DELETION OF *CHDH* (*CHOLINE DEHYDROGENASE)* IN MICE DOES NOT ALTER BRAIN MITOSIS AND APOPTOSIS

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Nutrition, Gillings School of Global Public Health.

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Abstract

Sai Lao: Deletion of *Chdh* (*Choline dehydrogenase)* in mice does not alter brain mitosis and apoptosis
(Under the direction of Dr. Steven H. Zeisel)

Choline is an essential nutrient. Large amounts of choline are delivered across the placenta to the developing fetus, contributing to brain development and, consequently, affecting memory performance during adulthood. Rodent studies have described specific epigenetic mechanisms, whereby choline deprivation altered the neurobiology of progenitor cells, modifying their proliferation and survival. Choline, via oxidation to betaine, provides one-carbon units to the methionine cycle and further, to all methylation processes part of the epigenetic control. We found that the choline dehydrogenase (*Chdh*) gene, responsible for this conversion, is expressed in the brain of adult and fetal mice. By deleting the *Chdh* gene, we investigated the effects of low betaine synthesis on fetal brain development. We assessed cell proliferation in $Chdh^{+/+}$ and $Chdh^{-/-}$ fetuses at embryonic day 17 (E17) by immunohistochemistry. Mitosis and apoptosis were not significantly altered by fetal genotype. Further studies will be needed to establish if gene-specific methylation is altered between *Chdh* wildtype and knockout mice fetuses.

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List of Abbreviations

AI	Adequate Intake
BADH	Betaine Aldehyde Dehydrogenase
BHMT	Betaine-homocysteine Methyltransferase
CDKN3	Cyclin-dependent Kinase Inhibitor 3
cDNA	Complementary Deoxyribonucleic Acid
CH_3	Methyl group
Chdh	encode Choline Dehydgrogenase
$Chdh^{-/-}$	Choline Dehydrogenase Knockout Mice
$Chdh^{+/+}$	Choline Dehydrogenase Wildtype Mice
CP-A	Caudoputamen-Amygdala
CPK	Creatine Phosphokinase
DAPI	4',6-diamidino-2-phenylindole Dihydrochloride
DNA	Deoxyribonucleic Acid
E17	Embryonic Day 17
IACUC	Institutional Animal Care and Use Committee
IHC	Immunohistochemistry
IOM	Institute of Medicine
KAP	Kinase-associated Phosphatase
LCM	Laser Capture Microdissection
MAT	Methionine Adenosyltransferase
mRNA	Messenger Ribonucleic Acid
NALFD	Non-alcoholic Fatty Liver

PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phophatidylethanolamine *N*-methyltransferase
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SNPs	Single nucleotide polymorphisms
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VLDL	Very-low-density Lipoprotein

Chapter 1 - Background

Introduction

Choline is an essential nutrient required for the proper function of nearly every cell, and is especially important for the developing fetus. It is a critical component of cellular membranes, neurotransmission, and methylation. In humans, the need for choline is highly individualized as functional single nucleotide polymorphisms (SNPs) in genes within the choline synthesis and metabolism pathways may alter dietary requirements [1, 2]. Previous human and rodent studies have focused on modulating maternal choline intake during pregnancy and the effect on fetal brain development [3-7]. However, the deletion of a key gene in choline metabolism, *choline dehydrogenase (Chdh)*, in the fetus may alter brain development regardless of maternal diet and genotype.

Human studies have provided mixed results on the effect of maternal dietary choline on measures of offspring brain development [3, 6, 7], but conclusions from rodent studies have been more uniform. Prenatal choline deprivation resulted in increased memory loss that persists throughout the lifetime when compared to choline control or choline supplementation and may result from potential epigenetic influences of choline [4, 5, 8-10]. Oxidation of choline by choline dehydrogenase generates betaine, the precursor to universal methyl donor S-

adenosylmethionine (SAM). Dietary choline may reduce SAM pool and subsequently reduce availability of methylation reactions which influence gene transcription.

As aforementioned, choline may influence brain development in numerous ways since it is critical to multiple processes. In this section, the importance of choline to general health, choline metabolism, and the epigenetic influence of choline will be discussed.

Choline and Health

In 1998, the Institute of Medicine (IOM) officially designated choline as an essential nutrient. Adequate intake (AI) for choline as established by the IOM, Food and Nutrition Board is 550 milligram/day for adult males and 425 milligrams/day for adult females. AI for pregnant and lactating women is 450 mg/day and 550 mg/day, respectively [12, 13]. Choline deficiency has been linked to several adverse conditions, such as non-alcoholic fatty liver (NAFLD) [14], skeletal muscle damage [15], neural tube defects [16], and increased breast cancer risk [12]. These conditions can be linked to the complex roles that choline plays in important metabolic pathways and as a key element in cellular integrity. The need for choline starts prenatally and continues throughout life.

Liver is an important site for choline metabolism and storage, and also requires choline for proper function. Fatty liver develops when triglycerides accumulate in the liver and has been linked to choline deficiency [14]. Choline's role in fat metabolism extends from its utilization for phosphatidylcholine (PC) synthesis; 70% of PC in the liver is generated from the choline pathway and 30% from phosphatidylethanolamine (PE) catalyzed by phophatidylethanolamine *N*-methyltransferase (PEMT). PEMT has an estrogen response element and therefore adequate intake levels for men and post-menopausal women are higher than premenopausal women. SNPs

in the hormonal regulation (estrogen response element) of PEMT may alter the endogenous synthesis of choline and thus require increased dietary choline, even for premenopausal women [17]. Phosphatidylcholine is an essential phospholipid for mammals and is necessary for synthesis of very-low-density lipoprotein (VLDL). Triglycerides are packaged and secreted from the liver in VLDLs for storage in adipose tissues or utilization [18]. Clearly, choline deficiency results in decreased PC synthesis resulting in decreased ability for triglyceride export and subsequently impair liver function. If choline deficiency persists NAFLD, cirrhosis, and hepatic carcinoma may develop [14].

Choline deficiency in humans has also been found to increase skeletal muscle damage as marked by increased serum creatine phosphokinase (CPK) and more fragile myoctyes, which is a result of muscle cell PC depletion [19]. Furthermore, muscle mitochondria contain a choline transporter, SLC44A1, which shows reduced mRNA and protein expression with choline deficiency. Similar to hepatocytes, choline deficient muscle cells accumulate fat [20] which may result from perturbed mitochondrial function and lipid oxidation [14].

Neural tube defects risks were found to be the lowest in women whose intake of choline was above the 75^{th} percentile. Choline metabolism intersects with folate metabolism in the pathway for methyl-group donation (*S*-adenosylmethionine formation) and it is suggested that this may be the route by which choline affects neural tube closure [16].

As evidenced here, choline is needed for proper function of multiple bodily systems with potentially severe consequences if adequate choline supply is not met.

