GESTATIONAL AND LACTATIONAL α-LINOLENIC ACID AVAILABILITY INDUCES EPIGENETIC CHANGES IN THE BRAIN OF MOUSE OFFSPRING

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Abstract

FULI HE: Gestational and Lactational α-Linolenic Acid Availability Induces Epigenetic Changes in the Brain of Mouse Offspring
(Under the direction of Dr. Mihai Niculescu)

It has been acknowledged that dietary n-3 fatty acids improve memory and learning, in part by altering gene expression in the brain. However, the exact mechanisms are far from being clear, especially for α-linolenic acid (ALA). Inadequate perinatal nutrition can induce persistent changes in offspring phenotype, acting through epigenetic process. We investigated the effects of maternal ALA intakes on the epigenetic regulation of memory-associated genes, in the offspring brains, at the end of lactation (postnatal day 19, P19). Postnatal ALA supplementation altered mRNA expression of *Mecp2, Ppp1cc, Reelin and Dnmt3a*, while ALA deficiency during gestation induced changes in methylation of CpG sites in either promoter or intron1 of these genes. In addition, bivariate analysis indicated some significant associations between CpG site-specific methylation and the expression of *Dnmt3a* ($p<0.05$). In summary, the interplay between ALA availability during gestation and lactation can differentially alter methylation and expression of genes involved in neurogenesis and synaptic plasticity, potentially affecting brain development and memory-related biological processes. However, further studies are needed to confirm the physiological effects of ALA.

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List of Abbreviations and Symbols

5-AZA	5-Aza-2-Deoxycytidine
5hmC	5-hydroxymethyl Cytosine
5mC	5-methyl Cytosine
AA	Arachidonic Acid
ALA	α-Linolenic Acid
ARC	Activity-Regulated Cytoskeleton-associated protein
BDNF	Brain-Derived Neurotrophic Factor
CFC	Contextual Fear Conditioning
CNS	Central Nervous System
CREB	CAMP Responsive Element Binding protein 1
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
Egr1	Early Growth Response protein 1
EPA	Eicosapentaenoic Acid
ERK	Extracellular signal-Regulated Kinase
FA	Fatty Acid
Fads2	Fatty Acid Desaturase 2
GADD45B	Growth Arrest and DNA-Damage-inducible, beta
HDAC	Histone Deacetylase
LA	Linoleic Acid

LTP	Long-term Potentiation
MBD	methyl binding domains
MeCP2	Methyl CpG Binding Protein 2
MS	Mass Spectrometry
NMDA	N Methyl D Aspartate
PP1	Protein Phosphatase 1
PPAR	Peroxisome Proliferator Activated Receptor
PUFA	Polyunsaturated Fatty Acid
RT-qPCR	Quantitative reverse transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
SREBP	Sterol Regulatory Element Binding Protein
TBS	Tris-Buffered Saline

Chapter I - Background

Introduction

The mechanisms underlying long-term memory formation have been one of the topics of interest recently. Adequate gene expression patterns are needed for this process, beginning in fetal life. Most studies focused on the role of DNA binding transcription factors involved in gene expression regulation. However, the epigenetic markings of chromatin, such as histone modifications and DNA methylation in the brain, have been recently recognized as an essential mechanism for brain functions, especially in learning and memory formation. The epigenetic changes allow nerve cells not only to respond to environmental stimuli and modulate their profile of gene expression, but also to establish and maintain their own identity [1-3].

Adequate nutritional intakes during gestation and lactation are thought to be important for offspring's growth, especially the brain development. During this period, the neonate's brain is experiencing a substantial acceleration in growth, cellular proliferation, and neuronal and glial differentiation [4]. Several studies have indicated that dietary omega-3 polyunsaturated fatty acids (n-3 PUFAs) are important nutrients for early life. Supplementation of the lactating mothers with n-3 PUFAs was proved to increase children's learning and memory processes [5].

It has been well documented that n-3 PUFAs improve memory and learning process. However, the exact mechanisms are still far from being clear. Several lines of evidence suggest that n-3 PUFAs affect gene expression in the brain, which can occur through interactions with specific or nonspecific ligands that bind to response factors acting on cis-regulatory elements of the genes. More recently, a clear understanding is developing concerning the importance of epigenetic-related molecular mechanisms in transcription-dependent long-term memory formation. Thus, in current study, we aimed to clarify whether epigenetic mechanisms, specifically DNA methylation, are involved in the n-3 PUFAs induced gene expression changes.

Dietary n-3PUFAs and Health

PUFAs and the their related complex lipids are important constituents of biological membranes and contribute to maintaining the structural and functional integrity of cells and cellular components [6, 7], especially docosahexaenoic acid (DHA; 22:6 n-3) and arachidonic acid (AA; 20:4 n-6), as the fundamental components of membrane phospholipids in neural cells. DHA and AA can be obtained directly from the diet or be converted for their precursors α-linolenic acid (ALA; 18:3 n-3) or linoleic acid (LA; 18:2 n-6), respectively, which must be obtained from dietary sources because most mammals lack the enzymes to synthesize them (Figure 1).

ALA is the parent precursor for the n-3 PUFAs elongation and desaturation pathways. This 18-carbon fatty acid possesses three double bonds and is commonly found in plant-derived dietary oils such as flaxseed, canola, and soybean oils. All the other common dietary n-3 PUFAs have longer chains, and are found mainly in fish and fish oils, including the 20-carbon eicosapentaenoic acid (EPA; 20:5 n-3) and the 22-carbon DHA [5]. It is important to

note that human and most other mammals express the enzymes necessary for the conversion of dietary ALA to any of the other members of the n-3 PUFAs family. One human clinical trial showed that consumption of ALA-enriched supplements for a 12-wk period was sufficient to increase EPA and DHA [8].

It has been acknowledged that dietary n-3 PUFAs improve memory and learning, and also alter the mRNA expression levels of several genes in the brain [9]. Short-term and long-term feeding of ALA enriched diet to rats significantly improved spatial learning memory, and altered proteome profiles in the hippocampus, including cytoskeleton and energy metabolism protein, thereby affecting neurogenesis and synaptic plasticity [10]. Although the beneficial health effects of n-3 PUFAs have been well documented, modern western diets enriched in saturated fatty acids and simple carbohydrates are often deficient in n-3 PUFAs. Thus, n-3 PUFAs are gaining ground in the supplement and functional food market. Most of the food industry uses vegetable oils, such as flaxseed oil, due to low cost. Thus, it is of more significance and practical to identify the role of ALA in brain development and cognition.

Flaxseed is a rich source of n-3 PUFAs, which includes a >50% ALA of total FAs content in the seed. It has been used in human studies aimed at mitigating neurologic and visual disturbances, folliculitis and growth retardation, and hemorrhagic dermatitis [11]. In sows and their piglets, dietary supplementation with different forms of flax, such as seed or oil, during late gestation and lactation has shown significant effects on immune resistance, and effects on fatty acids (FAs) profiles as an increased n-3 FAs in maternal milk, carcasses, and brain tissues in their newborn offspring [12]. Notably, a flaxseed diet during the perinatal period led to a negative growth of the fetus, although the offspring showed an improved

cognition. Thus, there must be caution in encouraging the maternal intake of flaxseed during pregnancy and lactation [13].

Effects of Dietary n-3 PUFAs on Gene Expression in the Brain

As we mentioned, dietary n-3 PUFAs can modulate gene expression in the brain or certain brain regions such as cerebrum and hippocampus [14, 15]. Some gene products, alone or in combination with the membrane effects of these PUFAs, exert their beneficial effect on neural functions such as learning and memory. Animal made deficient in n-3 PUFAs showed a significant decrease in the levels of DHA in their neural tissue, and this loss of DHA was accompanied by changes in neural function, including membrane-related events, metabolic events and cellular events [14, 16].

The final FA composition of brain is determined during embryogenesis, particularly during gestation and early postnatal life [4]. Thus, it is important to ensure the brain supplied with adequate PUFAs for its functions during this time, since permanent metabolic or genetic alterations can be initiated during perinatal period in the body and influence the individual's health later in life. Previous work from our lab found that maternal ALA availability during lactation enhanced neurogenesis in the dentate gyrus (DG) of the offspring at postnatal day 19 (P19), and its beneficial effects were offset by maternal ALA deficiency during gestation [17]. Kitajka, K., et al. also suggested that the supply of n-3 PUFAs during the perinatal period influenced brain gene expression later in life. DNA microarray analysis showed the expression of several genes being altered in the rat offspring brains fed n-3 PUFAs deficient diets in the perinatal period, regardless of the n-3 PUFA-sufficient diets later in life. These effects appear to be mainly independent of their effects on membrane composition [9].

The fact that ALA and DHA activate several genes in other tissues, like liver or adipose tissue, is well known [18-20], but the underlying molecular mechanisms of the direct effects of n-3 PUFAs diet-induced gene-expression changes in the brain have been addressed by very few studies [14, 21, 22]. Some studies suggest that PUFAs can directly interact with transcription factors, like Peroxisome Proliferator Activated Receptor (PPARs), Sterol Regulatory Element Binding Protein (SREBPs), which further induce the alteration in the expression of target genes [9]. Interestingly, among all tested FAs, ALA has the highest binding constant [23]. In current study, we investigated whether ALA could be related to some other indirect mechanisms which regulate gene expression, such as DNA methylation. Previously, we have found that maternal exposure to ALA during gestation and lactation altered fatty acid desaturase 2 (*Fads2*) DNA methylation in both maternal and offspring livers [24]. Groups receiving postnatal ALA supplement showed an increase in both *Fads2* promoter and intron1 DNA methylation, which was negatively correlated with *Fads2* gene expression. We speculated that ALA supplementation could induce epigenetic changes in other tissues, such as the brain.

Memory and Epigenetic Regulation

Memory is an abstract term that is not adequately or systematically defined. The most frequently used definition of memory is that it is the process by which information is encoded, stored, and retrieved. Specifically, a newly formed memory must first be acquired (encoding) and then be converted to a more persistent state in a process called consolidation (storing). Finally, stored memories are subject to retrieval when re-exposure to the initial environmental stimulus (retrieving) [25]. It has been known that alterations in protein synthesis, gene expression and structural properties of neurons and synapses are required in

all three stages. Within the neuron, synaptic depolarization activates complex molecular signaling cascades that coalesce at specific gene loci, and causes acute modulation of transcriptional efficacy. The resulting protein products are thought to produce stable alterations in cellular phenotype by affecting the structure and physiology of postsynaptic dendritic spines [26].

Epigenetic mechanisms have appeared as a central process in learning and memory (Figure 2). Traditionally, epigenetic mechanisms have been defined as a set of stable and heritable molecular phenomena that modify gene expression and do not involve alterations of the DNA sequence of a cell [27]. However, since epigenetic marks can be transient and are not necessarily heritable, especially when applied to lasting behavioral and cellular memory, a less comprehensive, but more appropriate definition has been brought up: "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" [28]. Due to their critical involvement in gene regulation, epigenetic mechanisms represent an attractive and reversible means for the brain to respond and adapt to specific environmental changes.

Epigenetic mechanisms are essential to normal development, as they provide the cellular memory necessary for perpetuating the correct cellular phenotype during mitosis. Epigenetic mechanisms participate in brain formation and regulate cell physiology and behavior, including learning and memory, drug addiction, depression and long-term responses to maternal care [3, 29, 30]. It is important to mention that certain memory tasks are associated with multiple regions, which may reflect temporally and spatially distinct phases of memory. In mammals, the formation and consolidation of many types of memories

occur in the hippocampus, after which many are subsequently incorporated into and maintained in cortical areas [31].

Epigenetic mechanisms include DNA methylation, histone modifications and RNA interference, all of which are inter-related [32]. Numerous experiments have been carried out to investigate the activity- and experience-dependent epigenetic mechanisms in the brain, which are outlined in Table 1. Most research targets the regulation of memory by histone modifications. For instance, artificially elevating levels of histone acetylation using histone deacetylase (HDAC) inhibitors enhances induction of long-term potentiation (LTP) in vitro and formation of long-term memory in vivo. Moreover, acetylation and phosphorylation of histone H3 are increased in vitro and vivo in the hippocampus following activation of NMDA receptors and extracellular signal-regulated kinase (ERK) [33, 34].

