation, causing downstream impairments in other forms of communication. Russo et al. (2008) found that children with autism had difficulty processing pitch cues from speech sounds. The study suggested that an individual with ASD's failure to process the pitch fluctuations in speech was the reason for the deficits in language interpretation.

Auditory Brainstem Response

One way to determine hearing sensitivity and the integrity of the auditory pathway is Auditory Brainstem Response (ABR) test. The test records the electrical response of the auditory pathway in response to an auditory stimulus. This test can be used on humans too young or unable to participate in regular hearing tests, which require verbal or physical responses, and on animal models (Norrix, Trepanier, Atlas & Kim, 2012). Figure 1 shows an example of a typical ABR response. The ABR latencies in the rat have not been fully determined, but the neuroactivity of mice ABRs probably reflects the neural activity from the auditory nerve (P1), the posterior anteroventral cochlear nucleus (P2), the anterior anteroventral cochlear nucleus and the trapezoid body (P3), the medial superior olivary nucleus (P4), and the lateral lemniscus and/or the inferior colliculus (P5) respectively (see Figure 2).

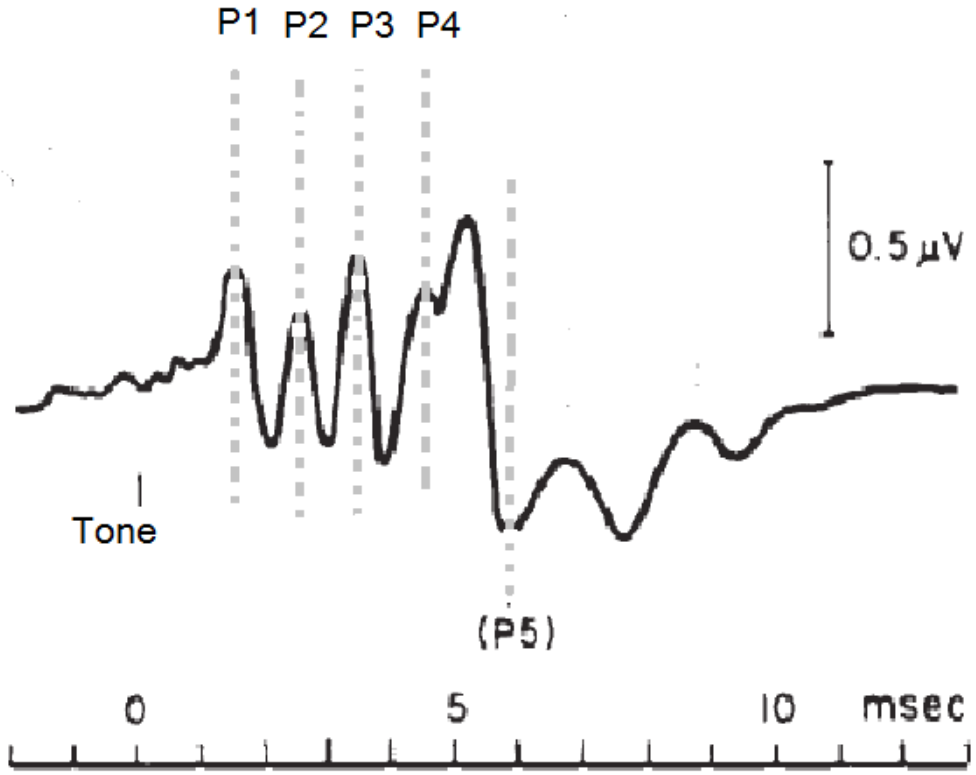

Figure 1. An example of a typical fast ABR response (1-10ms) recording. Where the response occurs within 10ms of the tone and the peaks are believed to correlate to specific points of the auditory pathway, including the auditory nerve (P1), the posterior anteroventral cochlear nucleus (P2), the anterior anteroventral cochlear nucleus and the trapezoid body (P3), the medial superior olivary nucleus (P4), and the lateral lemniscus and/or the inferior colliculus (P5).