Choline Metabolism

Choline is a quaternary amine that can be derived from dietary sources or synthesized *de novo* from PE catalyzed by PEMT to form PC and then catabolism of PC by phospholipases and lysophospholipases. Foods rich in choline are eggs, milk, and animal meat. Approximately 95% of choline in animals is in the form of PC with the remaining as free choline, phosphocholine, CDP-choline, and acetylcholine. Choline metabolism occurs mainly in the liver and kidney and directed into three main routes: betaine, PC, or acetylcholine synthesis [18].

Acetylcholine, an important neurotransmitter in learning and memory function [21], is synthesized from choline and acetate via choline acetyltransferase, and acetylcholine esterase catalyzes the reaction in the reverse direction [18]; thus, allowing for choline generation from this neurotransmitter when needed.

As previously mentioned, choline is mainly directed towards the formation of PC via the Kennedy Pathway [22]. Choline kinase phosphorylates choline to phosphocholine which is then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase. In the final step of PC synthesized by CDP-choline:1,2-diacylglycerol cholinephosphotransferase. Phosphatidylcholine can then be incorporated into lipoproteins, membranes, sphingomyelin, phosphatidylserine, and bile among others.

Betaine is an organic osmolyte necessary for normal kidney function and an important methyl donor for the conversion of homocysteine to methionine, which is the precursor for the universal methyl donor *S*-adenosylmethionine (SAM). Betaine is generated from choline in a two-step process. First, choline dehydrogenase within the inner mitochondrial membrane converts choline to betaine aldehyde. This is followed by the synthesis of betaine from betaine aldethyde within the mitochondria and cytosol by betaine aldehyde dehydrogenase (BADH).

Homocysteine and betaine are precursors for methionine synthesis which is catalyzed by betaine-homocysteine methyltransferase (BHMT) [23]. SAM is generated from methionine by methionine adenosyltransferase (MAT) and can be utilized in transmethylation reactions [24]. After donating its methyl group, SAM is converted to *S*-adenosylhomocysteine (SAH). Eventually, SAH results in homocysteine and betaine is required for remethylation of homocysteine.

Endogenous choline synthesis requires PE and three transmethylation reactions catalyzed by PEMT to form PC. Consequently, transmethylation reactions utilize SAM and the problem becomes increasingly apparent when choline deficiency occurs – reduced ability to produce betaine resulting in reduced SAM pool leading to reduced methylation reactions that control expression of important genes and decreased endogenous synthesis of choline, PC, betaine, and acetylcholine. Thus, the need for choline is increased during pregnancy due to requirements for proper function of maternal organs and for proper fetal development.

During pregnancy, choline is actively transported across the placenta to the fetus [25-27]. Maternal choline intake during pregnancy has been shown to affect the developing fetus. Dietary choline deficiency during pregnancy leads to impaired memory function [28], alter fetal brain angiogenesis [29], decreased progenitor cell proliferation and increased apoptosis in the fetal brain[4], and epigenetic alterations in the brain [5, 30]. Thus, the demand for choline is particularly high during this critical developmental period.

Choline and Epigenetics

Epigenetics has an ever-changing definition, but it is widely accepted as the study of processes that change gene activity without alterations to the DNA sequence. These epigenetic

modifications are heritable mitotically and/or meiotically and include DNA methylation and chromatin modifications. DNA methylation involves addition of a methyl group (CH_3) to cytosine, adenine, or guanine. Methylation of cytosine at CpG sites and CpG islands, CG dinucleotide rich regions, to from 5-methylcytosine are most widely studied due to their implications in carcinogenesis. If the CpG site is within the promoter of a gene, DNA methylation may alter the binding of transcription factors and thus prevent proper expression or inhibition of the gene. DNA stability and repair is also affected by methylation. Another type of epigenetic modification that was recently discovered is hydroxymethylation, which is the replacement of hydrogen by a hydroxymethyl group at the 5-position of cytosine. Hydroxymethylation is thought to be part of the demethylation process; therefore, methylation is potentially a dynamic and reversible process [31-37].

Choline contributes to epigenetic modifications via its role as a source of methyl-groups for SAM production [38], particularly homocysteine remethylation. Diets deficient in choline was shown to deplete hepatic SAM concentrations [39] and induce hyper- and hypo-methylation [38, 40]. In a C57BL/6 mouse model, perinatal choline deficiency altered offspring global and gene-specific methylation patterns leading to changes in mRNA and protein expression that inhibits the cell cycle [5]. Data here indicate the potential role of perinatal choline nutrition influencing the epigenetic control of gene expression during critical developmental periods. Thus, if choline dehydrogenase is critical to synthesis of betaine and subsequently the generation of SAM, *Chdh* deletion could potentially result in aberrant DNA methylation patterns affecting organ development.

Hypothesis

Choline has complex and varied roles at different stages of development for multiple tissues and dietary need is different for each individual based on genetic disposition. Utilization of a mouse model allows close examination of the role of choline in multiple organs during developmental periods. With the evidence presented above, a mouse model in which *Chdh* has been deleted will be used to determine the effect of this gene on neural progenitor cell proliferation in fetal samples. We hypothesized that deletion of a key gene in one-carbon metabolism and important pathway in methyl-group donation will result in aberrant methylation patterns influencing gene transcription and ultimately organ development and function.

For the current study, we studied mitosis and apoptosis of progenitor cells in the developing fetal brain at embryonic day 17 (E17) in a *Chdh* chimeric mouse model. The objective was to determine whether results from E17 $Chdh^{-/-}$ fetuses are similar to offspring from low-choline-fed dams. The results of this investigation provide better understanding of the differences between $Chdh^{+/+}$ and $Chdh^{-/-}$ in the fetal environment and will help to develop future research goals in determining the role of choline dehydrogenase in methylation.

Chapter 2 – Journal Manuscript

Introduction

Choline, a water-soluble essential nutrient found in foods such as eggs and animal meat, is involved in many biological processes including neurotransmitter and membrane biosynthesis and methyl-group donation [12]. In order to be used as a methyl-donor, choline must be oxidized to form betaine in a 2-step pathway catalyzed by choline dehydrogenase *(encoded by Chdh)* and betaine aldehyde dehydrogenase (BADH). In humans, there are functional single nucleotide polymorphisms (SNPs) in *CHDH* that decrease the formation of betaine from choline [23, 38].

