The Effects of the Type and Timing of Dietary Folate on Memory, Learning, and Gene Expression in Mice

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Acceptance of Senior Honors Thesis

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Abstract

Folate has been known as an important vitamin for several decades. It is vital in development and epigenetics and is especially known for its involvement in the prevention of neural tube defects in newborns. Due to this ability, a synthetic form of folate, folic acid, was mandated by the government to be reinforced into cereals and grains. This study used a mouse model to determine the effects folic acid and 5-methyltetrahydrofolate have on gene expression and behavior. Since folate is known to be important for epigenetics and neuronal development, this study examined the effects of folate by applying behavioral tests to test memory and learning and a microarray analysis to test for gene expression changes. It was confirmed that folate is important for properly functioning cognition in mice. Additionally, the microarray analysis showed clear gene expression changes between the folate replete mice and the folate deficient mice. Interestingly, it was observed that the natural form of folate, 5-methyltetrahydrofolate, may confer long term benefits compared to the synthetic form, although additional research is needed to confirm this.

Keywords: folate, folic acid, vitamin B9, 5-methyltetrahydrofolate, cognition, memory, mice, Barnes maze, novel object recognition, Y-maze, microarray

The Effects of the Type and Timing of Dietary Folate on Memory, Learning, and Gene Expression in Mice

Folate is the generic term for a family of B vitamins that have been shown to be important in the prevention of several different conditions, such as alcohol liver disease, lung cancer, and especially neural tube defects (NTD) in early pregnancy (Scaglione & Panzavolta, 2014). Scientists first realized the physiological importance of folate in 1962 when Victor Herbert consumed a folate deficient diet and suffered symptoms of insomnia, irritability, fatigue and forgetfulness (Williams et al., 2005). After he switched back to a folate replete diet the symptoms vanished (Williams et al., 2005). Since then it was discovered that folate is involved in various epigenetic processes such as DNA methylation and in the production of nucleotides and neurotransmitters (McGarel, Pentieva, Strain, & McNulty, 2015). Because of this folate is an important nutrient for brain development. Additionally, humans are incapable of producing their own folate and must obtain it from their diet (Scaglione & Panzavolta, 2014). Folate is naturally found in a variety of fruit and vegetables while a synthetic form is used in dietary supplements and food fortifications (Scaglione & Panzavolta, 2014).

Folate Linked Conditions

While NTDs are a serious problem, folate has been shown to significantly decrease the chance of them occurring (Sutton, Daly, & Kirke, 2008). This is because of the importance of folate during development due to its involvement in epigenetics through DNA methylation. Additionally, folate has also been linked with the prevention of alcohol liver disease and some forms of cancer (Hirayama, Lee, Terasawa, & Kagawa, 2010; Medici & Halsted, 2013). Although in the case of cancer, it seems that it is important that the folate be administered before it develops. Folate is also positively

associated with cognition in both humans and mice (Jadavji, Deng, Malysheva, Caudill, & Rozen, 2015; McGarel et al., 2015).

Neural Tube Defects

NTDs are malformations of the central nervous system that occur in utero and can cause serious disabilities (Sutton et al., 2008). NTDs happen during embryonic development when the neural tube unsuccessfully closes exposing the neural tissue underneath and leading to neural tissue damage (Crider, Bailey, & Berry, 2011). In the 1960s folic acid deficiency of pregnant mothers was identified as a major risk factor for NTDs in newborns (Crider et al., 2011). This is because folate is a very important nutrient for the developing fetus and is heavily used by it, contributing to an average drop of 50% in maternal folate concentration (McGarel et al., 2015). Periconceptionally taken folic acid was shown to prevent up to 70% of NTDs (Sutton et al., 2008). The two most common forms of NTDs are anencephaly and spina bifida (Sutton et al., 2008). Anencephaly is the most serious NTD as babies with anencephaly are born without large portions of their brain and rarely live past a few days. Spina bifida is a broad term that includes a wide range of malformations such as caudal lesions on the spinal cord or vertebrae (Mitchell et al., 2004). Those afflicted with spina bifida are at serious risk for orthopedic abnormalities, bowel and bladder dysfunction, leg weakness and paralysis, and sensory loss (Mitchell et al., 2004).

Alcohol Liver Disease and Cancer

In addition to helping reduce the risk NTDs, folate has been shown to play an important role in other diseases. Folate deficiency has been associated with the development of alcoholic liver disease (ALD) (Medici & Halsted, 2013). Chronic

alcoholism decreases intestinal absorption and renal reabsorption of folate which leads to folate deficiency which contributes to the development of ALD (Medici & Halsted, 2013). This was demonstrated in a study on micropigs which showed that micropigs who were given ethanol and a normal diet developed ALD much slower than those who were fed a folate deficient diet and ethanol (Medici & Halsted, 2013). Furthermore, it has been shown that supplementation of S-adenosylmethionine (SAM), which folate is needed to produce, compensates for decreased folate absorption and helps prevent ALD in animal models (Medici & Halsted, 2013).

Folate has also been demonstrated to play an interesting role in the prevention of cancer (Smith, Kim, & Refsum, 2008). One study showed that in women who were 55-74 years old that folic acid consumption greater than 400 mcg/day led to an increase in breast cancer as long as cancer cells were already present (Smith et al., 2008). However, if cancer was not present at the beginning of supplementation, than the increase of folic acid provided a protective effect against lung, colon, prostate, and breast cancer (Smith et al., 2008). The protection folate provides before cancer has started is due to its role in epigenetics. On one hand, If folate is plentiful before cancer starts it helps regulates gene expression properly and thus helps prevent the onset of cancer (Smith et al., 2008). On the other hand, since folate is needed for nucleic acid synthesis it is needed for cancer cells to proliferate which is why some anti-cancer drugs are anti folate drugs (Smith et al., 2008).

Cognitive Effect on Humans

It has been shown that increased folic acid supplementation during the first trimester of pregnancy is correlated with improved verbal performance, motor

development, and neurodevelopment in 4 year old children (McGarel et al., 2015). A randomized controlled trial (n of 39) demonstrated that mothers supplemented with folic acid in the second and third trimester gave birth to children who at age 3 scored higher on the cognitive portion of the Bayley's development assessment (McGarel et al., 2015). Folate has proven to be important for cognition after pregnancy as well. A study on North Indian children aged 12-18 months showed a link between low folate and vitamin B-12 levels and reduced cognitive potential (Strand et al., 2013). Another study on children ages 6-16 showed a correlation between higher serum folate and an improvement in reading scores and block design (Nguyen, Gracely, & Lee, 2013). Additionally, high levels of homocysteine, which is usually caused by a deficiency of folate, combined with low serum folate levels have been identified as risk factors for depression and various forms of dementia such as Alzheimer's disease (Miller, 2008; Reynolds, 2006).

Cognitive Effect on Mice and Rats

Folate has been shown to be important for cognition in mice as well as in humans. It was shown that folate deficient mice gave birth to mice with poor short term memories at three weeks and increased cellular apoptosis in their hippocampi (Jadavji, Deng, et al., 2015). It has also been shown that high levels of homocysteine due to folate deficiency combined with impaired DNA repair results in cognitive deficiency in mice (Jadavji, Farr, et al., 2015). Possible mechanisms for this observed cognitive decline could be related to the levels of plasma homocysteine which are elevated under folate deficient conditions (Jadavji, Farr, et al., 2015). Elevated homocysteine has been shown to impair spatial memory, long term memory, and short term memory in mice (Jadavji, Farr, et al., 2015). These cognitive impairments could be caused by a myriad of different factors as

elevated homocysteine can cause an increase in reactive oxygen species in the brain, negatively impact vascular function, promote cytotoxicity by oxidizing membrane proteins and lipids, inhibit nitric oxide, reduce levels of SAM, cause excitotoxicity in cells via recurring activation of the n-methyl-D-aspartate receptor, lower levels of acetylcholine, and elevate levels of apoptosis (Jadavji, Farr, et al., 2015).