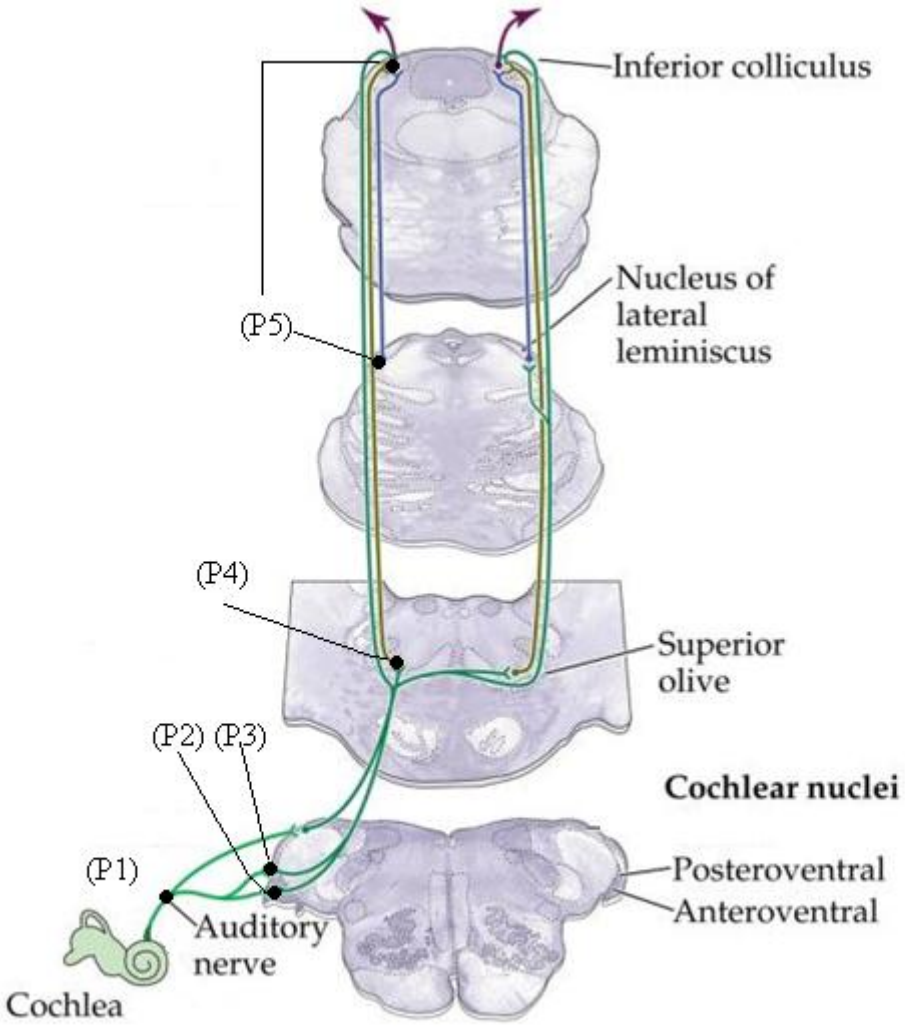

Figure 2. A representation of the human auditory pathway, with proposed points of origins for ABR response latencies . Retrieved from http://www.rci.rutgers.edu

Valproic Acid Model of Autism

One of the common animal models of autism is the valproic acid (VPA) model. VPA was first introduced as an anticonvulsant medication. The treatment itself is effective for controlling seizure disorders such as epilepsy, but the medication also results in a five to twenty five fold increase in the risk of spina bifida when taken during pregnancy (Ubeda, Alonso-Aperte, and Varela-Moreiras, 2002). VPA is a teratogen in humans, mice, rats and rabbits that causes

debilitating morphological and cognitive effects on a fetus if taken during pregnancy. In humans, these are combined under the term fetal valproate syndrome (FVS) and include low birth weight, mental retardation, autism and other abnormalities (Williams and Hersh, 1997). Infants with FVS tend to have developmental delays especially in language development and craniofacial abnormalities, respiratory and cardiovascular problems, abdominal wall abnormalities, and urogenital dysfunctions (Christianson, Chelser and Krombers, 1994). The animal model of autism has been developed using a single injection of valproic acid into the pregnant rat to incite cerebellar, and other damage to the central nervous system (CNS) around the time of neural tube closure (Ubeda, Alonso-Aperte, and Varela-Moreiras, 2002).

Folate and Folic Acid: Prenatal Nutrition

The drive to reduce poor nutrition worldwide has been an ongoing effort since the early 1990's; with the origins of the movement began even earlier. The effects of folic acid deficiency, anemia in children and adults and neural tube deficits like spina bifida and anencephaly during pregnancy are well documented. Folate and its stable synthetic counterpart, folic acid, are water-soluble B vitamins that are essential to proper neurodevelopment, folic acid is the component placed in vitamin supplements and fortified foods, while folate occurs naturally in foods. Both forms of the nutrient are important in the production of red blood cells, and in the prevention of anemia, folate is used in the process of methylation and helps to produce and maintain newly developed cells (Kamen, 1997). This is particularly important at times of rapid cell growth such as early development, especially around the time of neural tube closure. The time of very early pregnancy is crucial to normal neurodevelopment. Folate is most important to neurodevelopment of a fetus before pregnancy can be reliably determined, neural tube closure

occurs on day 21 of gestation for humans; thus it is difficult to ensure the consumption of recommended amounts of folic acid at this stage of pregnancy. To combat this, supplementation of folic acid during planned pregnancy has become relatively widespread, the recommended dose of this important micronutrient is 400 µg per day for adults and the recommended dose for women who are pregnant or may become pregnant is 600 µg per day.