Studies in rodents have shown that decreased availability of choline in maternal diet during pregnancy resulted in diminished neurogenesis and increased neural progenitor cell apoptosis in the fetal hippocampus, leading to diminished hippocampal (memory) function in the offspring [4, 41, 42]. Previously, we suggested that the mechanism whereby maternal dietary choline affects fetal brain neurogenesis was epigenetically mediated. We showed that, in IMR-32 neuroblastoma cells grown in low-choline medium, the promoter region of *CDKN3* (encoding cyclin-dependent kinase inhibitor 3) was hypomethylated [9]. This was associated with increased gene expression, increased protein product of this gene (kinase-associated phosphatase; Kap) with resulting inhibition of the G_1/S phase of the cell cycle and reduced cell proliferation. In

subsequent studies, using laser-capture micro-dissection (LCM), we harvested neural progenitor cells from the prime germinal zone of the dentate gyrus (hippocampus) of fetuses from dams fed a low choline diet and observed decreased gene-specific DNA methylation of the gene (Cdkn3) that encodes for Kap, correlating with increased expression of this protein [5]. Maternal dietary choline also modulated methylation of histones in fetal brain [43]. These epigenetic effects of a low choline diet provided a plausible explanation for the relationship between dietary choline and neural progenitor cell proliferation and apoptosis.

In order to test this hypothesis, we created a mouse in which *Chdh* was deleted [23]. This mouse cannot form betaine from choline [23] and therefore cannot use choline as a methyl-donor. We hypothesized that the knockout mouse would have changes in fetal brain that were similar to those seen when dams were fed a low choline diet - diminished neurogenesis and increased neural progenitor cell apoptosis in the hippocampus. We report that this was not the case - neurogenesis and neural progenitor cell apoptosis in the hippocampus were not affected by *Chdh* gene deletion.

Methods

Animal models and tissue collection

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA) and at the David H. Murdock Research Institute (Kannapolis, NC, USA).

We previously described how the *Chdh* knockout mouse was created [23]. Mice were housed in a 12-hour light-dark cycle and temperature controlled environment. For immunohistochemistry (IHC) studies, male and female $Chdh^{+/-}$ mice with mixed C57Bl/6 and

129 backgrounds were timed-mated. Throughout pregnancy, mice had *ad libitum* access to a defined diet (AIN-76A) containing 1.1 g/kg choline chloride (Dyets Inc., Bethlehem, PA, USA) and water. Dams were anesthetized with isoflurane, and fetuses collected on day E17 of gestation. Typical litter size for each dam is 6-8 offspring. Animal genotypes and sex of fetal samples were determined as previously described [23]. Fetal brains were collected as described previously [4], fixed in 4% paraformaldehyde overnight, paraffin embedded and then sectioned at 5 μm.

For gene expression experiments, adult male *Chdh* wildtype mice were anesthetized with isoflurane and liver and brain was extracted and immediately snap-frozen in liquid nitrogen. Methods for liver and brain collection from fetuses has been previously described [4]. Samples were stored at -80°C until analysis.

Chdh mRNA expression

Choline dehydrogenase mRNA expression was analyzed to determine if the gene is expressed in the fetal brain and its relative expression level to adult tissues. RNeasy Protect Mini Kit (Cat. No. 74124, Qiagen, Venlo, Netherlands) was used following manufacturer's protocol with to extract RNA from frozen liver and brain tissues of *Chdh* fetal and adult mice. Complementary DNA (cDNA) synthesis was synthesized with Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814, NY, USA) following manufacturer's protocol in 20 uL reaction volume with an Eppendorf Mastercycler epGradient (Hamburg, Germany) machine. Gene expression was assessed using quantitative real-time PCR (qRT-PCR). Materials used were TaqMan 2X Master Mix (Cat. No. 4369016, Applied Biosystems, NY, USA), 20X mChdh assay (Mm00549261_m1, Applied Biosystems, NY, USA)

and 20X mGapdh assay (Mm99999915_g1, Applied Biosystems, NY, USA). Quantitative RT-PCR was performed on an Eppendorf Realplex^2 Mastercycler epGradient S running Eppendorf Mastercycler ep realplex 2.2 (Hamburg, Germany). Gene expression data was analyzed by the comparative CT method ($2^{-\Delta\Delta CT}$)[44] with data normalized to *Gapdh* and then normalized to adult expression values.

Immunohistochemistry

Mitosis and apoptosis were assessed in at least three alternating 5 μm sections of the hippocampus from each of 4-6 fetal brains. Both hemispheres were analyzed in each section.

To assess mitosis in the fetal hippocampus, coronal sections of the embryonic day 17 (E17) fetal brain were probed for phosphorylated histone H3, a core protein of the nucleosome and marker for mitosis. The immunohistochemistry protocol used has been previously described [4]. Rabbit anti-H3Ser10ph (Cat. No. 06-570, Millipore, MA, USA) was used as the primary antibody. Goat anti-rabbit Cy3 conjugated secondary antibody (Cat. No. AP132C, Millipore, MA, USA) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. No. 32670, Sigma-Aldrich, MO, USA) were used to fluorescently label the primary antibody and all nucleated cells, respectively. Images were acquired at the University of North Carolina at Chapel Hill Microscopy Services Laboratory (Chapel Hill, NC, USA) using an Olympus BX61 (Tokyo, Japan) upright fluorescence microscope controlled by Improvision Volocity (PerkinElmer, Waltham, MA, USA) acquisition software on an Apple iMAC under OS X Version 10.6.8 (Cupertino, CA, USA).

DNA fragmentation is a hallmark of apoptosis. The ApopTag® In Situ Apoptosis Fluorescein Detection Kit (Cat. No. S7111, Millipore, MA, USA), a terminal deoxynucleotidyl

transferase dUTP nick end labeling (TUNEL)-based assay, enzymatically labels the free 3'-OH end on single and double DNA strand breaks for fluorescent detection. Manufacturer's protocol was followed with several modifications; xylene washes and sample rehydration was followed according to mitosis protocol as previously described [4]. Sections were incubated in 1x PBS-T overnight at 4°C following the stop/wash buffer step. Following the anti-dioxigenin conjugate incubation, samples were rinsed in PBS-T, then incubated in a DAPI working buffer protected from light, washed with PBS-T, mounted in 80% tris-buffered glycerol (pH 7.0) with No. 1 glass coverslip, and sealed with commercial nail polish. Images were acquired at the David H. Murdock Research Institute Microscopy Facility (Kannapolis, NC, USA) using a Zeiss Axio Imager.M1 (Oberkochen, Germany) and Zeiss AxioVision Relesase 4.8.1 (11-2009) (Oberkochen, Germany).

Image analysis

Images acquired for mitosis analysis were converted to JPEG format and viewed under Adobe Photoshop 6.0 Version 6.0.1 (San Jose, CA, USA) to manually count positive mitotic cells. Each positively stained H3ser10ph cell was identified as one count. Cells exiting M-phase of the cell cycle, visualized as two fused cells by IHC, were identified as two individual counts. Apoptotic cell counts were gathered manually using Zeiss AxioVision Release 4.8.1 (11-2009) software and each brain lobe was divided into three regions (hippocampus, neocortex, caudoputamen-amygdala) for analysis. For apoptosis, each fragmented cell was identified as one individual count. Hippocampus and neocortex ventricle length were measured with open source software Fiji [45] to determine major structural changes between genotype. SL was blind to the

genotype of the samples when counting the mitosis- and apoptosis-positive cells and measuring ventricle length.