Biochemical Pathways of Folate

Folate is extremely important in the methylation cycle and in the synthesis of precursors for nucleic acid (Scaglione & Panzavolta, 2014). Before folic acid can be used it has to be converted to tetrahydrofolate (THF) via a two-step reaction with a dihydrofolate intermediate by the dihydrofolate reductase enzyme (Scaglione & Panzavolta, 2014). Once folic acid is reduced to THF it then undergoes a reaction catalyzed by the enzyme serine hydroxymethyltransferase (SHMT) to produce glycine and 5,10-methylenetetrahydrofolate (Scaglione & Panzavolta, 2014). Afterwards, 5,10-methylenetetrahydrofolate is either involved in the formation of the DNA base thymidine, or it is converted by the riboflavin dependent enzyme methylenetetrahydrofolate reductase (MTHFR) to 5-methyltetrahydrofolate (5-MTHF) which is the most common form of folate found in plasma (McGarel et al., 2015). 5-MTHF is used to remethylate homocysteine to methionine via the B_{12} dependent enzyme methionine synthase which takes the methyl group off 5-MTHF converting it back to THF (Scaglione & Panzavolta, 2014). Methionine is then used to make SAM (Scaglione & Panzavolta, 2014). In protein and DNA methylation SAM is one of the primary methyl donors which is why folate is important in DNA methylation and thus DNA regulation (Scaglione & Panzavolta, 2014). After the methyl tag is removed from SAM to methylate its target, SAM becomes S-

adenosyl homocysteine which is converted into homocysteine which can then begin the process again (Scaglione & Panzavolta, 2014). Because folate is needed to convert homocysteine into methionine, homocysteine levels can be used as an inversely proportional indicator of folate level as without folate homocysteine will not be remethylated to SAM and thus its levels will build up (Scaglione & Panzavolta, 2014).

SAM itself has been shown to be important in mood regulation via its involvement with neurotransmitter synthesis (Williams et al., 2005). SAM has been demonstrated to raise the levels of 5-hydroxyindoleactic acid in the cerebrospinal fluid (Williams et al., 2005). 5-Hydroxyindoleactic acid is the primary metabolite for the synthesis of the neurotransmitter serotonin (Williams et al., 2005). Low levels of serotonin are correlated with depressive illnesses and folate deficiency has been shown to decrease serotonin activity.

The two commonly ingested forms of folate are 5-MTHF and folic acid. Folic acid is an oxidized synthetic form of folate not found in nature that is not metabolically active and must first be converted to the active THF form through a few steps one of which includes the MTHFR enzyme (Simmons, 2013). This can cause problems with those who have MTHFR deficiency and receive folic acid as their source of folate (Knowles, Morris, & Walter, 2016; Scaglione & Panzavolta, 2014). Folic acid is used in food fortification because it is more stable than most other forms of folate and can survive the baking process (Green et al., 2013). 5-MTHF is a biologically active form of folate and makes up over 90% of the folate found in plasma (Scaglione & Panzavolta, 2014). 5-MTHF has some known advantages over folic acid such as an improved

effectiveness at increasing serum folate (Scaglione & Panzavolta, 2014). Additionally, 5-MTHF does not need to be converted to an active form by the MTHFR enzyme.

Mandated folic acid reinforcement

Since NTDs were a serious but preventable problem many educating campaigns came about to warn women of the dangers of having children on a folate deficient diet and to encourage them to either eat folate rich food or to take supplements (Crider et al., 2011). Although these education programs encouraged women to take at least 400 µg of folic acid a day prior to pregnancy to decrease NTDs, many women either were ignorant of this advice or failed to comply with it (Crider et al., 2011). Additionally, half of pregnancies are unplanned and so many women wouldn't think to follow the advice and increase folate intake until it was too late (Crider et al., 2011). Although folate is naturally found in certain types of food many people still had folate deficient diets for a few reasons. One reason is due to the fact that naturally occurring folate is unstable and up to 30% of it is destroyed in food processing (Scaglione & Panzavolta, 2014). Another factor is that some people, such as those in lower socioeconomic groups, have limited access to folate rich foods such as fresh fruit and vegetables or are turned away by the higher price of such foods (Scaglione & Panzavolta, 2014). Because of all the preventable cases of NTDs due to insufficient dietary folate, the United States government began implementing mandatory fortification of cereal grains with folic acid in 1996 (Crider et al., 2011). This mandatory reinforcement was effective at reducing the occurrence of NTDs by around 19-32% (Crider et al., 2011).

Although the reinforcement of folic acid into various foods has had clear benefits, there are still some important questions about folic acid and folate in general that need to

be answered. Is folic acid completely equivalent in terms of physiological effects compared to other forms of folate? Aside from early development what long term effects does folate have?

Aspects of Study

While folic acid has proven effective at decreasing NTDs at birth, not as much research has been done on its longitudinal effects after birth. Additionally, there is the question of whether the differing pathways 5-MTHF and folic acid take to enter the folate cycle might cause them to have differing long term effects. This study will test the gene expression changes and learning and memory capabilities of mice over 18 months on various folate diets by using a DNA microarray test and the Spontaneous Alternation Y-maze, Novel Object Recognition, and Barnes Maze behavioral tests.

Behavioral Tests

One key aspect of this study are the behavioral tests performed to test the learning and memory of the mice involved. While genetic analysis of hippocampal tissue samples is useful, it is important to confirm that any changes in gene expression also cause a noticeable change in the capabilities of the mouse (Crawley, 2007). This is because behavior is the definitive manifestation of the central nervous system and any significant changes to it should be noticeable via behavioral tests (Crawley, 2008). Due to this there is an abundance of literature on tried and true methods for testing the motor functions, sensory abilities, learning and memory, social interactions, feeding and drinking, drug and alcohol self-administration, and traits related to depressions, schizophrenia, and anxiety of mice (Crawley, 2008).

In this study the Y-Maze Spontaneous Alternation test was chosen to test for working memory, the Novel Object Recognition Test was picked to determine learning and memory, and the Barnes Maze test was selected to examine spatial learning and memory. While these tests are not the only ones that could have been used, they were chosen because they were fairly inexpensive, less time intensive than similar tests, and not stressful for the mice. This was due to budget and time constraints and it was worried that an overly stressful test could cause physiological changes in the mice and affect the gene expression analysis performed later. For instance the Morris Water Maze is a gold standard for spatial memory and learning but it would have been more expensive, taken more time, and been more stressful for the mice than the Barnes Maze.