In this study, we were interested in another epigenetic mechanism, DNA methylation, that might be involved in perinatal brain development related to the process of memory formation, since a more recent and burgeoning field is discovering evidence implicating DNA methylation in mammalian performance (learning and memory) in behavioral tasks.

DNA Methylation in Memory Formation

DNA methylation is one of the primary epigenetic mechanisms for heritable gene silencing and regulation of gene expression. It has been show to play essential roles in genomic imprinting, X chromosome inactivation and maintenance of genome stability [2, 35]. This covalent modification of DNA involves conversion of cytosines at CpG dinucleotides to 5-methylcytosine (5mC). Approximately 60-70% of CpGs in the mammalian genome are highly methylated, except for the CpG-dense areas (some of them in the vicinity

of promoters), which are called CpG islands. Predominantly, CpG islands are kept in a demethylated state and function in part to regulate local gene expression [36]. The hypermethylation in CpG islands in promoter is usually associated with repression of the gene transcription. There are two models most acceptable in gene suppression effect: a)methyl cytosine prevents transcriptional activators from binding to the promoter region, or b)attracts transcriptional repressors that have methyl binding domains (MBD), such as methyl CpG binding protein 2 (*Mecp2*). These either directly influence transcriptional efficiency or indirectly affect chromatin structure by recruiting HDACs [37]. These two models have been complemented by recent studies which described active de-methylation via5-hydroxymethylcytosine (5hmC) [38, 39].

Dynamic DNA methylation events have been reported in the adult brain, particularly in the hippocampus. During learning, synaptic activity at hippocampal neurons initiates several signaling cascades, with changes occurring locally at the synapse in addition to the nucleus where transcriptional changes are made to plasticity-related proteins. These changes are supported, at least in part, by epigenetic modifications, including DNA methylation [40].

There are three DNA methyltransferases (DNMTs), the "maintenance methyltransferase" Dnmt1 and the "*de novo* methyltransferases" DNMT3a and DNMT3b, that have functional enzymatic activity in mammals. DNMT1 has a substrate preference for hemi-methylated DNA over unmethylated and interacts with the DNA replication machinery during the S phase of dividing cells. DNMT3a and DNMT3b can methylate the unmethylated cytosine of CpG dinucleotides on both strands, altering the epigenetic information content. These *de novo* methyltransferases play vital roles in establishing new genomic methylation patterns during cell differentiation [41]. DNMTs were found to be abundantly expressed in

neurons. Importantly, the three *DNMT* genes display differential expression profiles in the central nervous system (CNS). *DNMT1* is highly expressed in neurons from embryogenesis through adulthood [42, 43]. *DNMT3b* expression is observed in neural progenitor tissue only during early embryogenesis. *DNMT3a* is expressed from late embryogenesis to adulthood with a peak during the early postnatal period, then its level declines but remains detectable in the post-mitotic neurons of the adult brain [44]. These findings raised the possibility that DNA methylation patterns might be involved in dynamic changes in the brain for yet unspecified roles.

Alterations of neuronal gene expression patterns are required for long-term memory formation or for synaptic plasticity – the ability to change the strength of synaptic connections – in the CNS [45]. Since DNA methylation is required in neural activity-induced transcriptional changes, methylation might be of importance in the process of memory formation. However, whether the subsequent alteration of memory-associated gene expressions occur within the hippocampus in response to direct changes in DNA methylation of genes has yet to be determined.

In vitro, methylation inhibitor, zebularine and 5-aza-2-deoxycytidine (5-AZA) were used to inhibit DNA methyltransferase activity in the hippocampal slices of sacrificed mice. After inhibition, the promoter regions of the memory enhancer genes, *Reelin* and *brain-derived neurotrophic factor* (*Bdnf)*, induced rapid and dramatic decreases in DNA methylation, which was speculated to contribute to the failure of LTP in the hippocampal region of these sacrificed mice [46].

Further, Milier and Sweatt carried out an *in vivo* study to better illustrate that DNA methylation was crucial and necessary in memory formation [47].The researchers induced a group of adult male Spragure-Dawley rats with a contextual fear conditioning (CFC) test. After sacrificing, the DNA methylation pattern of the memory suppressor gene (*protein phosphatase 1* (Pp1) and the memory enhancer gene *Reelin* were analyzed in the hippocampus. They found a rapid methylation and down-regulation of *Pp1*, along with the demethylation and activation of *Reelin* after CFC. However, 5-AZA and zebularine infusion into the hippocampus immediately reversed the methylation patterns of these two genes, indicating a potential role in the dysfunction of long-term memory formation. In addition, with a similar methodology and the same type of rats, alterations were found in chromatin structure and in methylation, which affected differential regulation of the *Bdnf* gene expression, again suggesting the critical role of DNA methylation in the formation of memory [48].

More recently, *Dnmt1* and *Dnmt3a* double conditional knockout mice were engineered to illustrate that DNA methylation is required for long-term memory [2]. Results showed that the complete loss of gene expression of these two proteins in forebrain neurons led to an abnormal methylation pattern and deregulated expression of some other genes in the same area, causing deficits in the ability to learn and memorize. The neurons appeared smaller than those seen in the wild-type mice, though a neuronal loss was not identified.

It is important to note that DNA methylation and histone acetylation may work together to regulate long-term memory formation and synaptic plasticity. MeCP2 has been suggested to function as the media that connects DNA methylation and histone acetylation [49]. Substantial crosstalk between DNA methylation and histone modifications has been

reported [50, 51]. The dominant understanding is that the MeCP2 protein binds to DNA that has been methylated. Once bound, MeCP2 can interact directly with HDAC1 and 2 through its transcriptional repressor domain, suggesting that DNA methylation at certain loci indirectly silences gene expression through chromatin modification. However, a recent epigenomic analysis suggested this might not be the case [39].

MeCP2 is found in high concentrations in neurons and is associated with maturation of the CNS and in forming synaptic contact. Its mutations can cause Rett Syndrome, a debilitating neuro developmental disorder associated with learning and memory deficits from a young age. It is likely that the inhibition of MeCP2 would mimic the effect of DNMT blockade. Actually, deletion of *Mecp2* resulted in deficits in paired-pulse facilitation, a form of short-term plasticity [52], while over-expression of *Mecp2* led to enhanced short-term plasticity [51]. It is important to note that the gain-of-function genetic variation in *MeCP2* can also lead to MeCP2 duplication syndrome, which indicates that precise regulation of MeCP2 is a key requirement for the neuronal homeostasis.

The key players involved in dynamic DNA demethylation changes in neurons in the context of memory formation and storage remain unknown at present. Interestingly, Dnmt3a and Dnmt3b have been also implicated in active demethylation of DNA [53, 54]. In addition, another study found that the growth arrest and DNA damage-inducible protein 45 (Gadd45) family contributed to active DNA demethylation utilizing a mammalian cDNA expression library and methylated reported constructs [55]. Later, the isoform *Gadd45b* was reported to be robustly up-regulated in response to cell depolarization, indicating its function associated with activity-regulated DNA demethylation and postnatal neurogenesis [56].

DNA Methylation Marks in Memory

DNA methylation has primarily been considered as a transcriptional regulator that mediates gene expression. However, its regulation and function are likely to be far more complex in the brain. As mentioned above, DNMT inhibitors have significant effects on memory formation and storage and behavioral responses to drugs of abuse as well. The ability of DNA methylation to affect these behaviors raises the question whether those genes, which are important regulators of these experiences, contain active sites for methylation and/or demethylation of DNA. In fact, a large number of genes that have previously been shown to positively and negatively modulate behavioral memory contain dense CpG islands surrounding their promoter regions. Thus, these genes are potential targets for changes in the machinery that underlies DNA methylation in neurons.

In the current study, we selected 12genes that were previously reported to be associated with consolidation of synaptic plasticity or/and long-term memory formation, including 4 DNA methylation regulatory/machinery genes*, Dnmt1, Dnmt3a, Gadd45b* and *Mecp2* (Table 2)

Hypothesis

Several studies found that short-term and long-term feeding of ALA-enriched diets significantly improved memory and learning processes, and also altered mRNA expression levels of several genes in brain, thereby affecting neurogenesis and synaptic plasticity. However, most of the studies were conducted in the adult animal models. Importantly, the neonate's brain experiences growth spurt during gestation and lactation, thus the perinatal period is particularly important for brain development.

Our previous work revealed that the maternal ALA during gestation and lactation can alter the hippocampal development in the male offspring at the end of lactation at P19. As a continuation of this study, we aimed to determine the potential influence of ALA availability during gestation and lactation on brain development of male offspring, specifically on the memory process. **The hypothesis was that maternal ALA availability during gestation and lactation could induce epigenetic changes in the offspring's brain, which in turn, alter the expression level of several memory-associated genes.**
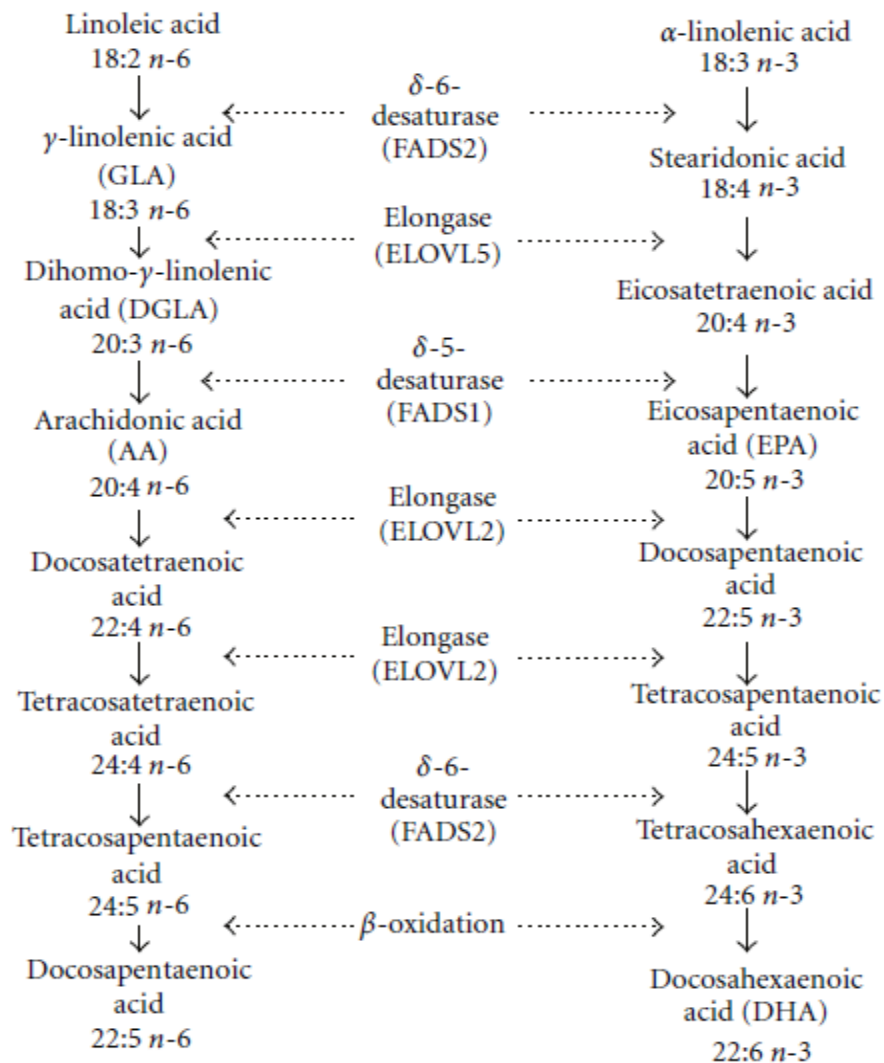
Figure 1.Metabolism of n-6 and n-3 PUFAs. (adapted from [57])