Statistics

For *Chdh* expression differences between adult and E17 fetal samples, mitosis data, apoptosis data, and global DNA methylation data, Student's t-Test ($\alpha = 0.05$) assuming equal variances (Excel 2010, Microsoft, Redmond, WA, USA) was performed to determine significance between groups in each experiment. P-values less than 0.05 were considered statistically significant for all statistical analyses.

Results

***Chdh* expression**

Chdh expression in fetal liver (Figure 1A) and brain (Figure 1B) of male wildtype mice was significantly lower than that of the same tissues in adult male wildtype mice. No *Chdh* was expressed in knockout mice. Results here verify that *Chdh* mRNA expression is present in fetal liver and brain.

Mitosis

Neuronal progenitor cells proliferate adjacent to the ventricle and then migrate to other areas of the brain. Therefore, positively labeled H3ser10ph cells were observed in close proximity to the ventricle in the E17 brains (Figure 2A, 2B). There was no statistical difference in the number of mitotic cells in the primordial hippocampal region; $Chdh^{+/+}$ (17.1 ± 2.3 H3ser10ph positive cells; n = 4) and $Chdh^{-/-}$ (17.5 ± 2.6 H3ser10ph positive cells; n = 6) or in the

neocortical region, *Chdh*$^{+/+}$ (17.0 ± 2.6 H3ser10ph positive cells; n = 4) and *Chdh*$^{-/-}$ (13.1 ± 2.1 H3ser10ph positive cells; n = 6) (Figure 2C). P-value was greater than 0.05 for both analyses.

Ventricular length was measured and was not different between genotypes (hippocampal ventricular length *Chdh*$^{+/+}$ 1295 ± 67 µm, n =4; *Chdh*$^{-/-}$ 1439 ± 92 µm, n =4; neocortical ventricular length *Chdh*$^{+/+}$ 1487 ± 51 µm, n =4; *Chdh*$^{-/-}$ 1561 ± 93 µm, n =4; Figure 2D).

Sample size (n) for mitosis, apoptosis, and global DNA methylation analysis represents the number pregnant dams from which fetal samples were collected. Several *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ samples were collected from the same dam.

Apoptosis

TUNEL analysis of apoptosis indicated cell death throughout the brain. There was no difference in numbers of TUNEL-positive cells in any of the 3 regions studied ((hippocampus, neocortex, or caudoputamen-amygdala; (Figure 3A-C). In the hippocampus region, *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ had 5.2 ± 0.4 (n = 6) vs. 4.9 ± 0.9 (n = 6) apoptotic cells, respectively. In the neocortex region, *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ had 9.3 ± 0.8 (n = 6) vs. 9.4 ± 1.7 (n = 6) apoptotic cells, respectively. In the caudoputamen and amygdala (CP-A) region, *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ had 4.3 ± 0.8 (n = 6) vs. 5.5 ± 0.7 (n = 6) apoptotic cells, respectively. P-value was greater than 0.05 for all three analyses.

There were no differences by genotype in ventricular length (*Chdh*$^{+/+}$ 1,417 ± 96 µm (n = 6) versus *Chdh*$^{-/-}$ 1,464 ± 72 µm(n = 6)) or neocortical ventricular length (*Chdh*$^{+/+}$ 1,641 ± 97 µm (n = 6) versus *Chdh*$^{-/}$ 1,615 ± 78 µm (n = 6)) (Figure 3D).

For mitosis and apoptosis data, Means and 95% confidence intervals for KO minus WT were computed for each measure and region from the mitosis and apoptosis data (Supplemental Table 1).

Discussion

Deletion of *Chdh* in mice did not significantly alter the founder population of progenitor cells during brain development when compared to *Chdh* wildtype in brain on day E17. In addition, in wildtype mice, we found that expression of *Chdh* in brain and liver was much lower in fetuses than in adult mice.

As discussed earlier, a potential epigenetic mechanism for the effects of choline on fetal brain neurogenesis and apoptosis has been suggested [5, 9, 43]. Because choline must be converted into betaine before it can serve as a methyl-donor [48], we expected that the effect of deleting this gene on fetal brain development would be similar to the effect of feeding a low choline diet to dams – decreased mitosis and increased apoptosis of neural progenitor cells in the developing E17 hippocampus. In future studies we plan to measure epigenetic marks on genes regulating cell cycle (i.e., *Cdkn3*) and histones with increased sample size and expect to see no difference by fetal genotype.

The *Chdh* mouse does not make betaine, and therefore accumulates more choline and the phosphorylated products of choline, including phosphatidylcholine, in tissues [23]. If the underlying mechanism explaining the effects of dietary choline on fetal brain neurogenesis and apoptosis involves the accumulation of choline or phosphatidylcholine rather than epigenetic marks, then we would expect the effect of deleting this gene on fetal brain development to be similar to the effect of feeding a high choline diet to dams – increased mitosis and increased

apoptosis of neural progenitor cells in the developing E17 hippocampus. This was not the case. Analysis of *Cdkn3* methylation and KAP in *Chdh*$^{-/-}$ fetal samples will provide a direct comparison against the choline deficient model.

Several functional SNPs in the human *CHDH* gene have been identified that may alter choline metabolism [1, 53]. Studying a knockout mouse model provides insight into the importance of the gene in multiple body systems, particularly the fetal brain in the present study. The current study has limitations. First, the mice model used is on a mixed 129/C57BL6 background. One study found inter-strain differences in mouse liver pathology when placed on a choline- and folate-deficient diet; C57BL/6J and 129S1/SvImJ were among the mouse strains used in the study [54]. Thus, it is possible that the mixed background of the mouse model may potentially mask differences. Secondly, we did not directly measure DNA and histone methylation and therefore cannot be sure that deletion of *Chdh* does not alter epigenetic marks, especially on genes critical to cell cycle regulation. Finally, we use mothers that were heterozygous for *Chdh*, and these mothers might have supplied betaine to the fetal brain. In future studies we plan to determine whether fetuses from *Chdh*$^{-/-}$ mothers have different brain phenotype than do fetuses derived from *Chdh*$^{+/-}$ dams.