Behavioral tests are not without their fair share of problems. Firstly, there is a great deal of built in variability between mice that can confound statistical detection of moderate phenotypical changes unless the number of mice is fairly large, 10-20 per group usually being sufficient (Crawley, 2008). Unfortunately, due to limitations of time, space, and funding only an N of 3 was achieved for each group. This was due to each litter being averaged and scored as an N of 1 to account for littermate similarities. Secondly, behavioral testing usually falls into one of two pitfalls in regards to scoring. Either the test is scored manually which is very time intensive and prone to subject variability between scorers or it is scored automatically via camera and computer software which is prohibitive due to its high cost (Patel et al., 2014). While the former method was used in this study, only one individual was responsible for scoring all the tests to eliminate any inter operator variability. Thirdly, behavioral tests are susceptible to a wide variety of environmental factors which includes vivarium noise levels, handling, and season among

others (Crawley, 2008). While there are difficulties in performing behavioral tests on rodents, as long as sufficient care is taken and methods are conducted appropriately, the results should be replicable by other researchers (Crawley, 2008).

Gene expression

As folate is involved in DNA methylation it is likely that its form or absence will affect DNA expression (Bhargava & Tyagi, 2014). When a certain protein is needed the appropriate gene will be transcribed or expressed to produce an mRNA transcript that will then be read to make the protein. Thus the various needs of a cell for certain proteins will be represented in the number of mRNAs that make that protein at any given time. One way the cell regulates the expression of genes is by DNA methylation. This is due to the fact that DNA methylation is involved in epigenetics which concerns how DNA and its genes are used. When a methyl tag is placed on a gene it turns the expression of the gene off or down. Thus by altering a source of methyl tags such as folate which is considered to be a major driving force of epigenetics, it is likely the expression of various genes will be altered (Bhargava & Tyagi, 2014). Because of this gene expression analysis of the hippocampus by microarray will be a significant portion of this study.

Materials and Methods

In this study a CD-1 mouse model was use with three litters being raised on 4 separate diets. The Y-Maze Spontaneous Alternation test was performed when the mice were approximately 4 months old. At approximately 4, 12, and 18 months the mice were tested using the Novel Object Recognition test. The Barnes Maze test was run when the mice were roughly 10 and 17 months old. Tissue samples were collected at around 4 and 18 months for microarray analysis.

Mouse model

The breed of mice used in this study was CD-1. Initially 12 adult females and 6 adult males were purchased. Half of the males and females were given a diet with folic acid while the other half was given 5-MTHF as Metfolin. After a couple weeks each of the males was allowed to breed with 2 females of the same diet. The result was 6 litters of pups from mothers on folic acid and 6 litters from mothers on 5-MTHF. After the pups were weaned, half of each set of 6 were switched to diets completely deficient in any source of folate. The result was four diets: folic acid, folic acid till weaning and then deficient, 5-MTHF, and 5-MTHF till weaning and then deficient. These diets will be referred to as FA, FA deficient, 5-MTHF, and 5-MTHF deficient respectively. The mice were given food completely deficient in folate and the B6 and B12 vitamins. All the mice were given B6 and B12 in their water and the FA and 5-MTHF mice were given their folate source in their water as well. The water was made and replaced every 2-3 days using B6 and B12 stock solutions kept at 4C and 5-MTHF and FA pills were added to the water. The mice were raised for approximately 18 months and behavioral tests were performed over this period.

Y-maze Spontaneous Alternation Test

The Y-Maze spontaneous alternation test is used to examine spatial working memory and relies on the proclivity of mice to explore where they have least recently been (Wolf, Bauer, Abner, Ashkenazy-Frolinger, & Hartz, 2016). This test was performed on all the mice at 4 months. The Y-maze was from Stoelting Co. It consisted of hard grey plastic with arm widths of 5cm, arm lengths of 35cm, and a wall height of 10cm. The maze was unbaited and the number of spontaneous alternations and possible

alternations were recorded and a percentage of spontaneous alternations calculated. A spontaneous alternation was defined as consecutive entry into all three arms and the alternation percentage was calculated by dividing the number of alternations by the number of possible alternations. A mouse was considered to have entered an arm when at least 3 paws were entirely inside it. The mice were given 2 minutes to run the maze and a second trial was performed 1 week after the first and the values for each litter were averaged together giving an n of 3 for each diet. If a mouse climbed out of the maze during the trial its results were left out.

Novel Object Recognition Test

A novel object recognition test was performed to determine long term memory in mice and relies on the inclination of mice to investigate a novel object for longer than a familiar one (Wolf et al., 2016). An 18 inch by 30 inch cardboard box was used with a plexiglass bottom for ease of cleaning. Three trials at approximately 4 months, 12 months, and 18 months were performed. The identical objects for the first trial consisted of two identical T-25s filled with dyed blue water and the novel object was a similarly shaped green Lego tower. The objects for the second trial consisted of two 50 ml conicals filled with dyed blue water and the novel object was a similarly sized green Lego construct. For the third trial two rubber green turtles were used as the identical objects and the novel object was a blue rubber dolphin. The mice were given 5 minutes the first day to become familiar with the two identical objects. Approximately 24 hours later they were placed in the box again with one of the identical objects having been replaced with the novel object. The mice were then given 5 minutes to examine the objects and the time

spent investigating each object was recorded. Results with a combined total of 9 seconds or less investigating either object were automatically discarded.

Barnes Maze

The Barnes maze was performed to test the mice's spatial memory and learning and relies on the tendency of mice to hide when anxious. The Barnes Maze table had a diameter of 92cm with 20 equally spaced holes around the edge each with a diameter of 5cm. It was made of plywood with a smooth blue lamination. The escape box and ramp were made of similar material and covered with the same lamination. Motivation to escape was provided by a 90 decibel white noise maker and overhead lights. The escape box with the ramp could be placed under any of the holes via a rotation arm underneath the table. Visual cues were provided by the asymmetry of the testing room and by 4 different colored notecards cut into the shape of a square, circle, triangle, and cross and placed equidistant around the table. These cues were provided so the mice would have something to give them a sense of direction that would let them remember the location of the escape cage from previous trials. The mice were tested at approximately 10 months and 17 months. The test was an abbreviated version run over 4 days. The mice were acclimated to the maze on day 1. This was done so they'd be used to the environment and table where the test was performed. Three trials were performed on each mouse on both day 2 and day 3 with approximately 15 minutes between each trial. The mice were given 3 minutes to find the escape cage on these days. After every trial the table and escape cage were washed with 70% ethanol to remove any olfactory cues that might affect the next trial. The probe trial for the 10 month time point was run on day 4 with the maze rotated 180 degrees from its original location and the mice were given 4 minutes to find

the cage. This method proved problematic and gave poor results so the probe trial on the second trial at 17 months was performed by removing the escape cage on the last day and allowing the mice 3 minutes to wonder around and primary latency was recorded.

Tissue Collection

When the mice were 4 months, 12 months, and 18 months old, 1 mouse from each litter was euthanized and tissue samples harvested. The primary method for euthanasia was CO_2 chamber and the secondary method was cervical dislocation. The hippocampi were isolated quickly after death and placed into RNA later homebrew (16.67 mM Sodium Citrate, 13.33 mM EDTA, 46.7 g/100 ml ammonium sulfate and a pH adjusted to 5.2 using H_2SO_4), allowed to soak in it for 1 day at 4C, and then stored at -80 C.

RNA Extraction

RNA was extracted by adding trizol to the tissue and homogenizing using a bead beater. Afterwards the samples were spun with chloroform and the aqueous phase kept. Isopropanol was added to the aqueous phase and spun again. The pellet was washed with 75% ethanol and vortexed and then spun again. The pellet was then air dried and resuspended in water and stored at -80 C.