The essential fatty acids LA and ALA are the metabolic precursors of n-6 and n-3 PUFAs, respectively. Once obtained from the diet, the precursors are converted by the successive desaturation and elongation steps to AA and EPA. EPA can be further metabolized to DHA through desaturation and peroxisomal β-oxidation. In response to dietary n-3 PUFA deficiency, AA is partly converted to DPA to substitute for DHA in membrane phospholipids.
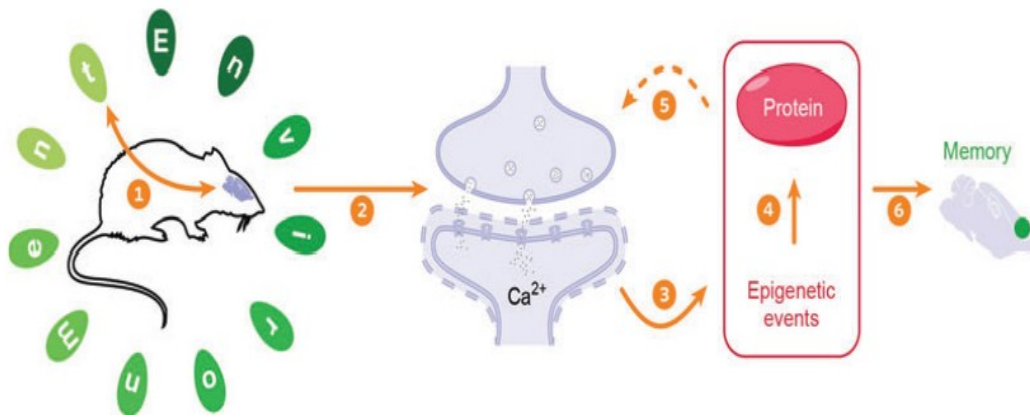
Figure 2.Summary of the basic structural and molecular events that take place during memory formation. (adapted from [31])

Associative learning can be induced by different environmental factors (1), which will lead to cellular changes in the brain in order to induce structural plasticity and increase synaptic strength (2). Epigenetic events is partly involved in the transcriptional regulation, in order to dynamically modulate protein synthesis through transcriptional activation or silencing (3, 4) to establish long lasting memory (6).Thus, epigenetic changes can mediate and be mediated by behavioral changes, synaptic plasticity and structural changes to potentiate long-term memory processes (3, 5).

Table 1. Summary of epigenetic processes in memory and memory-associated transcription

Mechanism	Findings
Histone acetylation	• HDAC activity was negatively associated with hippocampus (especially HDAC2) and cortex-dependent memory in mammals via CREB: CBP-dependent transcriptional activation, and may operate through induced of Nr4a1 and Nr4a2 transcription factors [58] • HDAC inhibition promoted associative memory consolidation and H3 acetylation in *Chasmagnathus* [59] • Contextual fear conditioning promoted H3 acetylation while latent inhibition training induces H4 acetylation [60] • Over-expression HDACs in the nucleus accumbens impaired conditioned place preference for cocaine, while HAD inhibitors increase cocaine place preference [61]
Histone phosphorylation	• H3S10 phosphorylation was associated with impaired spatial and contextual memory by deletion of msk2, and 2 impaired forced swim memories with deletion of Msk1 [62] • Activation of ERK/MAPK in vitro and contextual fear conditioning in vivo led to an increased histone H3 phosphorylation in hippocampal area [33]
Histone methylation	• Over-expression of the histone methyltransferase G9a in the nucleus accumbens impaired conditioned place preference, and opposite result was found in knockdown of G9a [63] • Contextual fear conditioning enhanced H3K4me3 and H3K9me2 marks, regulating genes including Egr1 and Bdnf [64]
DNA methylation	• Deletion of Mbd1 and Mecp2 impaired long-term memory in rodents [52] • DNMT inhibition impaired long-term memory consolidation in the hippocampus and remote contextual fear memory storage in the cortex • Fear conditioning induced dynamic alterations in DNA methylation at loci including Reelin, Ppp1cb, Bdnf and Egr1 in the hippocampus during memory consolidation [47, 48] • DNMT inhibition or knockout in the nucleus accumbens increased development of conditioned place preference for cocaine, whereas over-expression of Dnmt3a reduced cocaine place preference [65] • Remote memory storage is associated with lasting hypermethylation and down-regulation of Ppp3ca in the anterior cingulate cortex [66]

Table 2. Summary of target gene information

Gene	Findings
DNA methylation modifiers	
Dnmt1	• DNMT inhibition and genetic deletion of dnmt1 impaired LTP
Dnmt3a	• DNMT inhibition and genetic deletion of dnmt3a impaired LTP • Over-expression of dnmt3a increased spine density in nucleus accumbens
Gadd45b	• Associated with activity-regulated DNA demethylation and postnatal neurogenesis
Mecp2	• A transcriptional factor important for controlling gene expression through the interpretation and regulation of epigenetic markers • Mutation in Rett Syndrome • Deletion or truncation impaired LTP expression (reduction in paired-pulse ratio, increase in vesicle release probability, reduction in spontaneous neurotransmission, and enhanced inhibitory tone) • Over-expression led to enhanced short-term plasticity
Memory-associated genes	
Arc	• Required for consolidation of synaptic plasticity and formation of long-term memory • Regulate endocytosis of AMPA receptors in response to synaptic activity[67]
Bdnf	• Major regulator of synaptic transmission and plasticity at adult synapses in many regions of the CNS[48]
Creb1	• Enhanced expression in consolidation of long-term synaptic facilitation and long-term excitability in sensory neurons of Aplysia.
Reeln	• Regulate postnatal neurogenesis and enhances spine hypertrophy and LTP
Egr1	• Deficient mice have impairments in late phase hippocampal LTP and consolidation of some forms of long-term hippocampus- and amygdala-dependent memory
Pp1 complex: Ppp1cb/Pp1β & Ppp1cc/Pp1γ	• Inhibition of active mouse Protein Phosphatase 1 [Pp1] complex(es) increases LTP of mouse hippocampal tissue that is decreased by oligomeric AMYLOID β-42protein • A rapid methylation and down-regulation of Pp1 was found in mouse hippocampal region along with the demethylation and activation of *Reelin* after fear conditioning
Ppp3ca/Calicineurin	• Activity reduction of Calcineurin protein complexin brain from mouse increases long-term memory by mouse.

Chapter II – Journal manuscript

Introduction

Memory is the process that new information is first acquired (encoding), then converted to a more persistent state in a process called consolidation (storing) and finally, stored memories are subject to retrieval when re-exposure to the initial environmental stimulus (retrieving) [25]. It has been known that alterations in protein synthesis, gene expression and structural properties of neurons and synapses are required in all three stages [26].

Several studies have indicated that dietary n-3 PUFAs can improve memory and learning processes, and also alter the mRNA expression levels of several genes in the brain [9]. It is important to ensure the brain supplied with adequate PUFAs during gestation and early postnatal life, since the final FA composition of brain is determined and brain experiences a growth spurt at this time [4]. Permanent metabolic or genetic alterations can happen during perinatal period in the body that influence the individual's health later in life. Recent research has focused on the association between n-3 PUFAs and better cognitive development in infancy or slower cognition decrease in the elderly [68]. One study reported that rats with n-3 FA deficient diet achieved nearly the same level of brain DHA and spatial task performance as animals maintained for three generations on an n-3 sufficient diet, when

they were given DHA supplements from birth or weaning. Another study from our lab showed that in the mouse offspring on postnatal day 19, its brain development is differentially affected by maternal availability of ALA during gestation and lactation [17].

n-3 PUFAs are gaining ground as dietary supplements due to their potential benefits on health. There are many sources of n-3 PUFAs used for supplementation. However, most of the food industry uses vegetable oils containing ALA, instead of fish oils which have DHA and preformed EPA, because of low cost. Thus, it is of more practical significance to investigate the effects and mechanisms of ALA on physical health.

Flaxseed is a great source of n-3 PUFAs, which includes a >50% ALA of total FA present in the seed. It has been used in human studies aimed at mitigating neurologic and visual disturbances, folliculitis and growth retardation, and hemorrhagic dermatitis [11]. In sows and their piglets, dietary supplementation with different forms of flax, such as seed or oil, during late gestation and lactation has shown significant effects on immune resistance, and effects on fatty acids (FAs) profiles as an increased n-3 FAs in maternal milk, carcasses, and brain tissues in their newborn offspring [12].

The underlying molecular mechanisms of the direct effects of PUFA diet-induced gene-expression changes in the brain have been addressed by very few studies. Most studies suggest that PUFA can directly interact with transcription factors, like PPAR, SREBPs, by which further induce the alteration in the expression of target genes [9]. However, an accumulation of recent studies has emphasized the importance of epigenetic mechanisms, such as DNA methylation, playing a role in alteration of neuronal gene expression pattern, which is required for synaptic plasticity or memory formation [40].

DNA methylation is the covalent modification of DNA, involving conversion of cytosines at CpG dinucleotides to 5-methylcytosine. Approximately 60-70% of CpGs in the mammalian genome are highly methylated, except for the CpG-dense area in the vicinity of a promoter, which is called CpG island. Predominantly, CpG islands are kept in a demethylated state and function in part to regulate local gene expression [36]. A large number of genes that have previously been shown to positively and negatively modulate behavioral memory contain dense CpG islands surrounding their promoter regions. Thus, these genes are potential targets for changes in the machinery that underlies DNA methylation in neurons.

In the study, we hypothesized that maternal ALA availability during gestation and lactation could induce the alterations in the expression of several memory-associated genes in offspring brains, and these changes are related to epigenetic modifications, specifically DNA methylation, in the CpG islands spanning the promoter and the intron1 of these genes

Materials and Methods

In order to investigate the effects of perinatal maternal ALA exposure on the offspring's brain, female C57BL/6J mice were mated with C57BL/6J males and allowed the access to an ALA control diet containing soybean oil as fat, or an ALA deficient diet containing corn oil as the only source of fat before and during gestation, which was followed by either the same diet in gestation or an ALA sufficient diet containing flaxseed oil in the lactation period. Male pup brains were snap-frozen in liquid nitrogen and stored at -80 °C on postnatal day 19. DNA, RNA and protein were extracted from the frozen whole brain samples for DNA methylation, gene expression and protein expression analysis, respectively. The processes are detailed below.

Animals, Diets, and Tissue Collection

This study was approved by the University of North Carolina Institutional Animal Care and Use Committee. This work is a continuation of our previously published, and a detailed description can be found in previous published literature [17]. Briefly, mouse C57BL/6J females (10 wk old; Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned into two initial feeding groups (intervention 1, Figure 3.) for 30 days prior, and during gestation. One group was fed with a defined control diet (AIN-93G, DYETS, Bethlehem, PA) containing soybean oil as fat source (ALA control, C, n=12), while the other group was given an AIG-93G modified diet (DYETS) with corn oil as the only source of fatty acids (ALA deficient, D, n=12). After 30 days, the females were bred overnight with males that were maintained at all times on the C diet. One day prior to delivery date, the two groups of females were randomly split into two subgroups (intervention 2). Half from each group remained on the same diet (n=6), and the other half were switched to a modified AIN-93G diet containing flaxseed oil (ALA supplemented, S, n=6), until postnatal day 20 (P19, considering the date of delivery as P0). On P19, all mothers and their pups were sacrificed. The pups were decapitated, the brains were extracted, snap frozen in liquid nitrogen and stored at -80 °C. In this study, the brain samples from the male pups were used for further analysis. However, due to the lack of male pups, only n=5 male brains were included here in the C-C, C-S and D-S group each.

The fatty acid composition of each diet is indicated in Table 3. Particularly, the diets contained 18:2 n-6 (LA) and 18:3n-3 (ALA) with the following concentrations: D diet, 87.237 and 1.554 nmol/mg; C diet, 86.938 and 10.827 nmol/mg; S diet, 23.133 and 75.367 nmol/mg.

Real-time RT-PCR

RNA was extracted from the male offspring brains (all at P19) with QIAcube instrument (Qiagen, Valencia, CA, USA) using AllPrep® DNA/RNA mini kit (Qiagen), according to manufacturer's protocol. After the concentration and quality were determined with NanoPhotometer (Implen GmbH, Munich, Germany), Quantitative reverse transcriptase PCR (qRT-PCR) method was used for the assessment of gene expression. First, cDNA synthesis was performed using a QuantiTect reverse transcription kit (Qiagen), on an Eppendorf Mastercycle ProS (Eppendorf, Hamburg, Germany). The second part was performed on an Eppendorf Mastercycler Realplex^{2}epGradient S real-time PCR cycler, using the QuantiTect SYBR Green PCR kit (Qiagen). QuantiTect primer assays (Qiagen) were purchased for the following genes: *Dnmt1*, *Dnmt3a*, *Mecp2*, *Gadd45b*, *Ppp1cb*, *Ppp1cc*, *Ppp3ca*, *Arc*, *Bdnf*, *Creb1*, *Reelin*, *Egr1* and 18s RNA (used as internal reference for each sample). The real-time PCR reactions were run in triplicate, and data were retrieved as C_T values normalized to 18S and log$_2$ transformed for subsequent statistical analysis. Final data were expressed as ratios between sample, and the average of the C-C group for each gene.