Figures

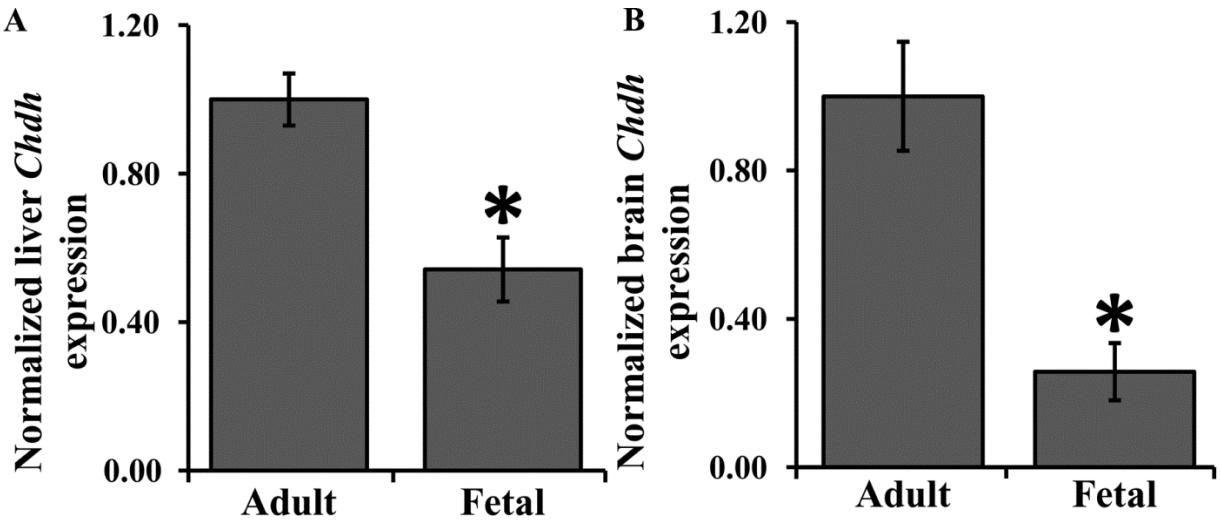


Figure 1. *Chdh* Expression
A) *Chdh* expression in fetal (n = 12) liver is 54.2% of adult (n = 14) liver. B) *Chdh* expression in fetal (n = 12) brain is 25.8% of adult (n = 12) brain. Error bars are standard error; Results are normalized to housekeeping gene *Gapdh* and then normalized to adult samples calculated with the comparative CT ($2^{-\Delta\Delta CT}$) statistical method. *P<0.05.

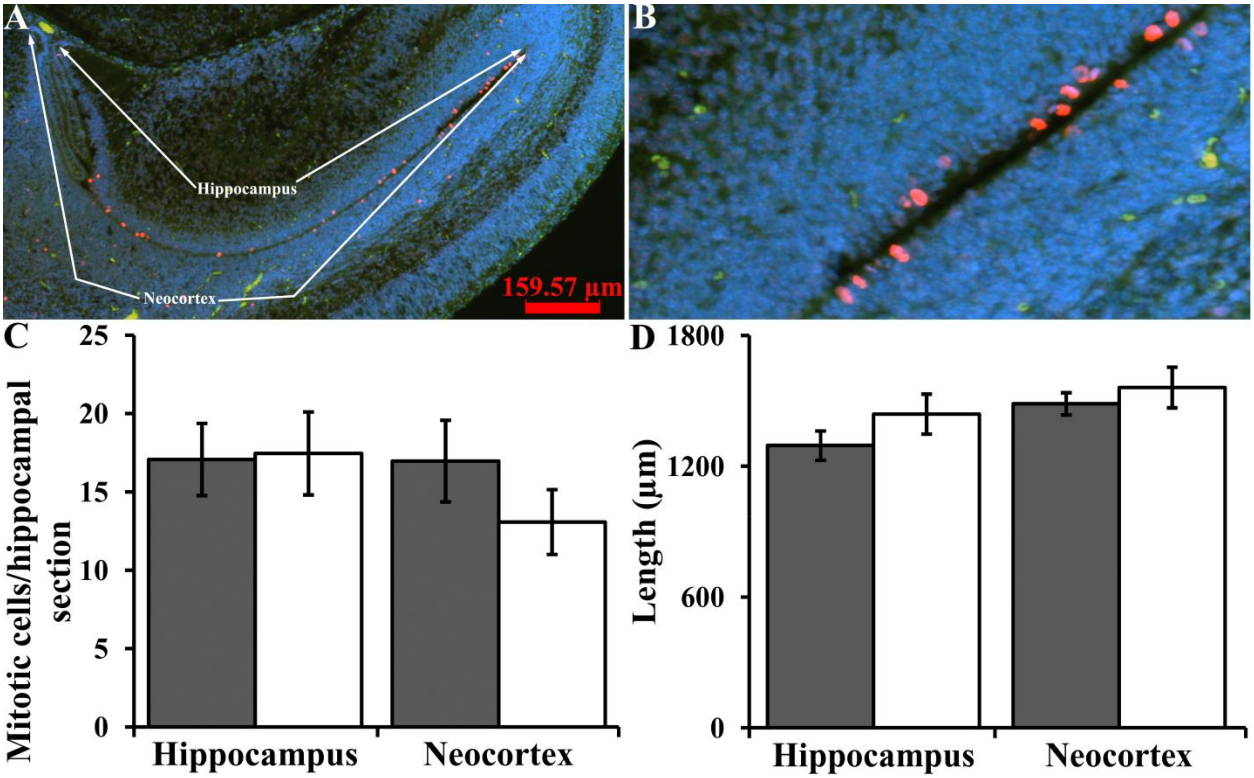


Figure 2. Mitosis in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ fetal brains

Coronal brain sections from E17 fetuses were analyzed by immunohistochemistry for H3ser10ph, a marker of mitosis. A) Mitotic cells in the hippocampus for *Chdh*$^{+/+}$ (n = 4) and *Chdh*$^{-/-}$ (n = 6) are 17.062±2.302 and 17.455±2.644, respectively. In the neocortex, *Chdh*$^{+/+}$ (n = 4) is 16.964±2.605 and *Chdh*$^{-/-}$ (n = 6) is 13.078±2.070. Student t-Test (α=0.05) indicates no statistical difference. P>0.05. Data are shown as (mean ± standard error). Error bars are standard error. B) Hippocampal ventricle length is *Chdh*$^{+/+}$ (n = 4) 811.561±42.156 and *Chdh*$^{-/-}$ (n = 6) 902.039±57.644. P>0.05. Neocortex ventricle length is *Chdh*$^{+/+}$ (n = 4) 931.557±31.922 and *Chdh*$^{-/-}$ (n = 6) 978.462±58.554. P>0.05. Data are shown as (mean ± standard error). Error bars are standard error. C) Immunohistochemistry stained coronal section of fetal E17 brain. Proliferating cells are red in color and visualized adjacent to the ventricle; these cells are distinct from endogenous vasculature (green) and are overlaid on nucleated cells (blue). D) Enlarged image to show H3ser10ph labeled cells (red), nucleated cells (blue), and endogenously fluorescent vasculature (green).