Folic Acid in Excess

Schmidt et al. (2012) collected self reported data for the intake of folic acid during pregnancy for both the mothers of 'typically developing children' as well as those with ASD. Her study found that mothers who reported an average folic acid intake at or above the recommended daily amount predominantly had children exhibiting typical development, implying an association between high levels of folic acid and a decreased risk of ASD (Schmidt, 2012). However, the wide range of reported intake of folic acid, from supplements and regular diet which had to be accounted for, emphasizes the difficulty in determining an optimal amount of folic acid. Mothers who reported a daily intake of folic acid that was in excess of the recommended dose had children in the typical development range; but whether this was an exaggeration of intake caused by the increased media attention towards folic acid consumption, or general difficulty remembering the exact or typical amounts of folic acid consumed is unknown. In contrast, Rodgers (2008) made a possible link between increased folic acid intake and the prevalence of autism. Rogers argued that increased folic acid consumption allowed for individuals such as those with an impaired enzyme called 5-methylenetetrahydrofolate reductase (*MTHFR*), which is necessary for the breakdown of folic acid for the process of methylation, to survive pregnancy (Rodgers, 2008). Typically such individuals would display a neural tube

defect or not survive to term. An increase in maternal folic acid intake allows children with nonfunctional *MTHFR* to survive pregnancy, but without a continuous high level of folic acid intake during early development, there is an increased chance of developmental delays and autism, essentially causing the child to regress to atypical development (Rodgers, 2008).

Women that have had a neural tube deficit (NTD) affected pregnancy, have been shown to also have decreased levels of several micronutrients during the first trimester (Smithells, R.W., Sheppard, S. & Schorah, C.J., 1976). Small trials and case studies have indicated a maternal supplementation of folic acid, 0.4 to 0.5mg per day in substantial excess of the recommended daily intake, decreased the recurrence of neural tube defects (Blom, 2009). As this micronutrient is found naturally in foods, artificially inserted into enriched foods and commonly taken as part of a supplement prior to and during pregnancy, the effects of consuming folic acid in excess need to be examined more closely.

Current Study

The purpose this study is to examine the behavioral deficits seen within the valproic acid animal model of autism after the maternal supplementation of folic acid. Studies of brain abnormalities in autistic individuals remain inconsistent. The effects of a maternal diet deficient in folic acid are well documented. However, few studies consider the role excess folic acid, beyond the possibility of the excess nutrient masking the deficiency of B_{12}. The effect of excess folic acid in an animal model of autism has not been widely researched. This study combined the known importance of folic acid in development and attempt to determine the effects of excess folic acid levels in the maternal diet on pup development. This study attempted to reproduce the VPA model of autism. Rats fed a specific diet of excess folic acid were tested for

the behavioral effects of both VPA and folic acid intake. A group of rats not exposed to VPA were used a baseline for the autistic like symptoms produced by VPA. Rats exposed to VPA but not folic acid were compared to rats fed an amount of folic acid in excess of the daily recommended pregnancy dose. All of the rats were tested in the areas of repetitive behavior through preference for familiar environments and auditory abnormalities, through the Auditory Brainstem Response (ABR). It is hypothesized that an excess supplementation of folic acid will cause a neuro-protective effect against the injection of VPA. Subjects that ingest folic acid in excess of the daily amount and are exposed to VPA are expected to not show significant differences in their preference for familiarity and ABR latencies from the non-VPA control group.

Materials and Methods

Subjects

4 pregnant female Spague-Dewley rats were placed into groups 2 dams were placed in the folic acid treatment group and 2 were placed in the non-folic acid groups on gestation day 5, denoted by the presence of a vaginal plug.

Diet

The recommended dietary intake of folic acid for rats, both pregnant and not, is 1.0 mg per kg of food. During pregnancy in humans, the recommended daily intake of folic acid is 1.5 times higher than non-pregnant adults (NIH). According to Oblak (2005), dams consume on average 19.6 grams of food every three days. The standard chow contains 1.2 mg/kg of FA, based upon the ratio of the recommended intake of pregnant women vs. non pregnant women a

daily supplement of FA was determined. The VPA, folic acid treatment and the control groups all received the recommended amount of FA or 1.0 mg per kg food in the standard chow. In addition a supplement of 0.31 mg/day of FA mixed in peanut butter was provided to the dams in the folic acid treatment group from gestation day 7 through gestation day 15. After day 15 the dams in all groups were fed the standard diet.

Housing

The rats were given free access to food and water, were on a 24 hour light cycle, and were given continuous exposure to an AM/FM radio. Litters were raised by their birth mother. Behavioral testing was performed on 24 animals, n=6 for the VPA and Control groups and n=12 for the VPA and FA group.