Bisulfite Pyrosequencing

DNA from male offspring brains (all at P19) was extracted with QIAcube instrument (Qiagen, Valencia, CA, USA) using AllPrep® DNA/RNA mini kit (Qiagen), following the specific protocol. Up to 1μg DNA from each sample was treated with sodium bisulfate using the same instrument and the corresponding protocol (EpiTect kit; Qiagen).

One Pyrosequencing DNA methylation assay was designed for *Mecp2* using PyroMark Assay Design 2.0 software (Qiagen). The other 8 PyroMark CpG assays were

directly purchased from Qiagen. These assays information, including the assay location, sequence to analyze, PCR primers, sequencing primers, and PCR conditions are indicated in Table 4. The amplification of templates was performed on an Eppendorf Mastercycler ProS using PyroMark PCR kit following the recommended protocol (Qiagen). The PCR condition was 95°C 15 min; 45 cycles of 94°C 30 s, 56°C 30 s, 72°C 30s; 72°C 10 min and 4°C hold. The biotinylated strand of the amplified DNA was subjected to pyrosequencing on a PyroMark MD machine (Qiagen), as described previously [69]. Each sample and sequence were run in triplicate, and only pyrosequencing reactions that passed the quality test were included in the analysis. For each sample and sequence, percentage methylated cytosine for each CpG site was expressed as the average of triplicates, and the values across all sites were averaged to express the average methylation across each sequence.

Western Blot

Male pup brains were homogenized in lysis buffer (1% sodium dodecil sulfate, 10mM sodium ortovanadate, and 10mM Tris-HCl). Total protein concentration for all samples was determined using the Lowry protein assay [70]. Proteins were separated by electrophoresis using polyacrylamide Tris-HCl gels (4-15%; Bio-rad Laboratories, Hercules, CA, USA) and then blotted on nitrocellulose membranes (0.2micron; Thermo Scientific, Rockford, IL, USA).

Immunodetection was carried out using SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) according to the dedicated protocol. This system uses vacuum assisted fluid distribution to rapidly and thoroughly permeate the blot with antibodies and buffers, and thus less antibodies and incubation time is required than normal

procedure. The membrane was blocked with 10x blocking buffer (Sigma) diluted with 0.1% Tween-TBS (TBST) prior to incubation with primary antibody Mecp2 (sc-20700; Santa Cruz Biotechnology) and Ppp1cc (#orb6773; Biorbyt Ltd) at a 1:200 concentration for ten minutes. Following primary incubation, membranes were washed three times in TBST, and incubated with the respective secondary at a 1:5000 concentration for another ten minutes. Membranes were then washed three times in TBST, incubated in SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) for five minutes and exposed to Autoradiography Film (MIDSCI, Missouri, USA) for 40 seconds.

The second immunoblotting with β-Actin (Rabbit mAb HRP conjugate #5125, Cell Signaling) for internal reference was performed with normal western blot procedure, after determining that the membrane was properly stripped with Restore Plus Western Blot Stripping Buffer (Thermo Scientific). Briefly, the membrane was blocked overnight at 4 °C with gentle shaking and then incubated with β-Actin antibody at 1:1000 dilution in blocking buffer for 2 hours at room temperature. The same luminal reagent and detection was performed as we mentioned before. Semi-quantitative analysis was carried out using Image J and normalized to β-Actin.

Statistical Analysis

All statistical analysis was carried out using SPSS 15.0 software (IBM). For DNA methylaton, gene expression, protein expression data, results were analyzed for equal variances, followed by 1-way ANOVA. For each variable, statistical significance between any two groups was tested using the Tukey test for multiple group comparisons ($P < 0.05$). In

addition, bivariate linear fit analysis was performed for the correlation between two

variables, with a threshold for significance of $P < 0.05$.
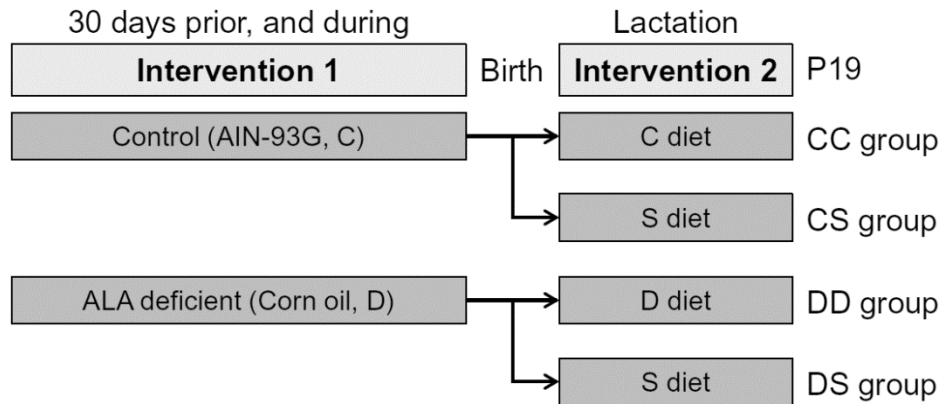
Figure 3. Study design

As described in Materials and methods, there were two internventions in the study. Intervention 1 consisted of the administration of specified diets for 30 days prior to and during pregnancy. Intervention 2 consisted of the administration of specified diets during lactation until P19, when dams and pups were sacrificed.

C, control AIN-93G diet containing soybean oil; D, ALA deficient diet (custom AIN-93G) containing corn oil; S, ALA sufficient diet (custom AIN-93G) containg flaxseed oil.

Table 3.Fatty acid composition of diets

		D diet (nmol/mg diet)	C diet (nmol/mg diet)	S diet (nmol/mg diet)
14:0	Myristic	0.124	0.251	0.151
14:1	Myristoleic	0.011	0.020	0.021
16:0	Palmitic	19.651	19.613	9.628
18:0	Stearic	2.910	2.519	2.365
18:1 n9	Oleic	34.435	33.229	29.756
18:2 n6	LA	87.237	86.938	23.133
18:3 n3	ALA	1.554	10.827	75.367
20:0	Eicosanoic	0.638	0.795	0.222
20:1 n9	11-Eicosenoic	0.352	0.328	0.214
20:2 n6	11,14-Eicosadienoic	0.030	0.067	0.049
20:3 n6	Dihomo-gamma-linolenic	ND	ND	0.037
20:4 n6	AA	ND	0.009	0.078
20:3 n3	11,14,17-Eicosatrienoic	0.187	0.559	0.200
22:0	Behenic	0.012	0.008	ND
22:1 n9	Erucic	ND	0.007	ND
20:5 n3	EPA	0.012	0.024	0.013
24:0	Lignoceric	0.228	0.169	0.139
24:1	Nervonic	ND	ND	0.010
22:5 n3	7,10,13,16,19-Docosapentaenoic	ND	ND	ND
22:6 n3	DHA	ND	ND	ND

ND, not detected

Table 4. Assay information for bisulfite pyrosequencing

Gene position	Assay	Sequence to analyze	Number of CpGs
Mecp2 promoter	*Self-designed*	*F:* AGTTTGGGTTTTATAATTAATGAAGGG *R [5-Biotin]:* ACCTTAACCATCCCACTCACAATCTC *S:* AGGTGTAGTAGTATATAGG *Sequence to analyze:* TTGGT**C**GGGAGGG**C**GGGG**C**G**C**GA**C**GTTTGT**C**GTG**C**G GGG	**7**
Mecp2intron1	Mm_Mecp2_01_PM	**C**G**C**G**C**GCAGCCCCAACTGG**C**GAAGCCCAGA**C**GA	**5**
Reelin promoter	Mm_Reln_01_PM	GGAC**C**CGACAGG**C**GAGCTT**C**GC**C**GGACTCTGTATTT A**C**G**C**GT	**6**
Egr1 promoter	Mm_Egr1_08_PM	CTCCACCCTGC**G**ACCC**G**CTCC**G**GCATC**G**C**G**AGC**G**C	**6**
Arc exon1	Mm_Arc_02_PM	AACTTGGAC**G**GCTAC**G**TGCCCACC**G**GC**G**ACTCACAG C**G**C	**5**
Ppp1cb promoter	Mm_Ppp1cb_01_PM	**C**GAAA**C**GCC**G**C**G**TGACT**C**GTAGGTGAGAA**C**GC**C**G	**7**
Ppp1cc intron1	Mm_Ppp1cc_03_PM	**C**GG**C**G**C**G**C**GGGTGGGTGG**C**GGGG**C**AGGCC**C**GGCC**G** GT	**7**
Dnmt3a promoter	Mm_Dnmt3a_04_PM	C**G**C**G**T**C**G**C**CACCCACAGCCAGGTGCC**G**C**G**GT	**5**
Dnmt1 promoter	Mm_Dnmt1_01_PM	G**C**G**C**ATG**C**G**C**G**C**AA**C**GGA	**4**

F: forward primer; R, reverse primer; S, sequencing Primer

Results

Maternal Exposure to ALA in Lactation Alters Expression of Memory Associated Genes and DNA Methylation Regulatory in Offspring Brains

qRT-PCR assays were performed to ascertain if memory-associated genes exhibited altered expression in the brain of offspring of male mice with different dietary treatment during gestation or lactation. Eight genes that have previously been shown to positively and negatively modulate behavioral memory were chosen for analysis. All of them contain dense CpG islands surrounding their promoter. Thus, these genes are potential targets for changes in the machinery that underlies DNA methylation in memory formation. Significant changes in transcript levels were detected for *Arc, Reelin, Egr1, Ppp1cb,* and *Ppp1cc* (Figure 4).ALA supplementation during lactation induced *Arc* under-expression in CS group (80% decrease), *Reelin* over-expression in CS (44% increase) and DS group (69% increase), *Egr1* and *Ppp1cb* under-expression in CS group (both around 75% decrease), and *Ppp1cc* over-expression in CS (46% increase) and DS group (56% increase), as compared to CC group.

The mRNA expression was also determined in *Dnmt1*, *Dnmt3a*, *Mecp2* and *Gadd45b* (Figure 4b). *Dnmt1* expression was suppressed in CS group (73% decrease), while *Dnmt3a* was suppressed in both CS (97% decrease) and DS group (93% decrease) and *Mecp2* was over-expressed in CS (33% increase) and DS group (53% increase), when compared to CC group. Those genes that did not exhibit significant differences between any two groups were not further investigated.

Effects of the Maternal ALA Treatment on DNA Methylation in the Offspring Brains

Because mRNA expression differences were identified in several genes, as well as DNA methylation machinery genes, we further determined whether the DNA methylation of these genes in the promoter or/and intron1 was altered by any dietary treatment. ALA deficiency during gestation induced a hypomethylation of *Mecp*2 promoter in the DD (45% decrease) and DS (40% decrease) group, as compared to CC group (Figure 5a). Interestingly, one CpG site (position 3) methylation was suppressed by postnatal ALA supplementation (55% and 80% decrease in CS and DS groups, respectively). We also identified alterations in several CpG sites in *Dnmt1, Dnmt3a, Egr1, Reelin and Ppp1cc* (Figure 5b).