DNA Microarray

The extracted RNA samples were then sent to Arraystar Inc for gene expression analysis. Due to time and budget constraints only the RNA for the 6 litters in the FA and FA deficient diets at 4 and 18 months were sent in. They quantified the total RNA in each sample by using the NanoDrop ND-1000 and assessed the RNA integrity using standard denaturing agarose gel electrophoresis. They ran the samples using Arraystar Mouse LncRNA Microarray V3.0 which determines the expression profile for both mRNA and

long non coding RNAs. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications made by Arraystar. The mRNA was purified from the total RNA using the mRNA-ONLY^{TM} Eukaryotic mRNA Isolation Kit from Epicentre. Data analysis was done using Agilent Feature Extraction Software to analyze the array images and using GeneSpring GX v12.1 software package (Agilent Technologies) for quantile normalization and subsequent data processing. After analysis, the data was sent back to us.

Results

Y-Maze Spontaneous Alternation Test

The Y-Maze tests were performed when the mice were 4 months old. The test was performed twice and the results averaged together. The spontaneous alternation percentages were nearly identical for all the different diets with values of 64.6% ± 3.5%, 64.4% ± 2.8%, 64.4%, ± 3.71%, and 63.0% ± 6.4% of spontaneous alterations for the 5-MTHF, 5-MTHF deficient, FA, and FA deficient diets respectively (Figure 1). Analysis of all the mice with either low or high number of entries as a potential grouping of outliers showed nothing noteworthy. No statistically significant differences between the mice on the different diets was observed.

Novel Object Recognition Test

Data for the novel object test were collected at 4 months, 12 months, and 18 months. The percentages of time spent inspecting the novel object at 4 months were 54.9% ± 6.0%, 51.1% ± 1.08%, 59.7% ± 0.4%, and 47.4% ± 4.7% for the 5-MTHF, 5-

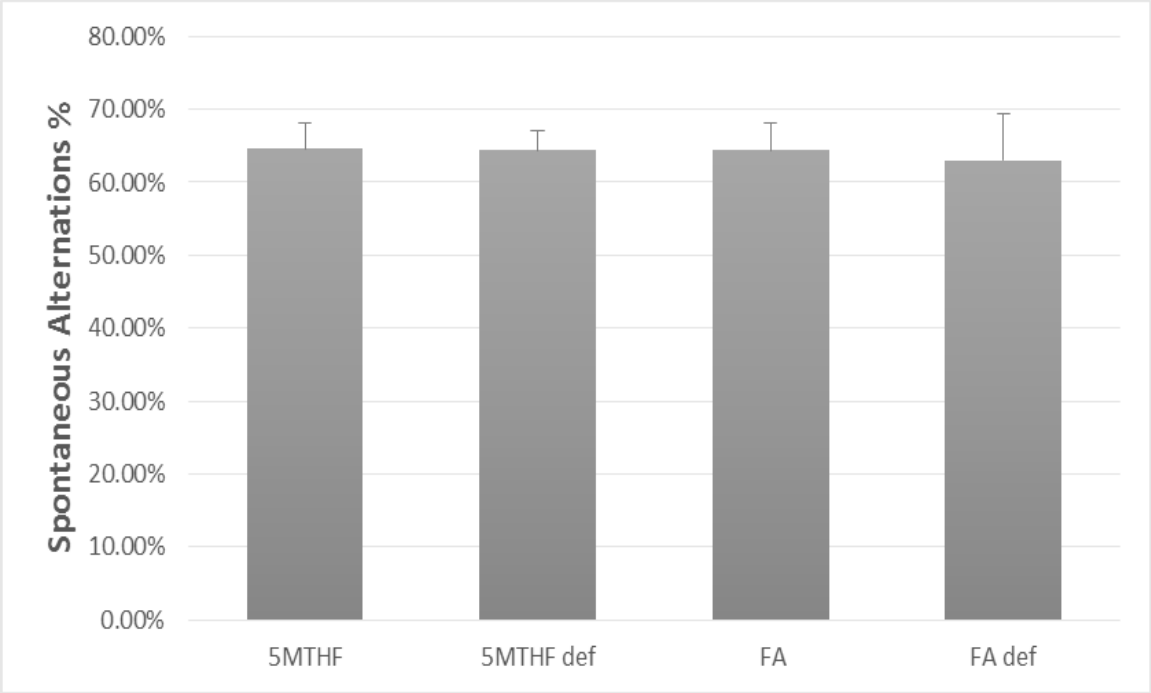

Figure 1. Y-Maze Spontaneous Alternation Test results at 4 months. The % of spontaneous alternations was recorded for each mouse over 2 minutes. Mice from every diet had similar percentages of spontaneous alternations.

MTHF deficient, FA, and FA deficient diets respectively (Figure 2). The percentages of time spent inspecting the novel object at 12 months were 61.0% ± 1.3%, 59.8% ± 0.5%, 63.5% ± 6.6%, and 57.3% ± 5.0% for the 5-MTHF, 5-MTHF deficient, FA, and FA deficient diets respectively (Figure 2). The percentages of time spent inspecting the novel object at 18 months were 61.5% ± 6.3%, 62.5% ± 1.9%, 76.3% ± 2.6%, and 52.9% ± 1.5% for the 5-MTHF, 5-MTHF deficient, FA, and FA deficient diets respectively (Figure 2). At 4 months there were plenty of mice and all 4 diets had each of their 3 litters represented giving an n of 3 with no problems. At 12 months less mice remained and a litter from both 5-MTHF deficient and FA were not included giving an n of 2. At 12 months the unrepresented litters for 5-MTHF deficient and FA only had 3 and 2 mice left respectively. In the case of the 5-MTHF deficient trials one mouse would not participate and another mouse was favoring the novel object but its results were discarded