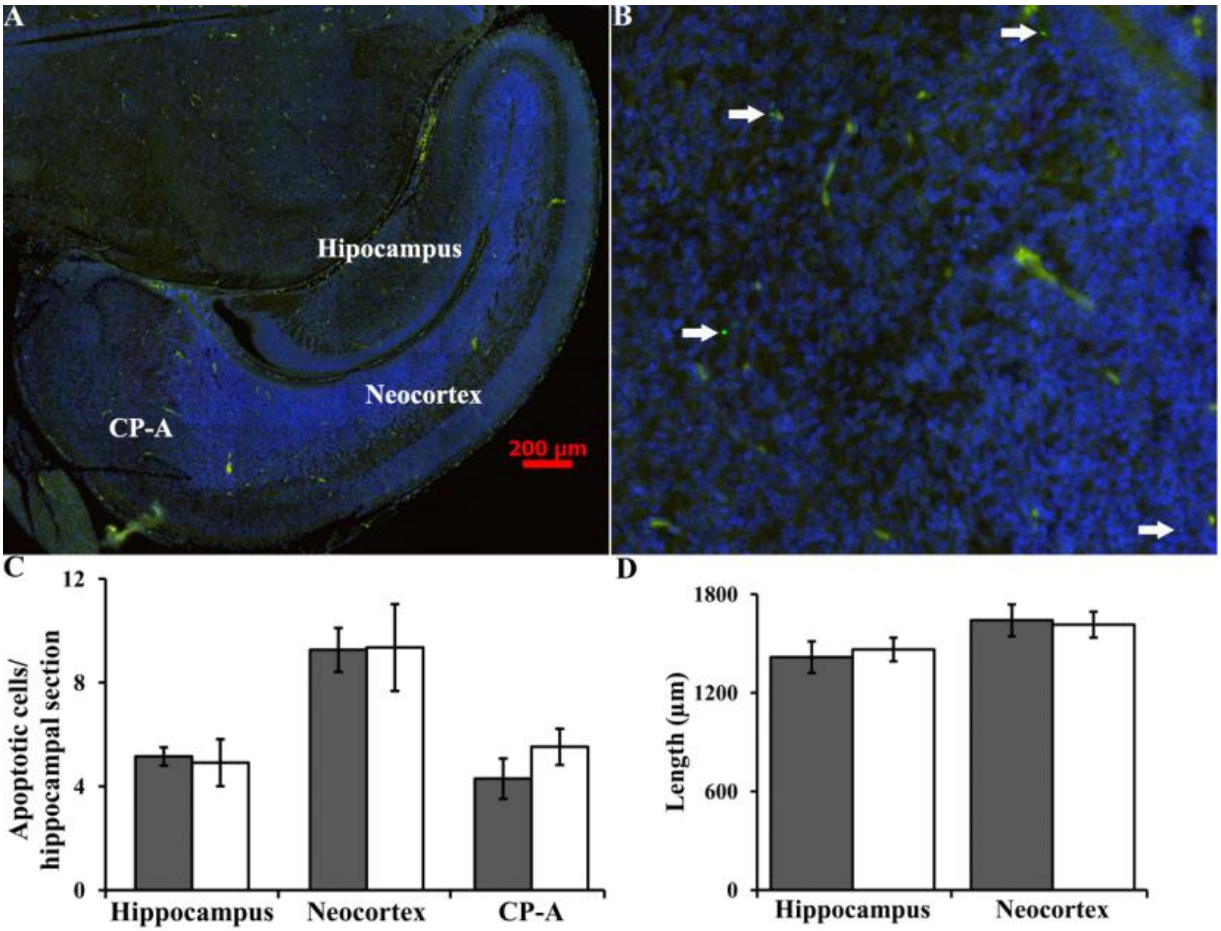


Figure 3. Apoptosis in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ fetal brains

Coronal sections from brains of E17 fetuses were analyzed by immunohistochemistry for markers of apoptosis using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling). A) Average apoptotic cell count in *Chdh*$^{+/+}$ (n = 6, dark grey bars) and *Chdh*$^{-/-}$ (n = 6, white bars) fetal brain sections. In hippocampus, neocortex, and caudoputamen-amygdala (CP-A) regions, positive apoptotic cell counts are 5.16 ± 0.35 vs. 4.91 ± 0.90, 9.27 ± 0.85 vs. 9.35 ± 1.67, and 4.30 ± 0.78 vs. 5.53 ± 0.70, respectively, for *Chdh*$^{+/+}$ vs. *Chdh*$^{-/-}$. Student's t-Test (α = 0.05) shows no difference between genotype. P>0.05 for all comparisons. Data are shown as (mean ± standard error). Error bars are standard error. B) The ventricular zone length on the hippocampal (1417.07 ± 96.34 µm vs. 1464.00 ± 72.14 µm) and neocortex (1641.26 ± 97.31 µm vs. 1615.34 ± 78.35 µm) were measured *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$, respectively. Student's t-Test (α = 0.05) shows no difference. Data are shown as (mean ± standard error). Error bars are standard error. C) Coronal section of brain lobe with ventricular zone. Positively labeled fragmented DNA are bright green in color. Nucleated cells are blue in color (DAPI) and endogenously fluorescent blood vessels are yellow, a mix of green and red. D) Positively labeled DNA fragments (white arrows) are shown in the enlarged image.

Supplemental data

Supplemental Table 1. Difference between KO and WT for each measure along with the 95% confidence interval

Measure	Region	P-value	Mean	95% CI of KO-WT
Mitosis	Hippocampus	0.92	0.4	(-8.4, 9.1)
Mitosis	Neocortex	0.27	-3.9	(-11,5 , 3.7)
Mitosis	Hippocampus Length	0.29	90.5	(-92.6 , 273.5)
Mitosis	Neocortex Length	0.56	46.9	(-131.6, 225.4)
Apoptosis	Hippocampus	0.93	-0.1	(-2.3, 2.1)
Apoptosis	Neocortex	0.73	0.7	(-3.7, 5.1)
Apoptosis	CP-A	0.22	1.5	(-1.0, 4.0)
Apoptosis	Hippocampus Length	0.47	89.3	(-177.6, 356.2)
Apoptosis	Neocortex Length	0.90	15.3	(-256.5, 287.0)

Means and 95% confidence intervals for KO minus WT were computed for each measure and region from the mitosis and apoptosis data.