We also performed bivariate fit analysis between Dnmt3a/Dnmt1 expression and the methylation percentage of these CpG assays, so as to determine the potential role of *Dnmt1* and *Dnmt3a* expression in the alterations in DNA methylation induced by maternal ALA exposure. A significant positive correlation was found between the *Dnmt3a* gene expression and position 3 (p3) CpG sites of *Mecp2* promoter assay ($R^2 = 0.243$, p=0.022; Figure 6a), and this CpG site was correlated moderately with *Mecp2* gene expression negatively ($R^2 = 0.136$, p=0.1; Figure 6b). Meanwhile, there was a negative correlation between *Mecp2* and *Dnmt3a* gene expression ($R^2 = 0.375$, p=0.004; Figure 6c). In addition, the methylation of another CpG site in *Mecp2* intron1 was negatively correlated with *Dnmt1* mRNA expression ($R^2 = 0.116$, P=0.03; Figure 6d). *Mecp2* was also reported to regulate gene expression of epigenetic marks. The expression of *Reelin* was marginally correlated with its CpG site methylation in promoter ($R^2 = 0.176$, p=0.058; Figure 8b) and Mecp2 expression ($R^2 = 0.957$, p=0.000; Figure 8c), while negatively related with Dnmt3a expression ($R^2 = 0.43$, p=0.002; Figure 8d)

Similar to what we found between *Mecp2* and *Dnmt3a*, there was a significant positive correlation between one *Ppp1cc* CpG site methylation and *Dnmt3a* expression ($R^2 =$

0.331, p=0.006; Figure 7a), and the mRNA expression of *Ppp1cc* was negatively associated with *Dnmt3a* expression (R^2 = 0.431, p=0.002; Figure 7c).

Mecp2 and Ppp1cc Protein levels do not correlate with Transcriptional Changes

We investigated whether the transcriptional changes would induce protein alteration in the offspring brains. Western blot results showed that there was no significant difference in Mecp2 and Ppp1cc protein levels between dietary treatments at the end of lactation (Figure 9).

Discussion

The findings of this study support the hypothesis that maternal ALA intake during pregnancy and lactation alters the expression of memory-associated genes, specifically in the offspring brain at P19, and that this is in part related with altered epigenetic regulation of the gene transcription.

Our previous work has shown that postnatal maternal ALA supplementation promoted neurogenesis in the hippocampus of the offspring [17]. The present findings indicate that this effect could be a result of increased expression in *Ppp1cc, Reelin and Mecp2* in CS and DS group. Reelin is essential for neuronal lamination and synaptic plasticity and a decreased transcript level of *Reelin* has been found as a result of ALA-deficiency [71]. The increased mRNA level of *Ppp1cc* is also in agreement with a previous report that its protein product, Pp1γ promotes neurogenesis by decreasing the phosphorylation and ubiquitination of Creb in hyperbaric oxygen therapy (HBOT) [72]. Mecp2 is a transcription regulator of gene expression that is an important epigenetic factor in

the maintenance and development of the CNS. Studies suggest that loss- or gain-of Mecp2 function exert bidirectional control (decreased vs. increased) in neurotransmission in cortical and hippocampal regions of the brain, probably through the regulation of synaptic gene expression [73]. Our results showed a significant correlation between *Mecp2* expression and *Ppp1cc, Reelin* mRNA level, indicating that *Ppp1cc* and *Reelin* may be potential target genes for Mecp2.

In contrast to the robust changes in expression, only few changes in CpG methylation were detected. Different from mRNA expression, the DNA methylation patterns were associated mostly with gestational ALA exposure, which suggested a particular important role of DNA methylation during early stages of embryonic and fetal development. It was also possible that other epigenetic mechanisms, such as histone modification, play a more important role during lactation period. Also, the *Dnmt3a* and *Dnmt1* expression did not necessarily predicate DNA methylation, suggesting the speed methylation of response to Dnmts may vary among different CpG sites. One limitation of our study is that we cannot differentiate the exact roles of ALA supplementation during gestation vs. lactation, for the lack of multiple time points, and a CD group (a control or supplemented diet during gestation, followed by a deficient diet in lactation).

The less significant changes in methylation may also be caused by a small sample size, and some limitations of the technique, especially the failure to separate 5hmC from 5mC by our current bisulfite-treated pyrosequencing. 5hmC is a marker for DNA demethylation. The presence of 5hmC in promoter regions was associated with high levels of transcription. More recently, Mecp2 was identified as the major 5hmC-binding protein in the brain and had similar high affinities to 5hmC- and 5mC-containing DNA [38]. Thus, we

could speculate, for instance, that Mecp2 binds to 5hmC in *Reelin* promoter, and in turn regulates its gene expression, since one CpG site was positively correlated with *Reelin* and *Mecp2* expression, while being negatively associated with *Dnmt3a* levels. This should be further validated by a complementary technique and be applied to a larger sample size, such MS-based approaches and Tet-assisted bisulfite sequencing (TAB-seq) that can detected 5hmC [74].

Although western blot results did not detect differences in Mecp2 and Ppp1cc protein level, it does not necessarily mean that their bioactivity has not been altered. More quantitative and specific assays should be performed. It is also important to note another limitation in the study: all the results were obtained using the whole brain of offspring. Evidence has indicated that gene expression differs across brain regions [75]. Studies using whole brains could result in false–negative results, owing to a dilution effect. Different brain areas are thought to be involved in specific types of memory. For example, the hippocampus is thought to be responsible for the spatial learning, while the amygdale is believed to participate in emotional memory [76]. Thus, protein measurement in these specific areas may present significant results, especially for *Mecp2, Reelin* and *Ppp1cc*, since they have been suggested to promote neurogenesis.

In summary, our results indicated that maternal ALA supplementation induced alterations in transcript levels and DNA methylation of several memory-associated genes. More study and experiments are required to determine the exact timing and mechanism of these events. A follow-up study should also be performed, in order to clarify the physiological impact of maternal ALA supplementation, from flaxseed oil and other plant

sources, during gestation and lactation on brain function, especially the memory and learning

process.

a. Memory-associated gene expression

b. Gene expression of epigenetic modifiers
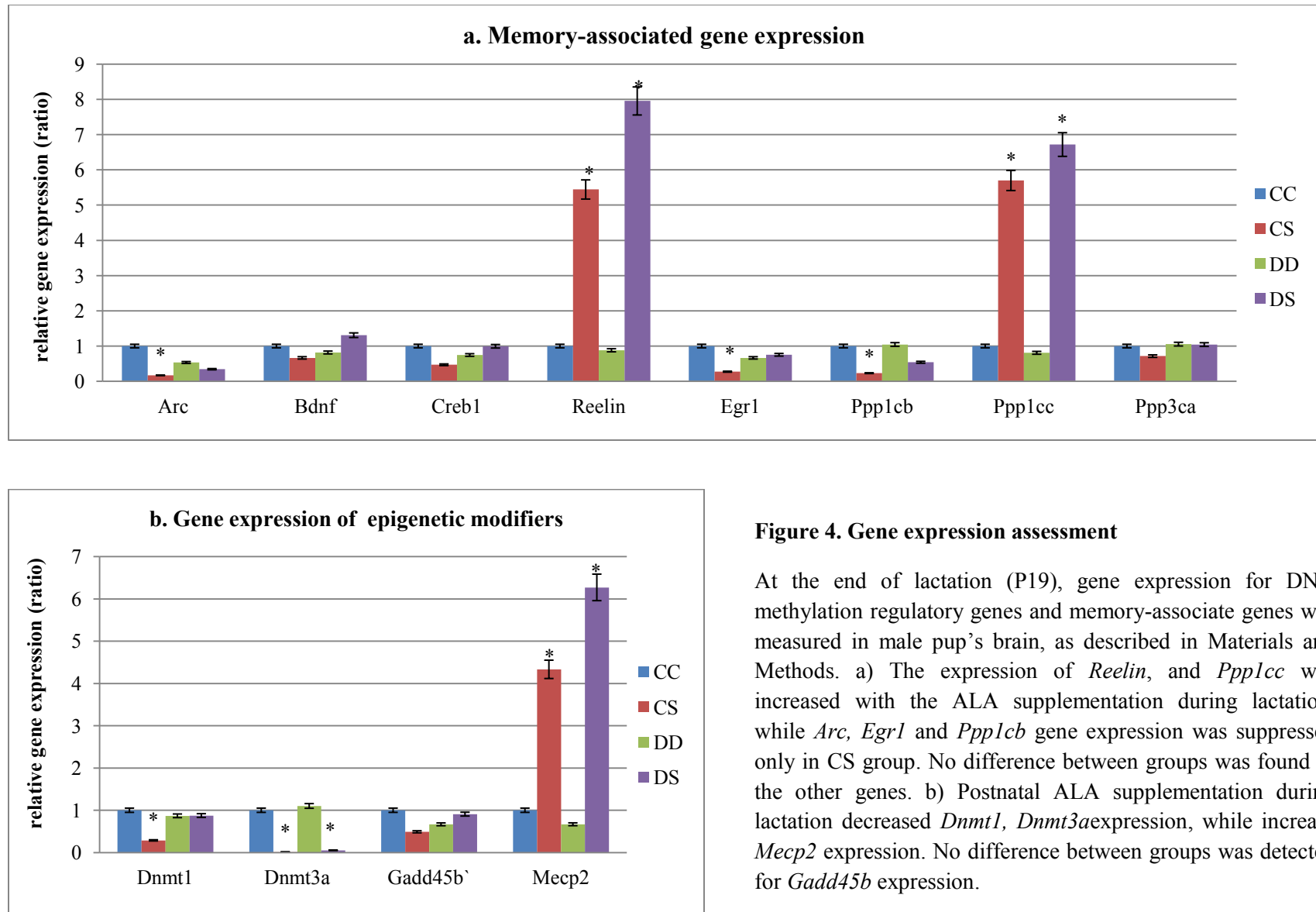
Figure 4. Gene expression assessment

At the end of lactation (P19), gene expression for DNA methylation regulatory genes and memory-associate genes was measured in male pup's brain, as described in Materials and Methods. a) The expression of *Reelin*, and *Ppp1cc* was increased with the ALA supplementation during lactation, while *Arc, Egr1* and *Ppp1cb* gene expression was suppressed only in CS group. No difference between groups was found in the other genes. b) Postnatal ALA supplementation during lactation decreased *Dnmt1, Dnmt3a* expression, while increase *Mecp2* expression. No difference between groups was detected for *Gadd45b* expression.
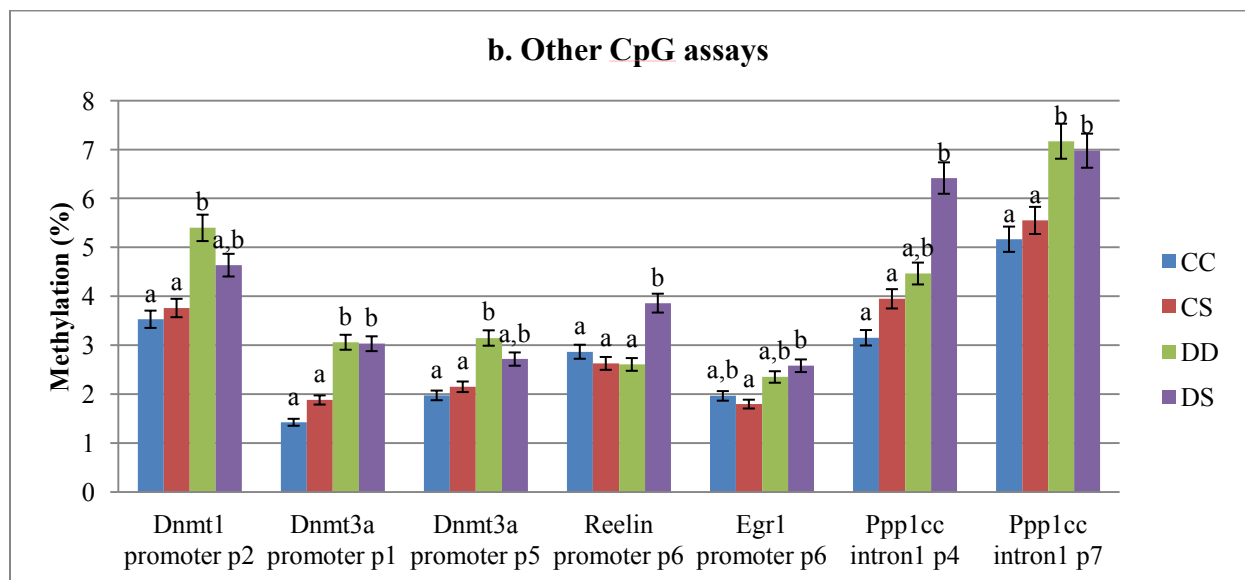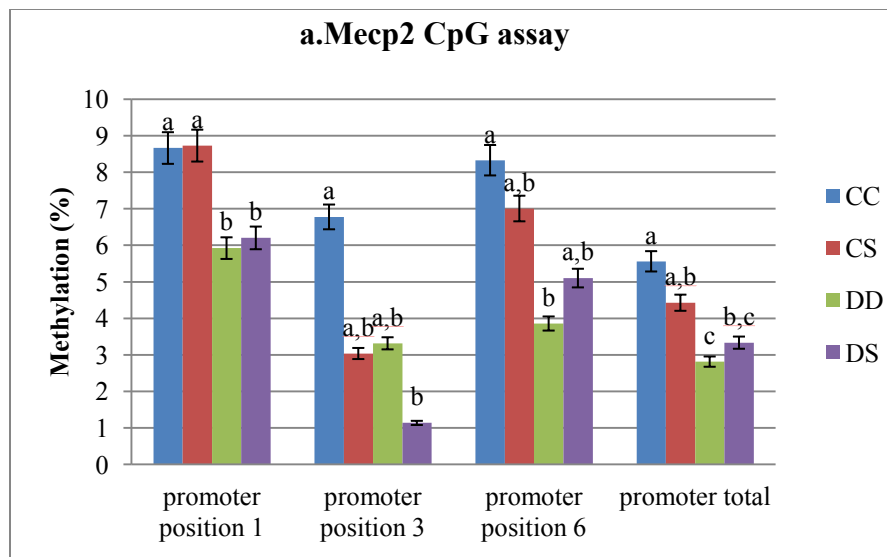
Figure 5.Bisulfite pyrosequencing for the assessment of genes CpG site in promoters or intron1