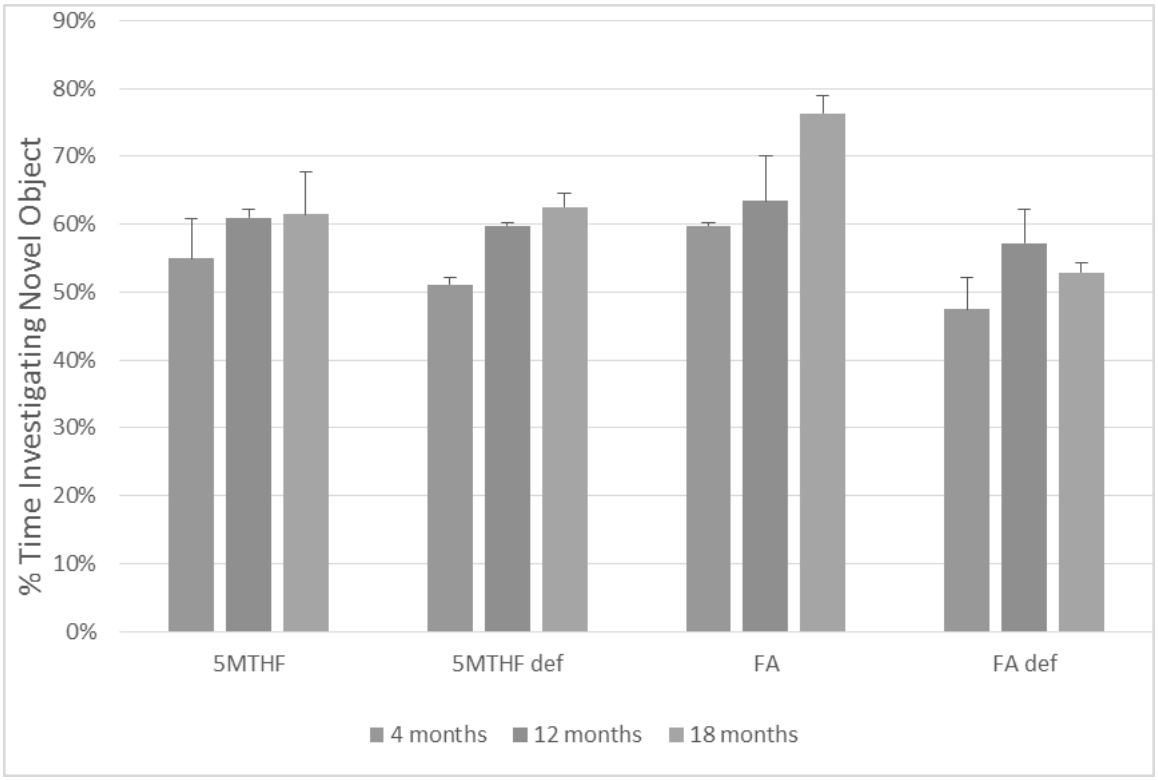

Figure 2. Novel Object Recognition Test Results at 4 months, 12 months, and 18 months. At 4 months the folate diets performed better than their deficient counterparts with FA's increase over FA having a P value of less than 0.05. At 18 months nothing statistically significant was observed although 5-MTHF deficient performed almost as well as the folate diets. At 18 months FA and 5-MTHF deficient performed remarkably well and surpassed FA deficient with P values less than 0.05.

due to stopwatch error. The remaining mouse's results were discarded due to being an outlier and likely unrepresentative of the litter and diet. The FA litter was discarded because the two mice performed far below the mice in the other two litters of the diet. At 18 months even less mice remained. In the previous two trials hard plastic objects had been used and the rubber objects picked for this time point proved problematic. At 18 months the objects were rubber bath toys and some of the mice chewed on them. Most of the mice who nibbled had fairly extreme values either for the old or new object which skewed the results. As such all results from mice who gnawed on the object were discarded which left many of the litters with only 1 mouse left. In the case of the 5-

MTHF deficient diet, one of its litter's results wasn't used as the one mouse left was below the 9 seconds cut off. The FA diet also had 1 litter left out of the results as it only had 1 mouse left which performed poorly compared to the other two litters and was only a fraction of a second away from the cutoff point of 9 seconds.

Barnes Maze Test

The first set of Barnes maze tests were performed over 1 month when the mice were approximately 9-10 months old. For this trial all the results of the mice from each litter were averaged together thus treating each litter as an n of 1 and giving an n of 3 for the diet. The primary errors (incorrect hole checks before the escape hole was found) and the primary latency (seconds before the escape hole was found) were recorded and averaged together at each trial for each diet.

The results for primary latency at 9-10 months from trials 1-6 of the 5-MTHF diet in order were 75 ± 25, 61 ± 11, 49 ± 20, 58 ± 41, 61 ± 22, and 22 ± 5 (Figure 3). The results of the 5-MTHF deficient diet in order were 94 ± 15, 73 ± 24, 84 ± 38, 66 ± 39, 84 ± 48, and 29 ± 10 (Figure 3). The results of the FA diet in order were 73 ± 20, 54 ± 10, 63 ± 23, 33 ± 8, 61 ± 21, and 32 ± 5 (Figure 3). The results of the FA deficient diet in order were 76 ± 15, 71 ± 20, 41 ± 14, 54 ± 12, 59 ± 2.0, and 52 ± 5 (Figure 3).

The results for primary errors at 9-10 months from trials 1-6 of the 5-MTHF diet in order were 11 ± 4.0, 6.7 ± 1.4, 5.6 ± 2.1, 7.7 ± 5.5, 6.2 ± 2.2, and 2.3 ± 1.1 (Figure 4). The results of the 5-MTHF deficient diet in order were 12 ± 3.5, 8.8 ± 2.6, 6.8 ± 0.60, 6.6 ± 1.9, 7.4 ± 1.3, and 4.4 ± 2.6 (Figure 4). The results of the FA diet in order were 8.6 ± 3.6, 5.8 ± 1.4, 6.8 ± 1.7, 4.3 ± 1.7, 6.4 ± 1.4, and 6.6 ± 2.3 (Figure 4). The results of the

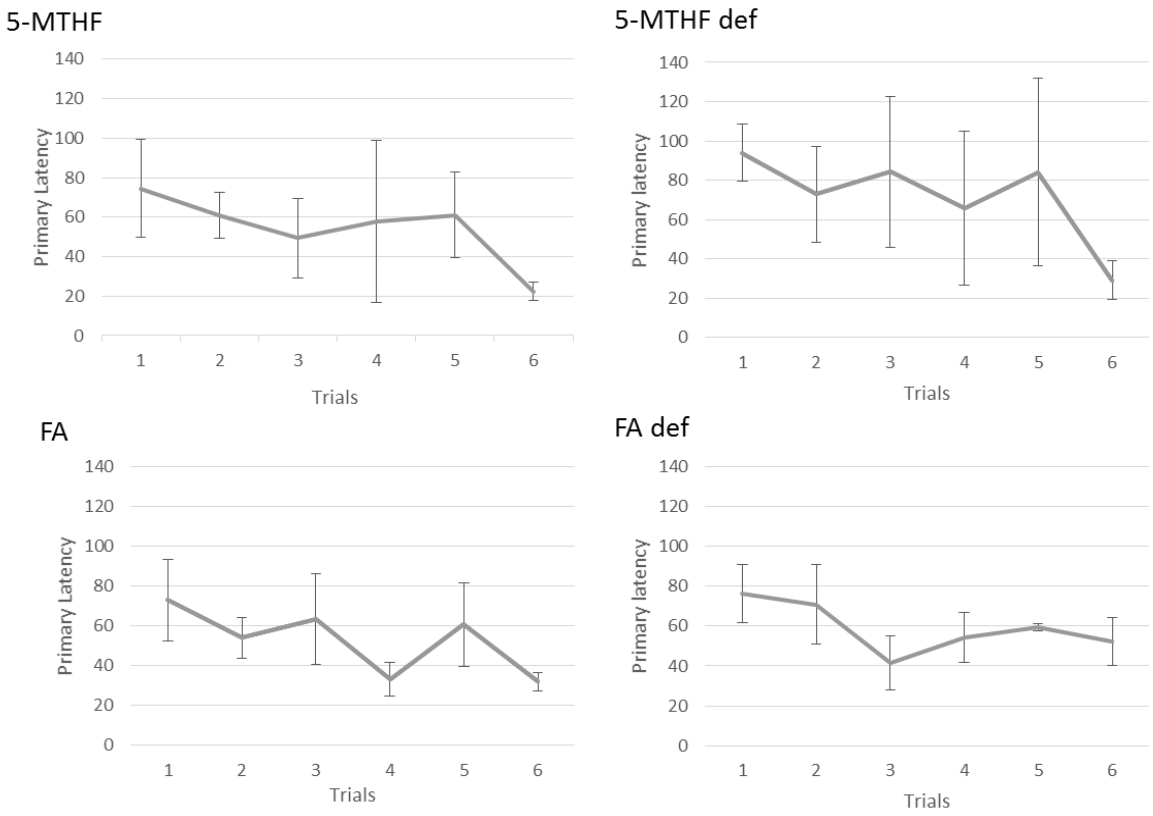

Figure 3. Barnes Maze Primary Latency at 9-10 Months. Primary latency, which is the time till discovery of the hole with the escape cage, was recorded for 6 trials with 3 trials being performed per day with approximately 15 minutes between each trial. Overall latencies decreases for all diets over the trials.