Valproic acid

On gestation day 12.5, the 2 dams fed folic acid in addition to the standard chow and 2 dam fed the standard chow received two intraperitoneal (ip) injection totaling 460mg/kg of valproic acid (VPA groups) or two injections of 1.0 mg/kg saline (non-VPA groups). The dams were be placed in separate cages after the injection and allowed to raise their own litters. The pups were weaned around postnatal day 28, by removing the dams from the cages. The dietary conditions housed separately by sex.

Behavioral Testing

Y Maze

Rats within each group underwent behavioral testing beginning at approximately 6 weeks of age. The Y- maze was used to determine the presence of a preference for familiarity. The Y-maze consisted of three arms, each measuring 25.5 cm long by 10.2 cm wide with 27.8 cm high walls. The central part of the y-maze was triangle in shape with 10.2 cm sides. The Y-maze was made of clear plastic and covered with black paper taped to the sides. The rats being tested were transferred to the testing room in their home cages. The rat sat in the home cage for 10 minutes, in order to become habituated. Each arm of the maze was labeled start arm, left arm, or right arm. The left or right arm of the Y maze was blocked and the rat was allowed to explore the other arms for 10 minutes, beginning in the start arm of the maze. After, this rat was taken out for 10 minutes, the left arm that was blocked was now unblocked. The unblocked left arm was thus the novel arm, and the right arm that was previously not blocked was the familiar arm. The rat was placed in the starting arm of the Y maze and allowed to explore the arms for 10 minutes. A rat was considered in the arm when both front limbs were within the arm. The time spent in each arm was recorded. The rat was taken out, put back into its home cage for 10 minutes, and the test was repeated, but for the second trial the novel and familiar arms were switched. With the left are being familiar and the now blocked right arm being novel.

ABR Testing

At approximately 8 weeks of age, 6 pups each pups from the VPA and control groups (n=12) and 12 pups from the VPA + FA group were tested for auditory brainstem (ABR) latencies. The

following protocol includes the finalized procedure determined to have the most consistent responses.

1. Calibrate the speakers to optimize sound and to determine the dB of the stimuli. The generally accepted optimal intensity is between 70 and 80 dB (Willott, 2005).

2. Adjust rate of stimuli to 3ms at a rate of 19 per second. Adjust frequencies to 200, or 1000 Hz. Each animal's ABR recordings run in ascending order from 200Hz or descending order from 1000Hz.

3. Anesthetize the animal using at a dose of .1ml/100g of body weight.

4. Place the anesthetized animal on a heating pad directly in front of the speaker for the placement of electrodes.

5. The subcutaneous electrodes should be placed at the vertex of the skull (ground) and behind the left ear (active). The use of three electrodes, at the vertex (active), behind the left ear (reference), and behind the right ear (ground) as stated in Church et al. (2007) had no discernible impact on the clarity of the response.

6. Determine that electrodes are not resting on the animal, nor is there any tension on the electrodes as both will interfere with signal.

7. Begin recording. Turn on auditory stimuli train. The stimuli is presented as a 3ms tone at 80 dB. The tone is presented at a rate of 19/second for 2 minutes.

8. Record at least 300 tones per frequency. At least 250 responses are required for averaging.

9. Stop the train, adjust frequencies, begin train. Repeat for at least two frequencies. If recording is good, repeat for frequencies 200, 400, 600, 800 and 1000 HZ.

10. After recording use cursers to specify the individual frequencies for averaging using Spike2 7.0 Waveform Average. The response should be adjusted to show in milliseconds. The ABR

latency should occur within 1-10ms of stimuli with a 0.3ms delay to account for the distance from speaker to animal.

Prior to ABR recording, each animal was anesthetized. The anesthesia, Ketamine, can influence the rat ABR latencies and/or amplitudes, but the effects are small, the thresholds are not altered and ABR quality is very good (Church and Gritzke, 1987). The ABR responses were recorded by two subcutaneous electrodes.

The latency of each ABR component was measured as the time between the activation of the tone to a wave's positive peak, and they included a 0.3 ms acoustic transit time between the earphone and the animal's pinnae.

Subjects Lost

Four rats, 1 female from the VPA group and the 3 males from the VPA group, were lost during the completion of the experiment due to illness. All animals were placed on antibiotics following the discovery of the illness. The animals lost had completed both the Y maze and the ABR procedure, and the information collected from those trials was included in the data set.

Statistical Analysis

The Y maze measurements were recorded in seconds and the data was statistically analyzed using a mixed ANOVA. A ratio of the time spent in the novel arm / time spent in the familiar arm was calculated in order to compare between groups. The results were examined using a mixed ANOVA ($\alpha = 0.05$) with trial number as the within subjects variable and the treatment group as the between subjects variable.