At the end of lactation period, offspring were euthanized, and brain DNA was used for bisulfite pyrosequencing of gene promoter or intron1. a) Gestation ALA deficiency decreased total DNA methylation in *Mecp2* promoter, regardless of ALA availability during lactation. However, one CpG site was hypomethylation groups with postnatal ALA supplementation (CS and DS). b) Two CpGs in *Dnmt3a* promoter and one CpG in Ppp1cc intron1 was hypermethylated in DD and DS group. The same trend was found in *Dnmt1* promoter p2 and *Egr1* promoter p6 CpG site. Hypermethylation was also induced in *Reelin* promoter p6 and *Ppp1cc* intron1 p4 CpGs. No difference was found in other CpG site between groups (data not show). Results for bars that do not share a letter differed significantly between the respective groups ($p<0.05$)
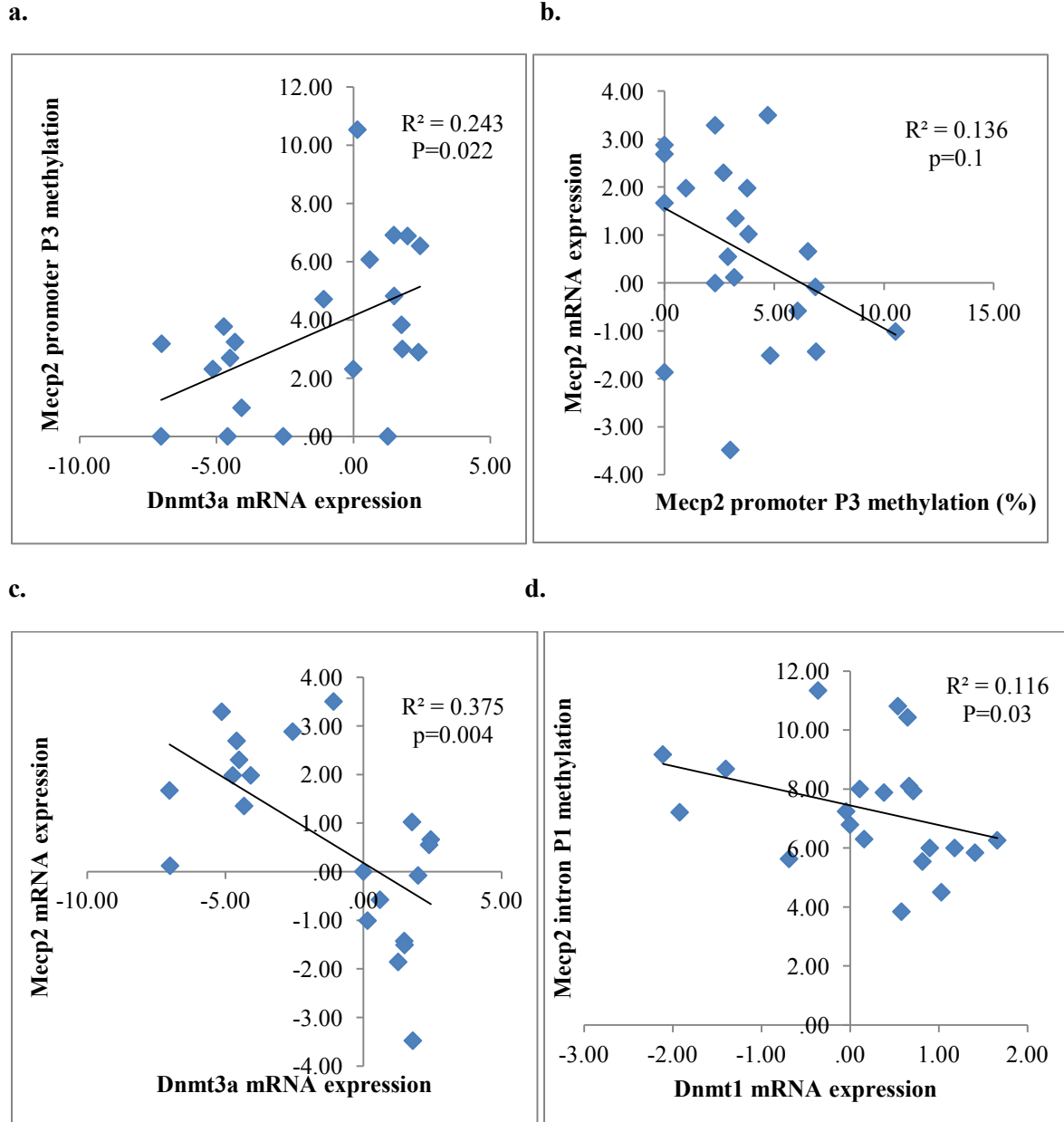
Figure 6. Linear regression for Mecp2

Bivariate fit analysis using a linear model was performed on paired variables. a) Mecp2 p3 CpG site methylation in promoter was positively correlated with Dnmt3a mRNA expression. b) This CpG site was also associated with its gene expression in off springs, though not significantly at $\alpha=0.05$. c) There was also a negative correlation between Mecp2 mRNA expression and Dnmt3a mRNA expression. d) Another CpG site methylation in Mecp2 intron1 was negatively related with Dnmt1 gene expression.

a.

R² = 0.331
p=0.006

Ppp1cc intron1 p3 methylatio (y-axis)
Dnmt3a mRNA expression (x-axis)

b.

R² = 0.142
p=0.09

Ppp1cc mRNA expression (y-axis)
Ppp1cc intron p3 methylation (x-axis)

c.

R² = 0.431
p=0.002

Ppp1cc mRNA expression (y-axis)
Dnmt3a mRNA expression (x-axis)

d.

R² = 0.959
p=0.000

Reelin mRNA expression (y-axis)
Ppp1cc mRNA expression (x-axis)
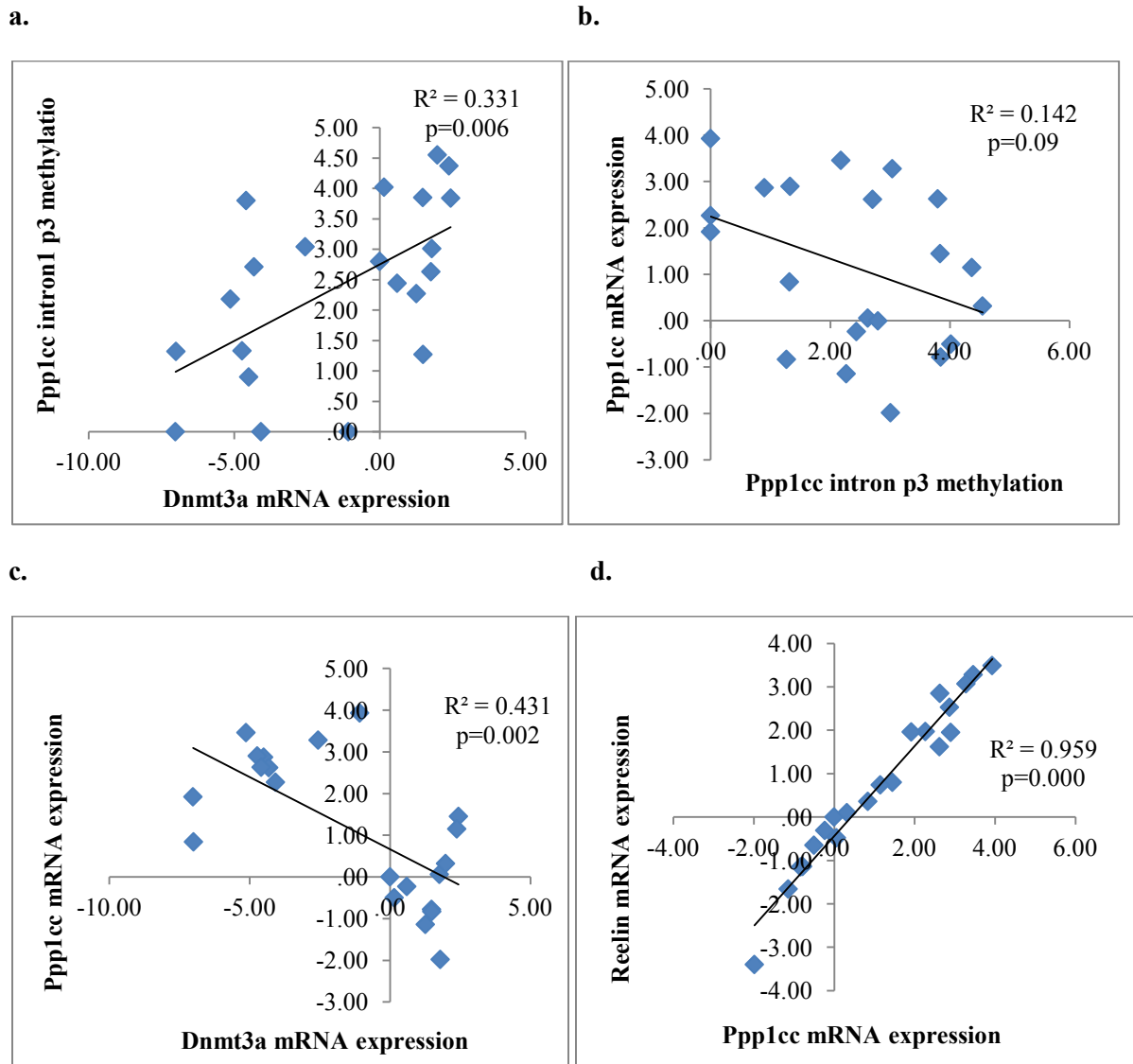
Figure 7. Linear regression for Ppp1cc