FA deficient diet in order were 9.8 ± 2.7, 9.6 ± 3.1, 5.2 ± 1.5, 4.1 ± 2.0, 6.4 ± 1.8, and 7.1 ± 1.7 (Figure 4).

The probe trial results for this time point are not shown as the method used proved problematic. The folate diets had less errors and a lower latency on both the first and last trial than their deficient counterparts. Additionally the 5-MTHF deficient mice outperformed the FA deficient mice. Unfortunately no significance was seen due to this test having a high degree of variability.

The second set of Barnes Maze tests were performed when the mice were about 17 months old and the primary latency and errors were recorded. At this there were far fewer mice left due to tissue collection and the occasional natural death. Due to having

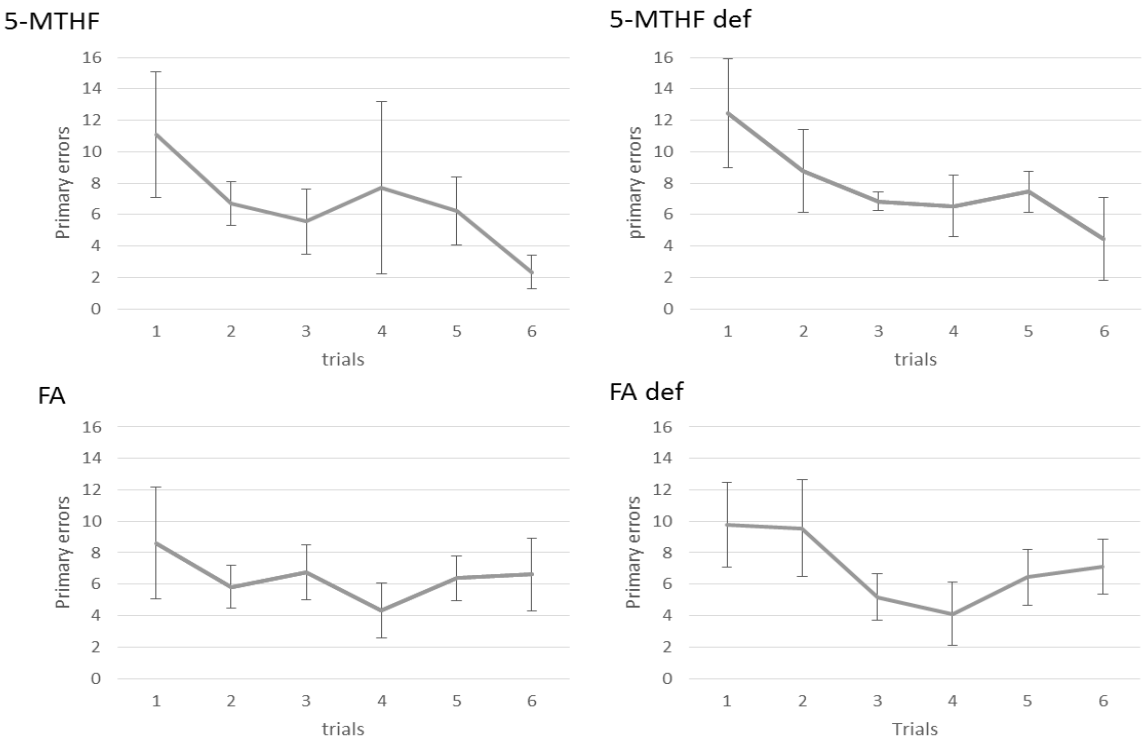

Figure 4. Barnes Maze Primary Errors at 9-10 Months. Primary errors, which are the mistakes made until discovery of the hole with the escape cage, were recorded over 6 trials with 3 being performed each day. Overall errors decreased for all diets from the first trial to the last.

fewer mice only 1 mouse per litter was used and the results averaged together per diet still giving the usual n of 3.

The results for primary latency at 17 months from trials 1-6 of the 5-MTHF diet in order were 23 ± 15, 54 ± 24, 33 ± 20, 33 ± 25, 23 ± 8.7, and 46 ± 16 (Figure 5). The results of the 5-MTHF deficient diet in order were 133 ± 47, 33 ± 13, 96 ± 49, 43 ± 19, 47 ± 39, and 52 ± 19 (Figure 5). The results of the FA diet in order were 76 ± 53, 61 ± 43, 74 ± 54, 40 ± 20, 61 ± 39, and 33 ± 25 (Figure 5). The results of the FA deficient diet in order were 143 ± 37, 180 ± 0, 136 ± 54, 79 ± 47, 76 ± 53, and 66 ± 38 (Figure 5).

The results for primary errors at 17 months from trials 1-6 of the 5-MTHF diet in order were 4.3 ± 2.8, 10.3 ± 4.3, 4.7 ± 1.7, 9.3 ± 8.8, 4.7 ± 2.7, and 9.7 ± 4.4 (Figure 6).

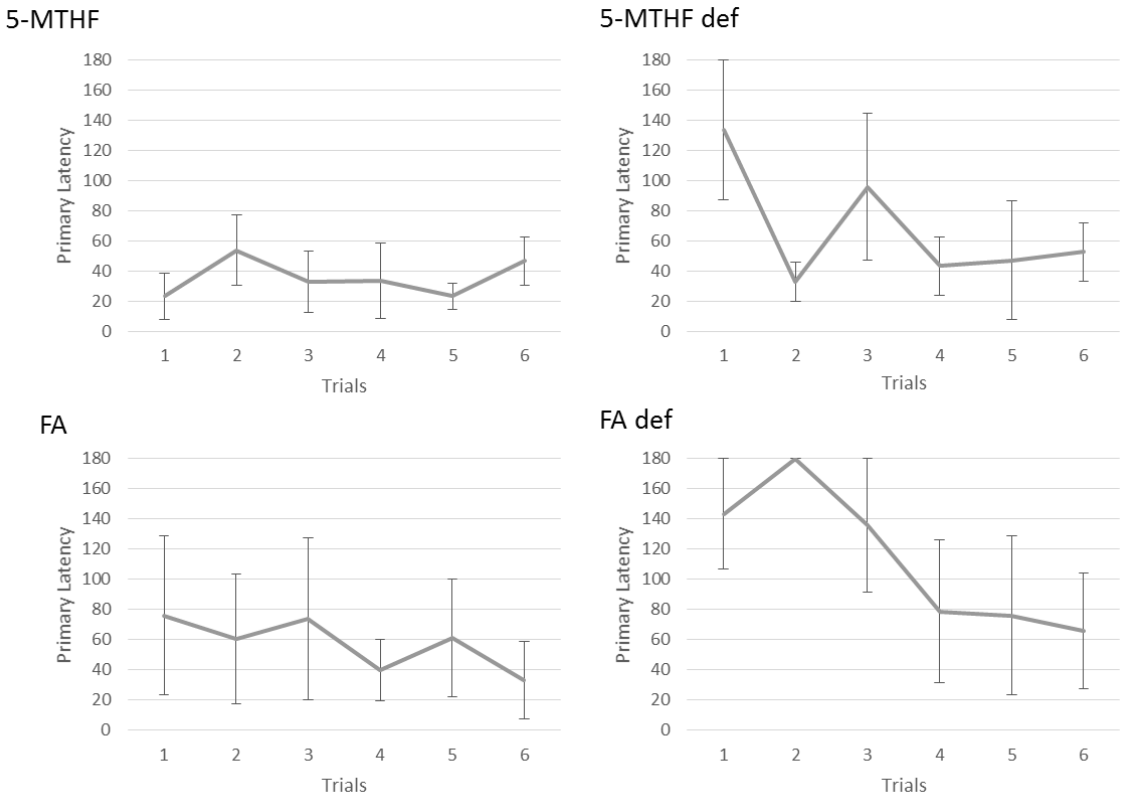

Figure 5. Barnes Maze Primary Latency at 17 Months. Primary latency was recorded over 6 trials with 3 trials being performed each day with approximately 15 minutes between each trials. The mice on the deficient diets had much higher latency for their first few trials compared to the mice on the folate diets who performed well from the beginning. Except for those on the 5-MTHF diet, all the mice performed better on their last day than on their first. The results probably display such a wide range of variability due to only having a few mice left in each litter left.