Results

Mixed ANOVA Comparing Treatment Groups across Y Maze Trials

A total of 24 subjects were used in the study. The ratio of time the animals spent exploring a novel environment versus a familiar one were analyzed using a 3x2 mixed-model ANOVA (Table 1). Analysis revealed the main effects of diet and Y-maze exploration trial had no significance diet $F(2,21)=2.323$, $p>0.05$, trials $F(1, 21) = 0.946$, $p>0.05$,. There was an interaction effect between diet and exploration of the Y-maze, The interaction $F(2,21) =3.877$, $p<0.05$, indicated significant differences in trial 1 between VPA ($M= 0.671$, $SD= 0.502$) and both the VPA + FA ($M= 1.045$, $SD=0.357$) and the Non VPA groups ($M= 1.076$, $SD=0.245$), in trial 2 there is a significant difference between Non VPA ($M= 1.129$, $SD=0.160$) and both the VPA +FA ($M= 0.943$, $SD= 0.204$) and VPA groups ($M= 0.849$, $SD= 0.240$). There was also significant difference in the VPA group between trials 1 ($M= 0.671$, $SD= 0.502$) and trial 2 ($M= 0.849$, $SD= 0.240$). The effect was weak($\eta^2 = 0.034$). Table 2 shows the means and standard deviations of the ratio of Y-maze exploration for each group and trial.

ABR Results

The ABR protocol was unsuccessful in producing an averaged ABR latency that was within the expected 1-10ms range (Figure 1, Figure 3). Early in the current study the ABR protocol included an event rate of 1,000 Hz (Figure 4). The response averaged 90ms in length despite changes in protocol. The event rate was raised to 50,000Hz (Figure 5). The response remained an average of 90ms, though the peaks became more clearly defined.

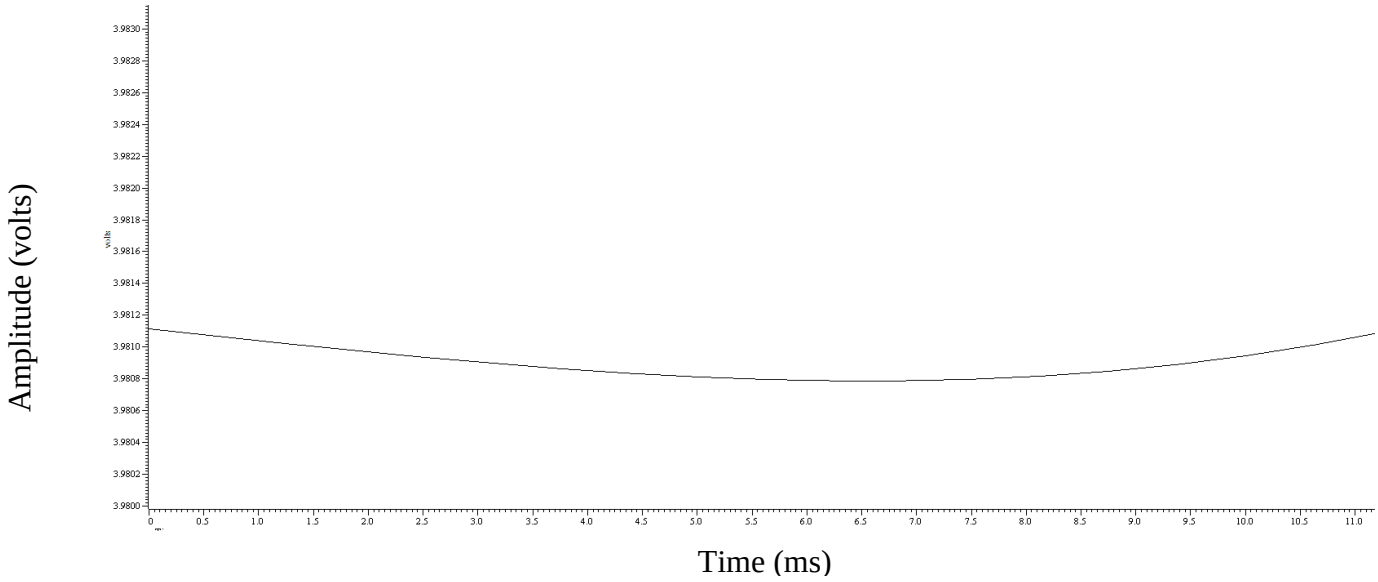

Figure 3. ABR recording at 1000Hz and 80dB at a sampling rate of 1000Hz, the 1-10ms response expected was flattened.