Same linear model was performed for *Ppp1cc* and *Dnmt3a*. a) Regression analysis indicated a significant and negative correlation between Dnmt3a mRNA level and methylation percentage of one CpG site in Ppp1cc intron1. b) The higher methylation of this CpG site was associated with its gene expression negatively only when significance level α was 0.1. c) However, there was an extreme significance in the negative correlation between Dnmt3a and Ppp1cc mRNA expression. d) Ppp1cc mRNA expression was positively associated with Reelin mRNA level
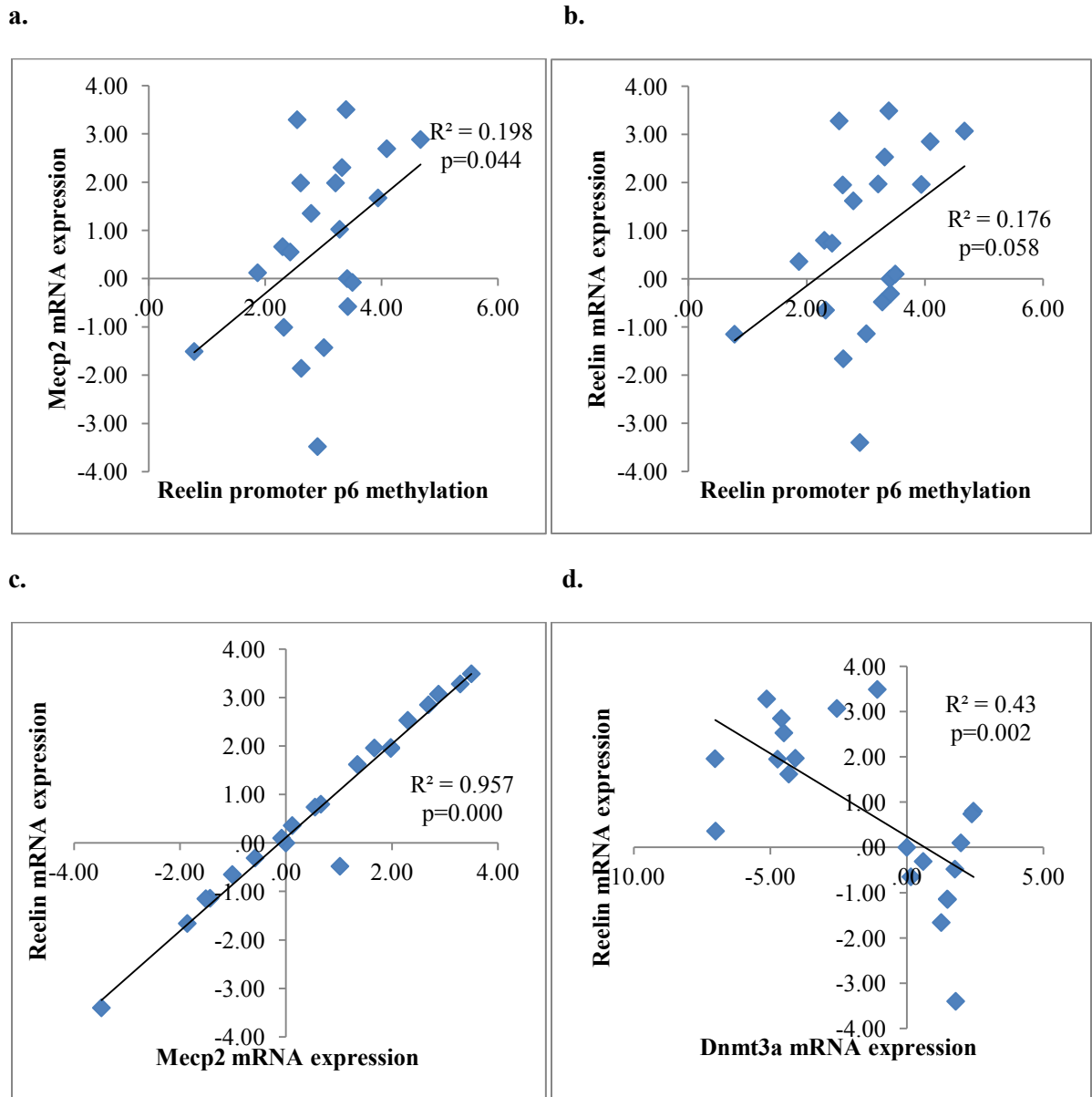
Figure 8.Linear regression of Reelin

a) There was a positive correlation between the methylation level of Reelin promoter position 6 CpG site and Mecp2 gene expression. b) The higher methylation of this CpG site was correlated with higher Reelin expression. c) Reelin mRNA expression was associated with Mecp2 mRNA expression positively, d) and Dnmt3a mRNA expression negatively.
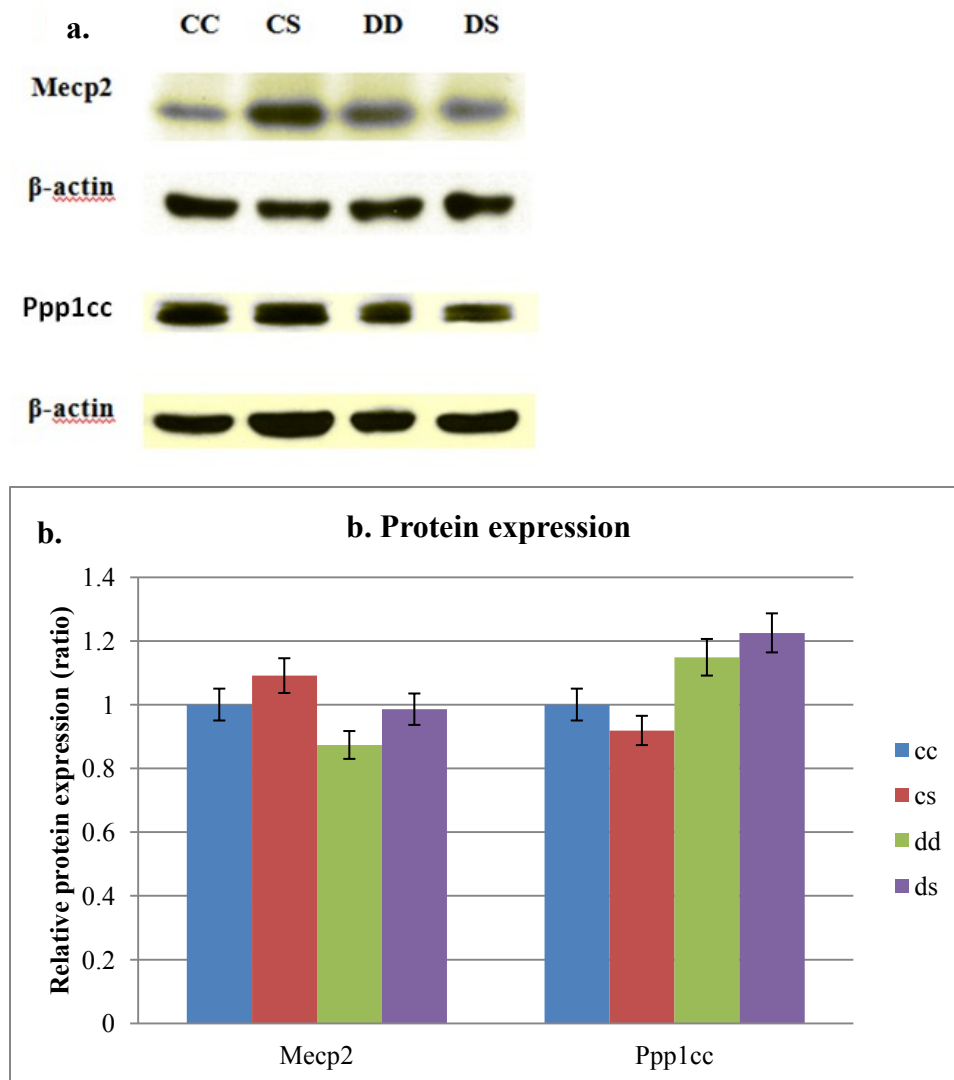
Figure 9. Protein expression of Mecp2 and Ppp1cc

Western blot analysis was performed to measure the protein level of Mecp2 and Ppp1cc. No difference was detected for this two protein expression in the offspring brains between the four dietary groups.

Chapter III- Discussion

n-3 PUFAs gained their reputation and are popular as a supplement in the last few years, especially in milk and infant food, because of potential health benefits, such as on brain development. Research performed with long chain PUFAs normally use fish oils that have DHA, because it is the most abundant n-3 PUFA in the brain. However, the food industries prefer to use vegetable oils containing ALA as a source of n-3 PUFA due to cost. Though ALA is the precursor for DHA formation in ALA, it can also function as a cells signal. Thus the effects of ALA itself on individual health should be further investigated.

This study focused on the role of ALA in brain development, specifically in synaptic plastic and memory process, during gestation and lactation period. Maternal ALA supplement induced significant alterations in transcriptional level of several memory-associated genes, which was in part associated with epigenetic changes. In this chapter, we would discuss the pertinent issues and research questions, as well as the limitations in the field of study.

Effects of Maternal ALA Availability on Gene Expression in the Offspring Brains

We investigated mRNA expression of 8 genes that have been reported to positively and negatively regulate memory and contain CpG islands around its promoter region. Five

genes were found to have a substantial alteration in mRNA levels in the brain of male offspring (Figure 4). Interestingly, for *Reelin,* and *Ppp1cc,* the effects were not offset by maternal ALA deficiency. According to our previous finding, there was an increased n-3 FAs levels in the offspring brains at the end of lactation in CS and DS group, and no differences in DHA content were found between any of the dietary groups of pups [17]. Thus, we can speculate that maternal ALA supplementation during lactation could have a direct effect on *Reelin* and *Ppp1cc* expression, which may change the memory capacity of the offspring.

Reelin is highly expressed in mammalian brain regions, including the hippocampus, the developing cerebellum, as the extracellular signaling molecule for the correct positioning of migrating neuroblasts. Reelin is essential for neuronal lamination and synaptic plasticity. In our current study, maternal ALA exposure during lactation significantly increased the *Reelin* transcript levels in the brain of the pups, which supports evidence that the amount of Reelin in the cortex and hippocampus of the newborn is reduced as a result of ALA-deficiency. Reduced concentrations of Reelin may cause a disruption in the cytoarchitecture of the laminar cortex [71].

Ppp1cc was another gene found to be up-regulated in both pup brains from CS and DS group. It encodes one of the catalytic subunit of Pp1. Interestingly, *Ppp1cb*, the gene encoding another catalytic subunits of Pp1c was significantly down-regulated in the CS group. The opposite response to ALA supplementation of the two subunits suggests that they have different interactomes that regulate specific cellular pathways. Pp1 has been implicated to be involved in protein phosphorylation process, generally as a suppressor of learning and memory and a potential mediator of cognitive decline during aging [77]. However, few studies have demonstrated the function of *Ppp1cc* separately. Lately, PP1γ, the protein

product of Ppp1cc, was reported to decrease phosphorylation and ubiquitination of CREB in HBOT, which promotes neurogenesis [72, 78]. The increase mRNA level of *Ppp1cc,* again supported our previous finding. Surprisingly, we didn't see significant changes in *Creb1* and its downstream genes *Bdnf*. One possible reason is *Ppp1cc* regulates other genes in neonate's brain that increase neurogenesis. There was a significant association between*Ppp1cc*and *Reelin* expression (Figure 7d), suggesting that *Reelin* may be a potential target gene for Ppp1cc.

Unlike *Reelin* and *Ppp1cc*, ALA supplementation significantly suppressed *Arc* and *Egr1*, but the effect was altered by gestational ALA deficiency, suggesting the importance of prior or gestational period in regulation of this gene. Arc is necessary for memory consolidation and induced selectively in the principal cells of the hippocampus and other brain regions by neural activity specifically associated with active information processing [79]. It is transcribed at low levels when an animal is resting and also reversely with aging. 7 weeks of krill oil (containing DHA) treatment to male rats induced *Arc* in the prefrontal cortex [67]. However, in this study, *Arc* expression was suppressed in the brain of offspring from the female mice with ALA supplementation, suggesting that ALA might work through a different physiological mechanism than DHA in terms of *Arc* induction. Also, we cannot exclude the possibility that pups with lactational ALA supplementation were more resting. One study has suggested that n-3 FAs may decrease aggressive tendencies [80]. Our result might also suggest a different expression profile of *Arc* in different brain regions, since no study has reported the impact of ALA intake on *Arc* expression in the whole brain.

Egr1 protein is a transcriptional regulator, and its activation is required for differentiation and mitogenesis. The induction of Egr1 in the brain has been associated with

neuronal activity and plasticity [81]. In 3T3 fibroblasts, AA (n-6 PUFA) significantly increased Egr-1 mRNA level, while EPA and DHA did not lead to the early gene mRNA accumulation [82]. Thus, the suppression of *Egr1* expression observed in CS group could be regarded as an n-3 FA antagonistic effect on n-6 FA induced Egr1 expression. Conversely, because ALA supplementation was associated to a relative n-6 deficiency, these changes could also be assigned to the n-6 pathways.