The results of the 5-MTHF deficient diet in order were 14 ± 2.6, 2.7 ± 0.7, 14 ± 6.2, 12 ± 5.9, 3.3 ± 7.7, and 13 ± 6.4 (Figure 6). The results of the FA diet in order were 11 ± 7.7, 6.3 ± 2.8, 9.3 ± 5.0, 8.3 ± 4.3, 7.7 ± 2.8, and 1.7 ± 0.7 (Figure 6). The results of the FA deficient diet in order were 10 ± 3.2, 11 ± 2.3, 14 ± 5.5, 5.3 ± 2.6, 5.3 ± 2.4, and 3 ± 2.1 (Figure 6). The results for the primary latency for probe test were 17 ± 5.8, 88 ± 41, 52 ± 42, and 83 ± 37 for the 5-MTHF, 5-MTHF deficient, FA, and FA deficient diets respectively (Figure 7).

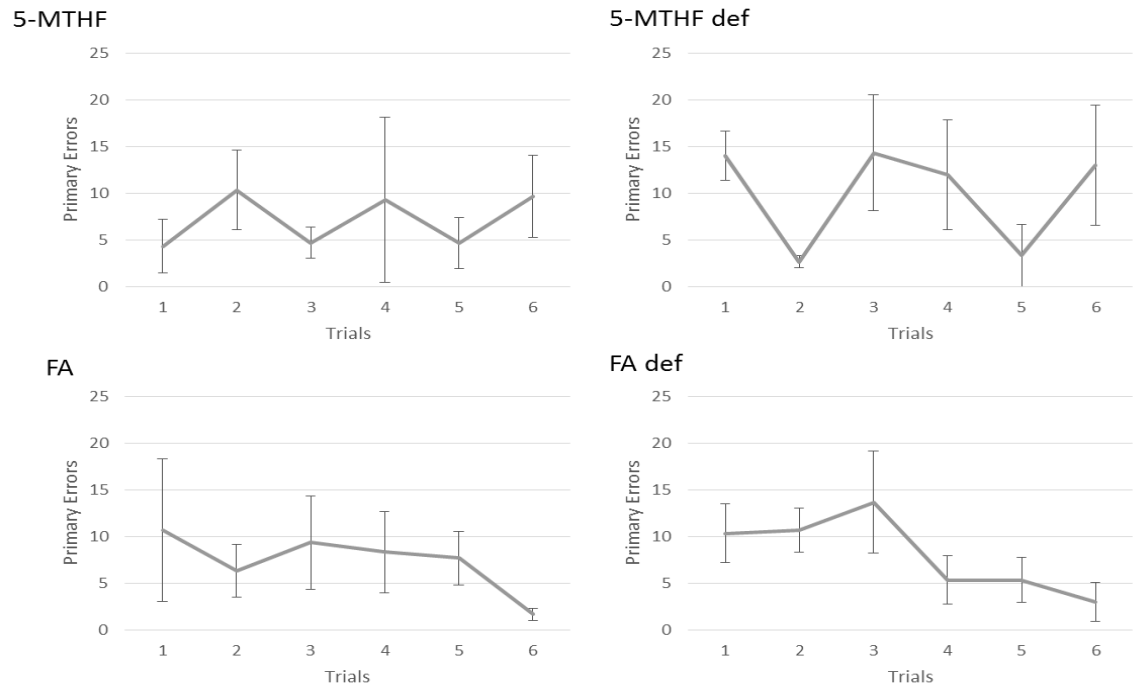

Figure 6. Barnes Maze Primary Errors at 17 Months. Primary errors were recorded over 6 trials with 3 trials being performed each day with approximately 15 minutes between each trials. The results display a wide range of variability due to the low amount of mice left in each litter at this time point, especially in the 5-MTHF and 5-MTHF deficient mice.

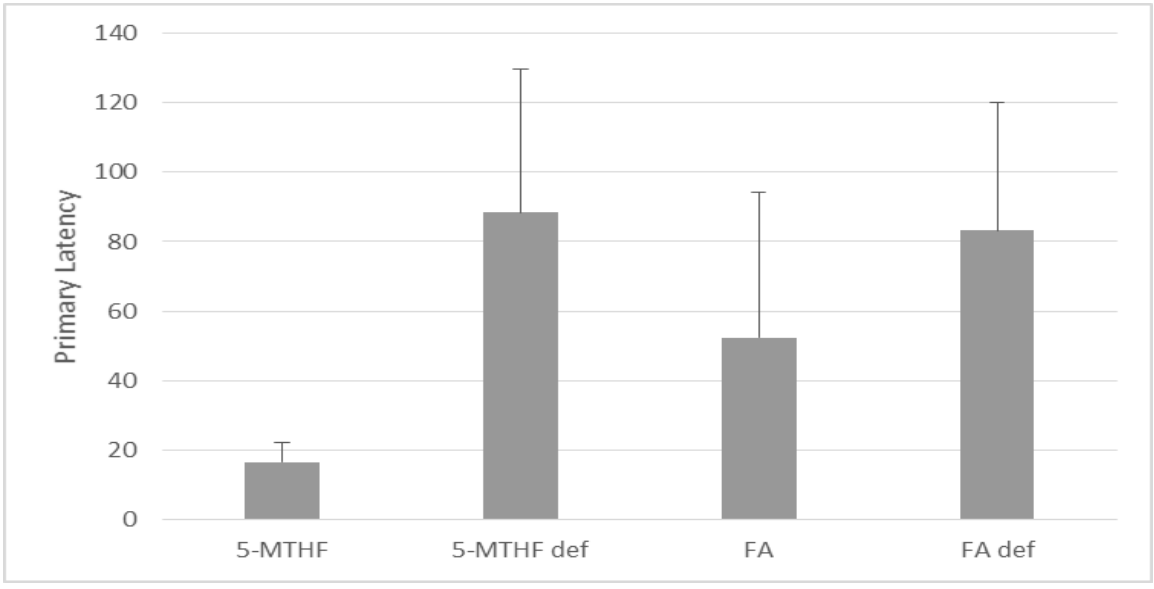

Figure 7. Barnes Maze Primary Errors From Probe Trial at 17 Months**.** Primary latency was recorded 1 day after the 6 trials performed at 17 months were finished. The escape cage was completely removed so primary latency was recorded as the time till the mouse checked the hole where it usually was located. Although there is a wide range of variability due to the low amount of mice left per litter, 5-MTHF did remarkably well.