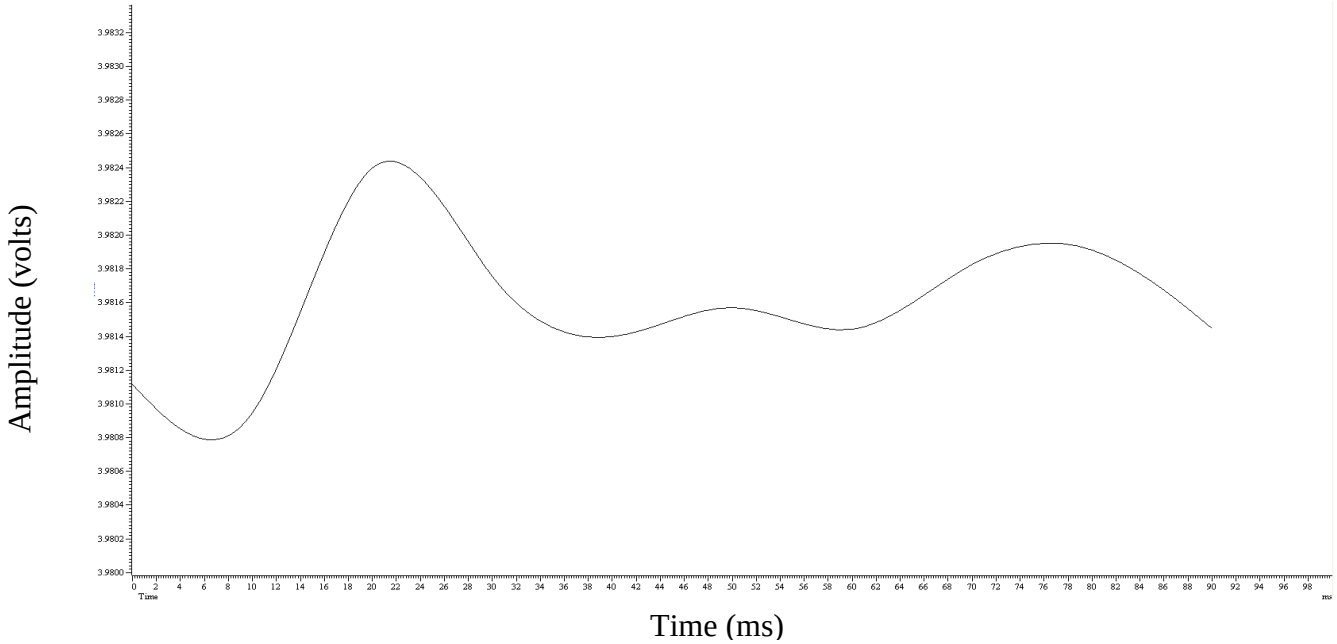

Figure 4. Early ABR latency of rat from VPA + FA group at 200 Hz and 80 dB. The latency was recorded using the experimental protocol. A total of 500 recordings were averaged. The resulting latency was much longer than the 1-10ms latency expected, extending nearly 90 ms.