Region-specific *Chdh* expression in adult brain

Animal model and sample collection

Samples for region-specific Chdh expression were collected from C57BL/6J mice. Brains were dissected into two hemispheres. One hemisphere was immediately clamped and snap-frozen in liquid nitrogen and was used to determine global *Chdh* expression.. The other hemisphere was dissected by brain region in chilled 1X phosphate buffered saline with 0.1% Tween-20 (PBS-T) solution under a dissecting microscope, each dissected region was placed in a microfuge tube, and then snap-frozen in liquid nitrogen. The following regions of brain were dissected and analyze for Chdh gene expression: olfactory bulbs, interbrain (also known as diencephalon, containing the thalamus, hypothalamus, subthalamus, epithalamus), isocortex FO (frontal pole and orbital cortex), isocortex SR (containing somatomotor and retrosplenial cortical areas), dentate gyrus region of hippocampus and cerebellum

Methods

RNA was extracted from frozen brain regions with RNeasy Protect Mini Kit (50) (Cat. No. 74124, Qiagen, Venlo, Netherlands) following manufacturer's protocol. Complementary DNA was synthesized with Bio-Rad iScript™ Reverse Transcription Supermix (Cat. No. 170-8840, Hercules, CA, USA) following manufacturer's protocol using an Eppendorf Mastercycler epGradient (Hamburg, Germany) machine. Gene expression was assessed using quantitative real-time PCR (q-PCR). Materials used were Bio-Rad SsoFast™ Probes Supermix (Cat. No. 172-5230, Hercules, CA, USA), Applied Biosystems 20X mChdh assay (Mm00549261_m1), and 20X mGapdh assay (Mm99999915_g1, Applied Biosystems, NY, USA). Quantitative RT-PCR was performed on an Eppendorf Realplex^2 Mastercycler epGradient S running Eppendorf Mastercycler ep realplex 2.2 (Hamburg, Germany). The whole brain samples were used to generate a standard curve with each qRT-PCR.

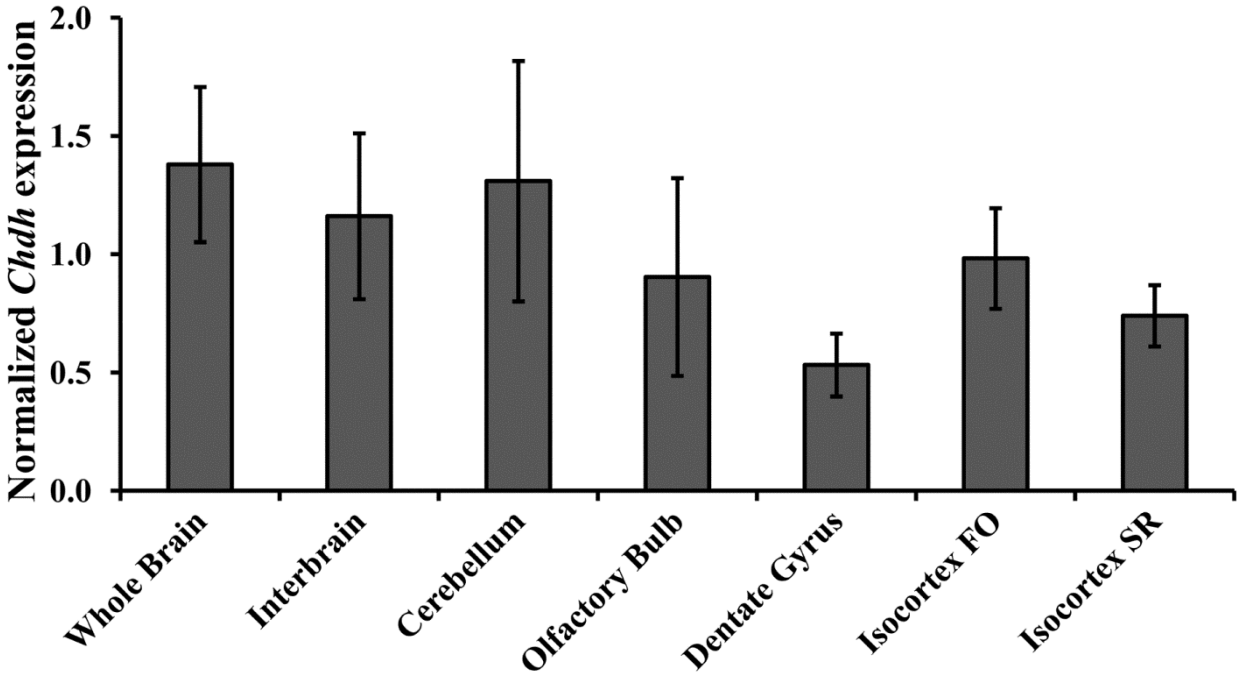
Data was normalized to *Gapdh* housekeeping gene for each brain region of each individual specimen were calculated following the Relative Standard Curve Method in the Applied Biosystems Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR[1]. Region-specific *Chdh* expression, statistical analysis was performed with REST 2009 software [46, 47]. A repeated measures ANOVA was used to compare the means of all six regions simultaneously and test for an overall difference. No significance was detected ($P = 0.18$).

Discussion

Since *Chdh* deletion resulted in no differences in fetal mitosis apoptosis we further questioned the role of choline in the more mature, complex adult brain. Neurogenesis begins

[1] www.cf.gu.se/digitalAssets/1125/1125331_ABI_-_Guide_Relative_Quantification_using_realtime_PCR.pdf

prenatally and continues throughout the adult life with limited neuron formation in the dentate gyrus postnatally [49]. *Chdh* deletion could prove to be more influential in adult neurogenesis because of the increased *Chdh* expression and activity in adult brain samples. Differential *Chdh* expression in different brain regions in the adult animal potentially illustrates the importance of betaine to the function to those areas. To determine if *Chdh* shows region-specific expression in the brain, adult brains of C57BL/6J mice (n = 4) approximately three months old were harvested and analyzed. ANOVA analysis showed no overall statistical difference across all six regions, but there seems to be a trend with lower *Chdh* expression in the dentate gyrus. This is pilot data and requires increased sample size for any definitive conclusions.



Supplemental Figure 1. Region-specific *Chdh* expression in adult mice brain

Brains from adult C57BL/6J animals (n = 4) were analyzed for region-specific expression of *Chdh*. Pairwise comparisons of normalized *Chdh* expression to *Gapdh* expression show significant difference between cerebellum and dentate gyrus (1.3 ± 0.5 vs. 0.5 ± 0.1, *P=0.02). Data are shown as (mean ± standard error).

Chapter 3 – Conclusions

Conclusions

The role of choline as an essential nutrient has only been recently identified, and it has been recognized that the recommended AI for choline is different for different populations. The need for choline is modulated by a variety of factors, such as age, gender, pregnancy, and genetic SNPs. Research has shown that choline is important for prenatal development, but the role of fetal choline metabolism has yet to be established. More importantly, does deletion of a key gene in choline metabolism, *Chdh*, in the fetus effect measurements of prenatal brain development even if the mother is supplied adequate amounts of dietary choline?

In this study we focused on identifying changes in progenitor cell proliferation between $Chdh^{+/+}$ and $Chdh^{-/-}$ fetuses harvested at E17 from $Chdh^{+/-}$ mating pairs with prenatal diets adequate in choline. We did not observe any significant changes in markers of mitosis and apoptosis between the wildtype and knockout *Chdh* fetal genotypes. In this section, we focus on more in-depth discussion of the study results, limitations, and suggestions for future studies.