Due to only having 1 mouse per sample there was a high degree of variability in the results at the 17 month time point. The mice on the 5-MTHF and FA mice both outperformed their deficient counterparts at 17 months on the Barnes maze tests. As can be seen in Figure 6 the mice on the 5-MTHF and 5-MTHF deficient mice performed very erratically which may be due to the low number of mice involved at that time point for the Barnes maze.

Microarray Expression Results

Gene expression analysis by Arraystar shows genes that have significant expression changes in the hippocampal tissues of the FA deficient mice compare to the FA mice. Significant in this case was defined as any gene having a fold change of 2 or greater and a p value of less than 0.05. By this analysis it was demonstrated that at the 4 month time point 147 genes were repressed and 32 activated and that at the 18 month time point 175 genes were repressed and 25 activated (Figure 8). It was also determined that of these genes only 44 were represented as significant at both time points.

Discussion

Y-Maze Spontaneous Alternation Test

The Y-Maze test showed no discernible difference between the mice of differing diets. This would indicate that the type of folate or its deficiency has no significant effect on working memory. Perhaps an effect would have been seen at a later time point. Unfortunately due to time constraints we could not perform these tests in conjunction with the others at 10-12 and 17-18 months but it is something that could be looked into at a later time.

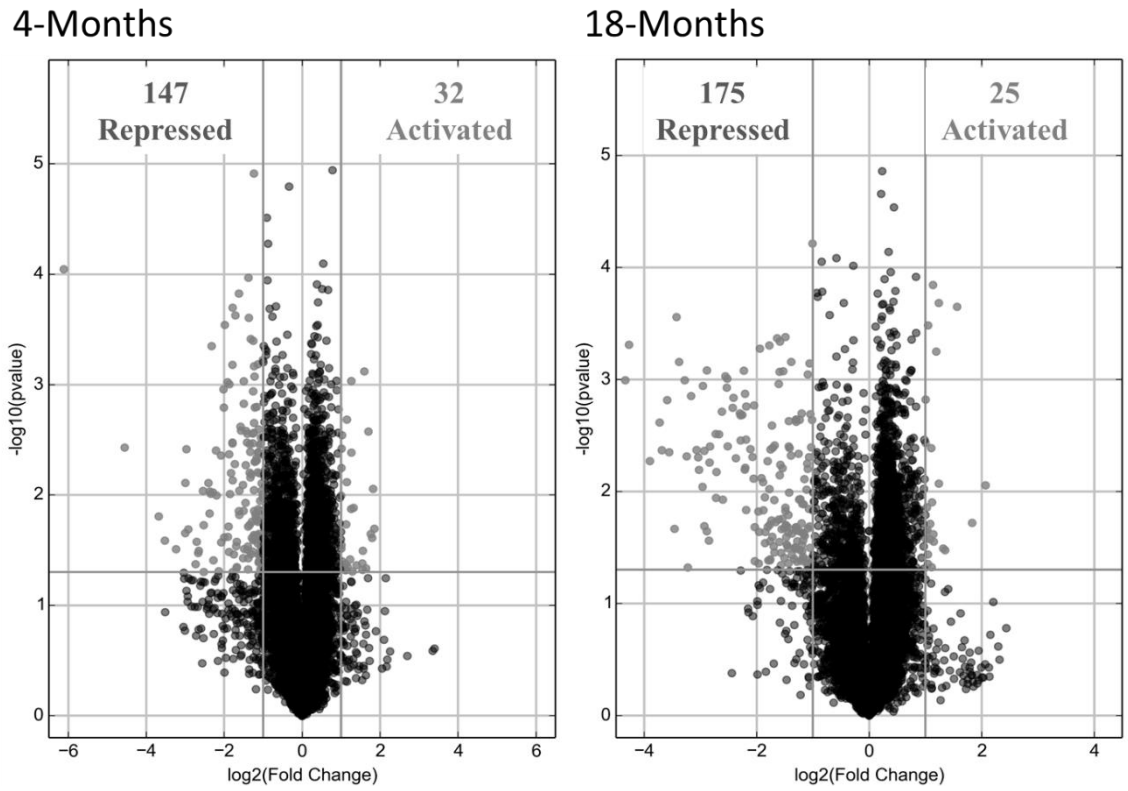

Figure 8. Volcano Plot of Gene Expression of FA Deficient Compared to FA at 4 & 18 Months. Hippocampal RNA expression from FA and FA deficient mice at 4 and 18 months was measured using micro array analysis to give the following volcano plots by comparing the FA deficient mice to the FA mice. Significantly repressed genes are shown in green and activated in red. Significance is fold value > 2 and P value < .05.

Novel Object Recognition Test

This test showed that folate, whether it is folic acid or 5-MTHF, is important for long term memory. The FA diet especially performed much better than its folate deficient counterpart. This would seem to indicate that although folic acid is important for healthy pregnancies and births, it is also important for long term learning and memory. What was also fascinating is that the 5-MTHF deficient mice performed better than their FA deficient counterparts. At 18 months the mice on the 5-MTHF deficient diet significantly outperformed the FA deficient mice. This might be due to differences in DNA methylation and development of the mice in utero and during weaning as this was the

only time these mice had access to either FA or 5-MTHF. Whatever the cause may be it seems that 5-MTHF has some kind of long term protective quality on the babies of the mothers who were given it over the babes of those given folic acid.

Barnes Maze

Although it is difficult to draw any hard conclusions from the Barnes maze tests due to a lack of statistical significance, there are some things to be considered. Again the folate diets outperformed their deficient counterparts as expected. What is somewhat interesting is that the 5-MTHF deficient diet outperformed the FA deficient diet at 9-10 months even if no statistical significance was seen. This somewhat confirms what was seen in the novel object results. Optimally this test would be done again with a larger cohort in order to improve significance.

Microarray

The microarray analysis and the generated volcano plot definitely demonstrated that folic acid is involved in gene expression. Of interest is that the majority of the genes that changed in the FA deficient samples were repressed. By removing the mice's folate source we are reducing their ability to methylate and shut off genes. Because of this we would have expected more genes to have increased in expression in the deficient mice. Instead the opposite happened. By what mechanism this occurs and how it affects behavior would definitely be of interest. Additionally it would be interesting if the genes that changed between the 5-MTHF deficient and 5-MTHF mice were different than the ones seen between the FA and FA-deficient mice.

Conclusion

Folate has been and will probably continue to be a common vitamin supplement. The question that could be asked is whether that supplementation should continue to be in the form of folic acid or if it should be switched to a different type of folate. While working memory wasn't shown to be affected by folate deficiency, as demonstrated by the Y-maze test, long term memory was definitely impacted as seen in the results from the novel object test. Additionally, the mice given 5-MTHF only before weaning performed better than their folic acid counterparts on the novel object test. The Barnes maze test showed nothing significantly although it some regards it tentatively confirmed what was seen in the novel object test results. The gene expression results from the microarray analysis confirmed that folate plays a role in epigenetics but strangely produced expression changes opposite of what was expected. While it is hard to say conclusively from this study that 5-MTHF is more beneficial than folic acid, I believe it does warrant more study on the topic.

The most obvious future work that could be done would be to perform a microarray analysis on the mice on the 5-MTHF and 5-MTHF deficient diets and look for specific gene differences between samples that might account for the behavioral differences. If the results proved interesting then perhaps it would be worth looking into the DNA methylation of the different samples. Additionally, it would be beneficial if the behavioral aspect of the study could be repeated with more litters to provide a larger N value and hopefully more statistical significance.

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