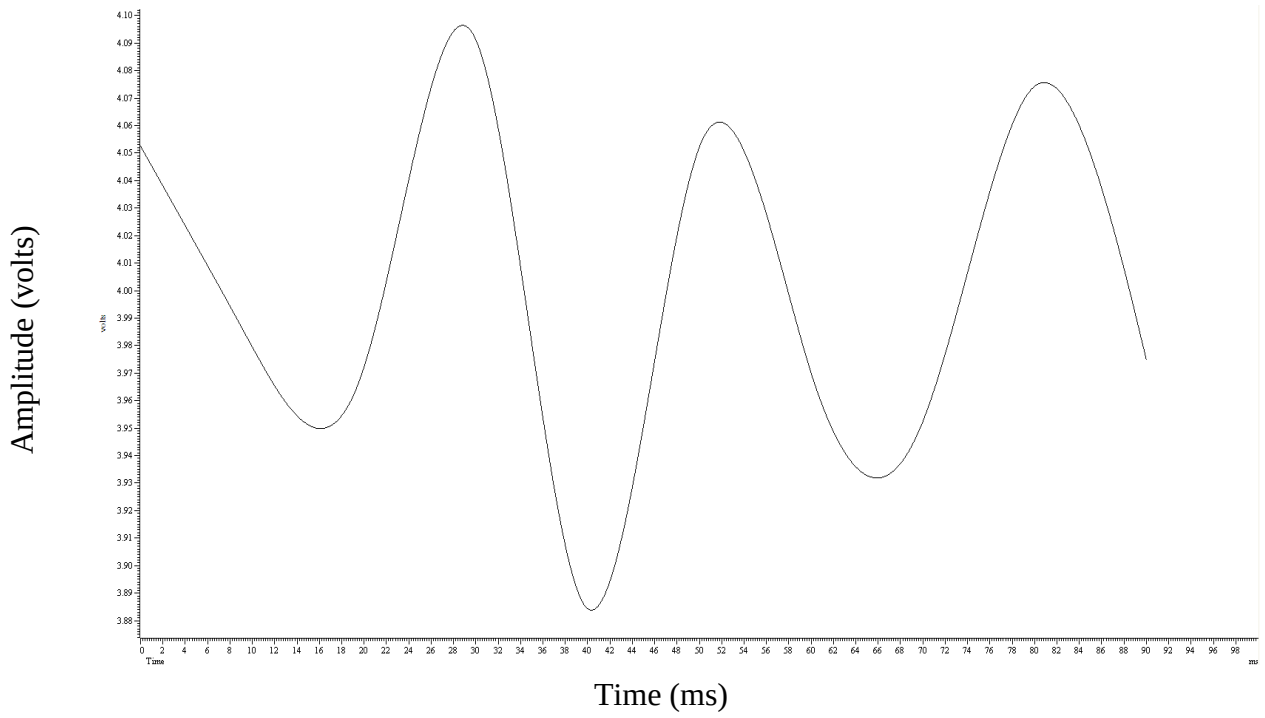

Figure 5. ABR latency of rat from control group at 200 Hz and 80 dB. The response was recorded using the experimental protocol A total of 300 recordings were averaged. The resulting latency was again much longer than the 1-10ms latency expected, the peaks of the recording were much clearer after increasing the event rate.

Discussion

Y maze

The Y maze was used to test repetitive behaviors in order to determine if folic acid had an effect on the preference of familiar or novel exploration. Preference for familiar or novel was measured by blocking the left or right arm of the Y maze, allowing the rat to explore, then unblocking the arm and recording how much time was spent in each arm (seconds). Entering the previously blocked arm, the rats explored a novel environment rather than the familiar unblocked arm. Rats that spent significantly more time in the familiar arm were considered to be undergoing a repetitive behavior because they remained in an arm that they had explored previously. Conversely, rats that spent more time, or even just an equal amount of time, in the novel arm compared to the familiar arm were indicative of not having a repetitive preference to an arm and are open to exploring all aspects of the Y maze. A rat would normally move to explore the second arm of the maze in subsequent trials. VPA model animals usually display a preference for the non-novel arm, suggesting repetitive behavior.

Trends, indicate the VPA + FA group and the control group, Non VPA, have similar exploration times in both the familiar and novel arm. The VPA group had the lowest ratio of time spent exploring, indicating that this group spent the most time, though not significant, in the familiar arm. Overall, the control group explored both the novel and familiar arms for about the same amount of time. Control rats explored the novel arm for a significantly longer amount of time than VPA rats for both trials, and explored more than the VPA + FA group in trial 2.

Although the main effect of diet was not significant, this current study indicated a trend where folic acid supplementation reduced the repetitive behaviors expected in VPA rats. Much

like human based studies, the use of folic acid supplements during early pregnancy are associated with a decreased risk of autism (Surén P., Roth, C., Bresnahan, M., Haugen, M., Hornig, M., Hirtz, D., Lie, K.K & Lipkin, W.I., 2013; Blom, 2009). The VPA + FA group did significantly better than the VPA group in the first trial, performing close to Non VPA levels. As the VPA +FA group was not significantly different than the VPA group in trial 2 but was significantly different than the Non VPA animals the results remain inconclusive as to the protective nature of folic acid supplements. As the VPA +FA group did perform similarly to the controls suggests that there is a protective effect. The causality and significance of the current and previous findings remain unclear.

ABR

Early ABRs recorded in the current study did not resemble expected latencies of 1-10ms (Figure 3). The waveform sample rate, or event rate, was set to 1000Hz, theoretically high enough to represent data from lower frequency stimuli. However when the event rate does not record samplings fast enough without becoming aliased, smoothing out the wave function due to having too few data points (Figure 3. The sampling rate was adjusted to 50,000Hz and the ABR protocol was repeated, a latency similar to figure 4 was expected, as the sampling rate was enough to gather data points without losing the response curve. The result, while more pronounced than the previously generated recordings remained nearly 90ms long (Figure 2).

As the ABR protocol was unsuccessful, the current study was unable to determine if the auditory pathways of the VPA rat were significantly different than the auditory pathway of control rats. Nor was the current study able to determine the effects of folic acid on the auditory pathway. The protective effects of folic acid in development suggested by studies of folic acid intake (Smithell, et al., 1976, Blom, 2009) could lead to a protective effect in other areas of brain

development including the auditory pathway. The neural tube is not the only structure forming in early development, the inner ear and brain pathway for audition is one of the many tissue specializations that occur during the first trimester (Moore & Linthicum, 2007). An excess of folic acid, which plays a role in the process of methylation (Rogers, 2008), in the developing auditory pathway could prevent structural abnormalities, as the process of DNA methylation alters gene expression. However, the opposite could also be possible. Where an excess of folic acid may cause an adverse change in gene expression leading to elevated auditory thresholds, an indication of lower auditory perception or a delayed ABR response. A delayed response indicates a delay in signal transduction. The data remain inconclusive as to whether folic acid in excess would cause a protective or detrimental effect on the auditory pathway. Had ABR recordings been successful, the comparison of the control, VPA and VPA +FA groups may have been able to indicate whether the folic acid had a protective effect against the VPA in which case the latencies of the VPA + FA group would be comparable to the latencies of the control group. Conversely, if folic had an adverse effect on the development of the auditory pathway the latencies of the VPA + FA group would have shown a delay.