Effects of Maternal ALA Availability on DNA Methylation Machinery in the Offspring Brains

DNA methylation and demethylation was reported to actively participate in regulating synaptic plasticity and learning and memory processes via regulation of gene transcription. Thus, we also measured the mRNA level of *Dnmt1*, Dnmt3a, *Gadd45b* and *Mecp2*.

Postnatal supplementation of mothers with ALA from flaxseed oil reduced *Dnmt1* expression when it followed gestational ALA deficiency, while decreased *Dnmt3a* expression regardless of the gestational ALA status.Dnmt1 and Dnmt3a are important DNA methyltransferases that are expressed in postmitotic neurons. By conditionally deleting *Dnmt1* and *Dnmt3a* exclusively in postnatal postmitotic neurons, abnormal long-term plasticity was shown in the hippocampal CA1 region and deficits in learning and memory, together with a significant decrease in DNA methylation in neurons [2].

In humans, mutations in DNA methylation machinery are associated with mental retardation disorders. One notable example regards *MeCP2* mutations. *MeCP2*, methyl-CpG-binding protein 2, is the first identified protein that binds to methylated CpGs and recruit transcriptional repressor complexes, and it is also an important epigenetic factor in the maintenance and development of the central nervous system. The loss-of-function and gain-

of function alterations in *MeCP2* expression results in the neurodevelopment disorders Rett syndrome and MeCP2 duplication syndrome, respectively [73]. In the current study, *Mecp2* was significantly increased by ALA supplementation during lactation, no matter the gestational ALA diet. These results indicate that maternal ALA intake during gestation and lactation induces DNA methylation alterations in the brains of the offspring. However, without a follow-up test in these pups, we couldn't determine whether the increase in *Mecp2* induced by ALA supplement was benefit or detrimental for brain function.

Gadd45b is thought to be associated with activity-regulated DNA demethylation and postnatal neurogenesis [56]. We did not find differences in its expression between dietary groups.

Perinatal Manipulation of ALA Intake Induce DNA Methylation Changes in Offspring Brains

We hypothesized that epigenetic modifications were involved in the gene expression alteration induced by different maternal ALA availability. Bisulfite pyrosequencing assays were conducted to measure DNA methylation in promoter or intron1 areas of the genes with significant changes in mRNA level (Figure4).Within a CpG island, successive CpGs can display different degrees of methylation, which may represent the result of interference between the methylation process and the local chromatin conformation [83].

It is important to note that few CpG sites have been analyzed for each gene. It is possible that these CpG sites did not necessarily represent the global methylation patterns. Moreover the method we used in the current study cannot discern 5mC from5-hydroxymethylcytosines (5hmC), which is hypothesized to stimulate gene expression

through DNA demethylation. Notably, 5hmC may be especially important in the brain, as the most abundant levels are found in central nervous system [84, 85]

Significant methylation changes in CpGs were found in the promoter regions of *Mecp2, Dnmt1, Dnmt3a, Reelin, Egr1* and intron1 of *Ppp1cc* (Figure 5). It is a surprise to us, because we never thought of nutrition in terms of this interaction. For instance, for *Mecp2* promoter p3 CpG site, if supplementation in lactation comes in a deficiency background in gestation, you have the most reduction in DNA methylation than any other combination of diets. This CpG site is an example that there is interplay between gestation and lactation in terms of methylation.

There is another case where the interplay of diet has different results. For *Ppp1cc* intron1 p7 CpG site, what counts is only the deficiency in gestation, because there is no change between DD and DS, which means it doesn't matter in lactation whether you have a supplement or deficient diet. Only the deficient diet in gestation plays a role. This indicates that this methylation of this site is established in gestation and it's done at birth. Nothing in lactation will influence that.

It seems overall the DS treatment and sometime the DD treatment are the strongest in terms of modulating DNA methylation. This suggests that the establishments of DNA methylation patterns are dependent on gestational ALA intake. There may be a fine tune in some CpG sites, which further changes during lactation. As it has been presented previously, n-3 PUFAs induce the expression of various genes involved in diverse functions, in different brain regions, and in an age and time-dependent manner. Therefore, ALA may take very different routes to alter transcription, depending on the cell-specific context and the target

gene. It seems that epigenetic marks are particularly vulnerable during early stages of embryonic development, which is a critical period for their establishment and maintenance.

Using linear regression, the hypermethylation of one CpG site in *Mecp2* promoter was associated with an increased expression of *Dnmt3a*. This hypermethylated CpG site was also correlated, although marginally significant, with a suppressed expression of this gene, which supported a known mechanistic view about DNA methylation. In addition, there was a negative correlation between *Dnmt3a* and *Mecp2* (Figure 6). It can be speculated that Dnmt3a induces methylation in the promoter CpG site in *Mecp2*, which partly contributes to it gene repression. Similar results were also found in Ppp1cc (Figure 7). Thus, these two CpG sites can be potential epigenetic marks targeted by ALA supplementation. Interestingly, *Dnmt1* was only negatively correlated with one *Mecp2* intron1 CpG methylation and most of the changes in *Dnmt1*and *Dnmt3a* expression did not necessarily predict the DNA methylation changes in CpGs. Similarly, our previous study also found a negative correlation between *Fads2* promoter hypermethylation and *Dnmt3a, 3b* expression in maternal and offspring livers [24]. These results together indicate that the DNA methylation level of the gene might not be related with its expression immediately at the end of lactation period. However, we were not able to determine the timing of these changes or the exact roles played by ALA availability during gestation or lactation, for the lack of multiple time point.

Mecp2 Regulation of *Reelin* through Epigenetic Mechanisms

Mecp2 is a key player in recognizing methylated DNA and interpreting the epigenetic information encoded in different DNA methylation patterns. As expected, there was a significant positive correlation between Reelin promoter p6 methylation and Mecp2 expression. However, unlike other CpG sites, the hypermethylation of this position is

correlated with an increase in gene expression (Figure 8a, 8b). As we mentioned before, 5hmC is a marker for DNA demethylation. The presence of 5hmC in promoter regions was associated with higher levels of transcription [86]. It is important to note that 5hmC is most abundant in neurons, suggesting that it is a stable epigenetic mark to carry out its functions in the brain [38]. Thus, we speculate that 5hmCcould be the dominant methylated cytosine form in this *Reelin* promoter CpG site, which was also suggested by the negative correlation with *Dnmt3a*, the *de novo* methylation enzyme. Recently, Mecp2 was identified as the major 5hmC-binding protein in the brain and its binding affinity was similar for 5hmC- and 5mC-containing DNA [38].

Reelin has been previously reported to be a primary target of Mecp2. ChIP assay on whole brain documented that Mecp2 binds to the *Reelin* promoter in normal mice. The transcript level of *Reelin* was decreased in the brain from wild type mice than that of Rett Syndrom (Mecp2 loss-of function) mice, indicating a negative association between the two genes [87]. The predominant model of Mecp2 function is that it binds to CpG methylated promoters and recruits histone deacetylase along with co-repressor activators, thereby silencing gene transcription [88]. It is important to note that this model is based on studies with artificial genetic modifications, such as knock-out or knock-down *Mecp2*. In contrary, *Reelin* mRNA expression was positively correlated with *Mecp2* mRNA expression (p=0.000) in our study. One possible explanation is that other ALA-induced changes in gene expression overcome the Mecp2 effects on gene repression at the end of lactation. However, it is also likely that the increase in *Reelin* observed in the Rett syndrome model resulted from a compensatory mechanism/negative feedback for *Mecp2* dysfunction. This is supported by an

integrated genome-wide promoter analysis of Mecp2 and gene expression, which revealed

that 63% of Mecp2-bound promoters were actively expressed [39].

No Alterations of Mecp2 and Ppp1cc Protein induced by Maternal ALA Exposure

Although we reported significant transcriptional alterations in *Mecp2* and *Ppp1cc*

between the four groups, there was no difference in both protein levels detected by western

blot. A key assumption in studying mRNA expression is that it is informative in the

prediction of protein expression. However, this is not the case for all genes. The most

accepted explanation for the lack of significant correlation finding is due to technological and

biological reasons. Western blot is one of the most common procedures for protein detection;

however it is much less accurate and less quantitative than high-resolution MS. Though

western blot can determine whether a protein has been modified, such as phosphorylation,

with specific antibodies, it cannot indicate the protein activity of interest. Thus, it is possible

that Mecp2 and Ppp1cc could exert different influences in the offspring brain between CS,

DS groups and CC, DD groups, according to their posttranslational regulation. Biological

processes such as transcriptional or post-transcriptional splicing, regulation and protein

complex formation, might affect the relative quantities of mRNA and protein of various

genes to various degrees. Thirdly, several studies have suggested the mRNA-protein

expression correlations vary in different biological categories of gene ontology. For example,

the highest correlation was achieved for genes of the extracellular region in terms of cellular

component while the lowest correlation was obtained for genes of regulation in terms of

biological process [89].

Indications of current study and Future Directions

In contrast to the robust changes in expression, we detected the relatively small changes in CpG methylation of some memory-associated genes in the offspring brains at the end of lactation. Interestingly, based the our results, it seems that the effects of lactational ALA supplementation on mRNA expression was not offset by gestational ALA deficiency, while the DNA methylation pattern was determined most by gestational ALA exposure. These data indicate that ALA may affect transcription in a complex way, and DNA methylation is particularly important during early stages of embryonic and fetal development. Altered gene expression could be brought about and perpetuated by transcription factors (such as PPARs, SREBPs), or epigenetic mechanisms other than DNA methylation (such as histone modifications), during lactation period. Because our study did not include groups with a supplemented or a control ALA diet during gestation, followed by postnatal ALA deficiency, we cannot differentiate the exact roles of ALA supplementation during gestation vs. lactation. In addition, multiple time points are also needed to learn about the timing of the response, especially when we found the DNA methylation level of the gene might not be related with its expression immediately at the end of lactation period.

Among the CpG sites whose DNA methylation patterns were modified by maternal ALA intake, only two CpG sites seem to be potential targets of ALA for epigenetic modifications in *Mecp2* promoter and *Ppp1cc*, respectively. The few significant results may arise from a delayed response, unrepresentative CpG sites, lack of statistical power, as well as some limitations of the technique, of which, the failure to separate 5hmC from 5mC is the biggest issue. We speculate that 5hmC in the *Reelin* promoter recruits Mecp2 and in turn regulate its gene expression. This should be further validated by a complementary technique

that be applied to a larger sample size. Several new approaches have been developed to detect 5-hmC , such MS-based approaches, Tet-assisted bisulfite sequencing (TAB-seq) [74]. In addition, since normal epigenetic state varies between cell types, further investigation of epigenetic mechanisms requires consideration of specific type or types of cells to be studied, such as neuron cells in this case [90].

It's the same for protein measurement. Though no difference was detected for Mecp2 and Ppp1cc protein level, it doesn't mean that their bioactivity hasn't been altered in a certain type of cells or areas. Our previously study reported that gestational and lactational ALA availability changed cell proliferation and early neuronal differentiation within DG of the offspring. Thus, more quantitative and sensitive assays, such as Immunohistochemical technology, should be performed in this specific area, especially for Mecp2, Reelin and Ppp1cc, since they've been suggested to promote neurogenesis.

Unlike genetic mutations, epigenetic profiles are potentially reversible [30]. Therefore, epigenetic approaches for prevention and treatment, such as nutritional supplementation may be developed to counteract negative epigenomic profiles [91]. Our results indicated that maternal ALA supplement induced alterations in transcript level and DNA methylation of several memory-associated genes at the end of lactation. Further study and experiments are required to determine the exact timing, interplay and mechanism of these events. Although a direct correlation between maternal ALA availability and variable DNA methylation in either childhood or adult brain cells has not been demonstrated, this study provides a basis for future research into the relationship between perinatal exposure to ALA and the physiological effect on learning and memory in the adulthood of the offspring. Moreover, taking F0 as the exposed pregnant mother, observations of F1 through F3

generations are essential to determine whether this epigenetic mechanism is responsible for a transgenerational effects, which is a major step forward in the understanding of complex brain function [92]. Last but not least, it is always important to determine the dose effect by respecting the needs of the organism, when the results are extrapolated to humans.

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