Choline, Mitosis, and Apoptosis

We showed that *Chdh* deletion did not result in changes in mitosis as measured by phosphorylated histone-3 (phospho-histone H3), a marker of mitosis that is located within the

nucleosome and critical for chromatin condensation maintenance during mitosis. This IHC method also enables visualization of different stages of mitosis based on cell shape and color intesnity. Mitotic cell counts along the hippocampal and neocortical ventricular zone of the fetal brain did not reveal any significant differences in progenitor cell proliferation between *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ fetuses.

Apoptosis, as measured by fluorescent TUNEL IHC, also revealed no difference between number of apoptotic cells in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ fetuses. The TUNEL assay enzymatically labels fragmented ends of single and double stranded DNA allowing for fluorescent identification. In the beginning stages of apoptosis, the cell shrink, chromatin condense, and DNA fragmentation occurs. This is followed by membrane blebbing and nucleus fragmentation. In the final stages of apoptosis, the cell fragments in to apoptotic bodies and subsequently consumed by phagocytes[55]. TUNEL IHC is able to capture apoptosis at these different stages, resulting in clusters (early apoptosis) of fluorescent fragments or isolated fragments as they break off from the intact cell (late apoptosis).

Choline is transported across the fetus and maternal plasma betaine has been shown to be positively associated with fetal plasma betaine [56]; thus, it is likely that choline and betaine can be supplied by the mother to the fetus. The results shown in Chapter 2 may reflect the fact that pregnant dams were on a diet sufficient in choline, and therefore the fetal environment was not low in choline or choline metabolites. Although the *Chdh*$^{-/-}$ fetal brains may lack *Chdh* and the ability to metabolize choline, choline metabolites were readily supplied by the mother resulting in normal brain development. Dietary studies restricting maternal choline during pregnancy resulted in reduced mitosis, increased apoptosis, and methylation reflected the maternal diet and subsequently sources of choline metabolites, such as betaine and PC. Disruptions in betaine

supply affect the availability of SAM, the universal methyl donor, leading to aberrant methylation patterns subsequently affect progenitor cell proliferation. Alterations in brain development during critical periods may greatly affect brain function in later life.

Chdh Expression

Fetal mouse brains showed significantly reduced *Chdh* expression compared to adults. A study in a rat model showed that *Chdh* activity in the liver of the progeny does not substantially increased until well after birth and weaning. Clow *et al.* (2008) study demonstrated that liver *Chdh* activity steadily increased after birth and plateaued around 35-42 days post-birth. Kidney *Chdh* also gradually increased after birth. *Choline dehydrogenase* activity was not measured in the brain [57]. Thus, it can be speculated that much of the choline and betaine found in fetal tissues is a result of maternal diet and not endogenous synthesis by the fetus.

Limitations

Aside from the limitations presented in the discussion section of Chapter 2, there are several others to note. First, the effect of maternal diet was not assessed. The fetus relies heavily on the mother for its nutrition, and thus fetal development may reflect the mother's ability to metabolize such nutrients as choline. SNPs (in humans) or gene deletion (in rodent models) of the pregnant mothers may alter nutrient availability. For instance, perturbed choline metabolism in the mother may result in less betaine, SAM, and PC for the fetus leading to altered programming in organ development. Additionally, we did not provide measures of gene-specific DNA methylation, particularly for genes that control cell proliferation. Assessment of DNA methylation patterns, in addition to the present data, will enable mechanistic hypotheses of

choline dehydrogenase and epigenetic reprogramming. Last, we only analyzed progenitor cell proliferation in the fetal brain. The brain consists of multiple cell types (i.e., astrocytes, ogliodendrocytes) that contribute to brain development. Astrocytes are the most abundant cell type in the mammalian brain and contribute to structure and formation of the blood-brain barrier. Additionally, astrocytogenesis is influenced by epigenetic mechanisms [58]. It is also hypothesized that astrocytes regulate energy supply for neurons, and thus survival [59].

Future Studies

Results from the current study generate several questions that could prompt further studies. Here I briefly propose future studies to address these questions, particularly the role of methylation and epigenetic regulation on progenitor cell proliferation.

Proposed Study 1

The current study will have to be replicated before any subsequent studies. This will confirm the findings herein and provide framework for further projects. Following the same study design, *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ fetuses will be harvested from *Chdh*$^{+/-}$ mating pairs fed AIN-76A with 1.1g/kg choline chloride. Fetal brains will be fixed for immunohistochemistry analysis of mitosis and apoptosis as described in Chapter 2. In addition to TUNEL analysis of apoptosis, there will be double staining of activated caspase-3 as previously described [4]. Double staining will better identify cells undergoing apoptosis. If results from this proposed study confirms the current study, it will increase sample size and provide sufficient evidence for further studies..

Proposed Study 2

Epigenetic regulation has been implicated in choline deficient models and this proposed study will determine if differential DNA methylation is present in *Chdh*$^{-/-}$ fetuses compared to *Chdh*$^{+/+}$ fetuses. *Chdh*$^{+/-}$ mating pairs will be maintained on diet AIN-76A with 1.1g/kg choline chloride to determine the role of the *Chdh* gene on DNA methylation. Fetal brains harvested from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ will be fixed, sectioned at 5 µm, and mounted on special membrane slides for laser-capture micro-dissection (LCM) of Ammon's horn ventricular and subventricular zones and the dentate gyrus for bisulfite modification and pyrosequencing. One gene of interest in this study is *Cdkn3*, an important regulatory gene in mitosis, particularly the G1/S-phase transition[60]. DNA methylation changes between fetal genotypes will validate (or counter) results seen in choline deficiency during pregnancy. Global DNA methylation and Kap protein expression will be determine by immunohistochemistry. Methods for these analyses have been previously described [5].

Results here combined with those in the current study and Proposed Study 1 will help to elucidate the impact of fetal *Chdh* deletion on brain development.

Proposed Study 3

Maternal diet has been shown to influence mitosis, apoptosis, and DNA methylation. To elucidate the interaction of the gene *Chdh* and diet, it would be of interest to assess progenitor cell proliferation, apoptosis, and methylation in E17 brains of *Chdh*$^{-/-}$ fetuses from *Chdh*$^{+/-}$ dams under normal and low dietary choline during pregnancy. Methylation pattern of genes that regulate cell cycle cyclin-dependent kinase inhibitors proteins, Kap, p15Ink4B, and p27Kip1 will be assessed. Behavioral tests in the adult animals under these in-utero conditions will help to

determine memory function as a result of maternal diet and gene deletion. Comparisons made between the fetal groups will determine the role of maternal diet during critical developmental window for the brain, even if an important gene in choline metabolism is deleted.

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