Limitations

The lack of viable ABR recordings limit the study in terms of relating back to human and other animal studies. The ABR protocol assumes the auditory pathway for mice an rats are comparable, there is a possibility of the peaks of an ABR corresponding to slightly different areas of the auditory pathway. The protocol also uses frequencies that are on the lower range of the rat's auditory range. Previously published studies use frequencies usually no lower than 2000Hz, attempting the ABR protocol at higher frequencies may produce a rapid latency, 1-

10ms, that was not found in the current study. The sample sizes were small, the power of the statistical analysis would be greater with a larger sample, with the exclusion of outliers in the behavioral data the conclusions that can be drawn from the current study are tentative. The weak interaction effect of treatment and Y maze trials indicates that further research should be done to determine if the interaction was due to individual differences in the animals. Future research should explore the relationship between animals treated with folic acid and non treated animals to determine if excess levels of folic acid increase levels of cognitive behaviors beyond the controls or if the excess has an adverse effect on normally developing animals.

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Appendix A. ABR Procedural Troubleshooting

The ABR is an electroencephalographic response measured with subcutaneous electrodes. A sequence of short tonal pips or clicks is presented to an anesthetized animal very quickly; each of these clicks evokes waves of neural activity in the brainstem that are computer-averaged so they are differentiated from non-auditory background voltages. The results follow the neural activity of the auditory nerve. The ABR latencies in the rat have not been fully determined, but the neuroactivity of mice ABRs probably reflects the neural activity from the auditory nerve (P1), the posterior anteroventral cochlear nucleus (P2), the anterior anteroventral cochlear nucleus and the trapezoid body (P3), the medial superior olivary nucleus (P4), and the lateral lemniscus and/or the inferior colliculus (P5) respectively (Figure 5).

The ABR latency procedure used in this experiment was a conglomeration of ABR mouse protocols and troubleshooting. The basis of the procedure was borrowed from Church, Jen, Stafferton, Hotra & Adams (2007) and Willott (2005). As this was an untested protocol, a significant amount of troubleshooting was necessary before the protocol produced reliable recordings. The recordings, when analyzed were determined to be unsuccessful recordings of the fast response ABR (Figure 3).

ABR recordings were initially taken at multiple decibel levels (between 120 and 70 dB) to determine optimum recordings. Recordings taken at 100dB and high showed high levels of background ‘noise’ likely due to the animal moving. Responses at 80 dB were determined to provide the cleanest data, and were used for all other recording sessions.

When recording ABR latencies it is important to maintain the body temperature of the rat as a change in temperature of 0.5°C can alter the ABR recordings (Rossi and Britt, 1984). The heating pad used in this experiment was an electric heating pad and thus interfered with the recordings. So, the heating pad was used to maintain the temperature of the animal during setup and then it was turned off during recordings.

The speaker should be equidistant from both pinnae to avoid a sound travel delay between the ears. The decibel level was determined before each session and the animal was placed approximately 10cm from the front and center of the speaker.

An average should consist of at least 250 responses; it is recommended that at least 300 responses are recorded per frequency (Willott, 2005). To account for anomalies and./or interference, the stimuli was presented for 2 minutes, or 2280 tones per sound frequency.

Appendix B. Tables

Table 1 *Summary of ANOVA for the Effects of Treatment on Exploration*

Source	*SS*	*df*	*MS*	*F*	*P*	η^2
Between Groups						
Treatment (A)	0.751	2	0.375	2.323	0.123	
Error	3.395	21	0.162			
Within Groups						
Trials (B)	0.020	1	0.020	0.946	0.342	
Interaction (A_xB)	0.163	2	0.082	3.877*	0.037	
Error Within	0.443	21	0.021			
Total	4.772	47				

*$p < 0.05$

Table 2. *Table of Means for Effect of Predisposition on Y-Maze Exploration*

	Exploration of Environment			
	Trial 1		Trial 2	
	M	*SD*	*M*	*SD*
Treatment				
VPA and FA	1.045	0.357	0.943	0.204
Non VPA	1.076	0.245	1.129	0.160
VPA	0.671	0.502	0.849	0.240

Note: Numbers ≥ 1 indicate a longer amount of time spent exploring a novel environment instead of a familiar environment.